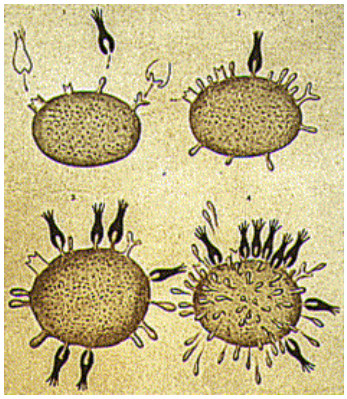
UCL Université catholique de Louvain

Antibiotic accumulation and efflux in eukaryotic cells: a journey at the frontier of pharmacokinetics and pharmacodynamics

"corpora non agunt nisi fixata"



Ehrlich's "magic bullet" theory

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Promoteur : Prof. Paul M. Tulkens

Co-promoteur : Prof. Marie-Paule Mingeot-Leclercq

I have thus come to the end. I am conscious of the fact that they are gaps in the work I have presented. But I did want to show you that we are getting to grips with the problem of obtaining an insight into the nature of action of therapeutic substances, the conception of which must consist in the recognition de sedibus et causis pharmacorum.

Paul Ehrlich, 1908, Nobel Lecture

Il y a précisément 10 ans, je défendais ma thèse de doctorat en sciences pharmaceutiques. Que de chemin parcouru depuis !…
En feuilletant l'album de cette décennie, je revois en effet avec émotion les personnes et les institutions qui ont contribué à mon épanouissement scientifique.

Au fil des pages, s'inscrivent en filigrane :

Le Recteur et les Autorités de l'Université catholique de Louvain : je leur exprime ma profonde gratitude pour la qualité de la formation scientifique reçue et la promotion exigeante mais stimulante d'une recherche de haut niveau. Qu'ils voient dans ce travail le témoignage de mon attachement et de mon dévouement à notre Université.

Le Fonds National de la Recherche Scientifique : je lui suis reconnaissante pour la confiance qu'il m'a témoignée en m'accordant son soutien financier depuis de nombreuses années, et plus particulièrement en m'octroyant le mandat de chercheur qualifié qui m'a permis de réaliser ce travail.

A l'avant-plan, je voudrais placer mes promoteurs, qui m'ont toujours assuré l'appui nécessaire pour progresser et m'ont accueillie comme leur collaboratrice avec bienveillance, estime et sympathie.

Le Professeur P.M. Tulkens m'a toujours fait confiance, n'hésitant jamais à m'impliquer dans des projets d'envergure. Son enthousiasme visionnaire et sa vaste culture scientifique m'encouragent à la persévérance. Une dizaine d'années sous sa houlette de chef d'Unité m'ont permis de découvrir sa nature d'expérimentateur, son esprit d'entreprise et son sens de l'ouverture.

Le Professeur M.P. Mingeot-Leclercq a fait preuve à mon égard d'une disponibilité à toute épreuve. Sa rigueur scientifique, ses compétences polyvalentes et ses questions pertinentes m'incitent à la réflexion. Près de quinze ans passés à ses côtés m'ont permis d'apprécier son œil exercé de chimiste, sa main de gestionnaire, son oreille attentive et amicale, autant de qualités qu'elle met à présent au service de l'Unité.

Je les remercie chaleureusement pour la formation complémentaire qu'ils ont pu me donner et espère que ce travail sera pour eux le reflet de ce qu'ils m'ont appris et m'ont permis de devenir. Je voudrais aussi souligner leur investissement inconditionnel à la direction successive de l'Unité. Ils ont su, chacun à leur manière, en faire un lieu de travail accueillant.

Une page spéciale de l'album est réservée au Professeur P. Courvalin et à son équipe : le séjour post-doctoral effectué dans son laboratoire à l'Institut Pasteur a certainement profondément marqué la suite de mon parcours universitaire par la qualité de son environnement scientifique. Je lui dois une reconnaissance particulière pour son accueil, sa disponibilité et son ouverture, qui m'ont permis d'acquérir des compétences nouvelles et de développer des projets de recherche novateurs.

Une large vue panoramique évoque ensuite des rencontres tous azimuts, avec des chercheurs d'autres laboratoires. Grâce à leur expertise, j'ai eu la chance de pouvoir développer ces dernières années des projets multidisciplinaires, dans le cadre exaltant de la recherche sur les nouveaux antibiotiques. Je remercie en particulier les équipes des Professeurs A.M. Corbisier, Y. Glupczynski, J. Poupaert et E. Sonveaux (Université catholique de Louvain), M. Prévost et M. Rooman (Université libre de Bruxelles) et J.M. Frère (Université de Liège). Je pense aussi aux collègues qui ont apporté leur concours à la rédaction des articles de revue présentés dans cette thèse, principalement les Docteurs E. Balzi, J.C. Pechère, P. Plésiat et Y. Van Laethem.

Je voudrais également associer à ces collaborateurs les membres de l'International Society for Anti-infective Pharmacology, qui m'ont appris les fondements de la pharmacodynamie anti-infectieuse et ont régulièrement enrichi mes réflexions lors de discussions aussi animées que conviviales.

Dans le cadre plus proche des tours Ehrlich et Van Helmont, je voudrais aussi remercier mes collègues du département des sciences pharmaceutiques de m'avoir accueillie parmi eux et de me faire confiance pour les activités de recherche et d'enseignement que j'assure.

Une série de clichés me rappelle enfin les nombreux post-doctorants, doctorants et étudiants qui ont laissé leur empreinte au laboratoire, tant par les recherches qu'ils y ont accomplies que par la richesse de leur diversité culturelle. Je les remercie pour leur présence amicale, mais aussi pour leur travail, qui sous-tend les concepts développés aujourd'hui et suscite les questions pour les recherches de demain.

Je pense en particulier à M. Barcia-Macay, N. Caceres, S. Carryn, M. Heremans, S. Lemaire, J.M. Michot, J.P. Montenez, J. Saffran et C. Seral, dont le travail expérimental est étroitement lié aux concepts développés dans cette thèse, à E. Ampe et A. Spinewine, qui apportent la dimension clinique indispensable à l'application de nos recherches fondamentales et apportent une contribution efficace à la mise en place d'un enseignement moderne de la pharmacologie, à A. Allabi, L. Avrain, N. Baririan, V. Basma, H. Ben Abdelaziz, M. Bensliman, N. Bles, L. Blot, C. Bruno, A. Boucly, S. Burton, H. Chanteux, E. Charvalos, E. Colacino, C. Dax, E. Delbecq, M. Dellale, L. Efron, M. el Mouedden, N. Fa, C. Gerbaux, T. Happaerts, P. Jacquemin, A. Kerkhofs, L. Kottis, C. Lepers, N. Mesaros, J. Nachega, M. Nyindu, A. Olivier, Y. Ouadhriri, I. Paternotte, M. Renard, N. Santos Duarte, S. Sauvage, H. Servais, A.S. Snoeck, N. Tchapkui, G. Thirion, D. Tyteca, I. Tytgat, M.J. Uwamahoro, D. Van Ackeren, S. Van den Bril, G. Van der Essen, S. Van de Velde, E. Viaene, C. Wanko, J. Zanon et E. Zarrinpashneh qui, au cours des dix dernières années, m'ont permis de m'ouvrir à différents aspects de la pharmacologie anti-infectieuse.

Je ne voudrais pas oublier N. Aguilera, M. Breugelmans, M.C. Cambier, O. Meert, F. Renoird, et M. Vergauwen, dont les compétences complémentaires permettent l'avancement harmonieux de projets diversifiés

Dans l'album de ces dix ans de ma vie, une photo de famille est incontournable.

Mes parents ont toujours accordé la priorité au bonheur de leurs enfants. Ouverts et attentifs, ils nous ont assuré une enfance et une jeunesse heureuses et nous ont encouragés dans nos études. Aujourd'hui, ils continuent à suivre avec un vif intérêt mon cheminement universitaire.

Mon frère Pierre et son épouse Françoise partagent depuis des années les événements de ma vie et les activités récréatives nécessaires au repos du chercheur… mais la meilleure distraction m'est offerte par mon filleul Quentin et sa petite sœur Florence, qui m'émeuvent par leur tendresse.

Je leur dédie ce travail du fond du coeur, en guise de remerciement pour leur présence fidèle si nécessaire.

L'album se termine aujourd'hui par une page de choix, en hommage aux Professeurs P. Courvalin, A. Dalhoff, M. Delmée, H. Derendorf, Y. Glupcynski, E. Sonveaux et F. Zech, ainsi qu'au Doyen de la Faculté de médecine, Monsieur le Professeur J.J. Rombouts, et au Secrétaire académique, Monsieur le Professeur M. Crommelinck, qui ont accepté de faire partie du jury de cette thèse. J'ose espérer qu'ils l'auront jugée digne d'intérêt et leur sais gré pour le temps qu'ils ont bien voulu consacrer à son évaluation.

Si les pages qui suivent sont encore blanches, l'album est loin d'être achevé. Le prochain chapitre sera assurément consacré à ces jours chargés pour moi de l'émotion particulière que l'on ressent lorsqu'on termine un travail que l'on a vraiment pris à cœur et qui fait dès lors un peu partie de soi-même. J'espère donc qu'il contribuera, même de façon modeste, au progrès de la science et jettera les bases des volumes encore à venir …

TABLE OF CONTENTS

I. General introduction: Studying cellular pharmacokinetics of antibiotics: does it matter ?

Based on concepts developed in the following publications

Reviews:

- F. Van Bambeke, D. Tyteca, Y. Ouadrhiri & P.M. Tulkens. 1999. Optimisation des traitements antibactériens sur base des propriétés pharmacodynamiques des antibiotiques. *Louvain Med*. 118:43-63.
- F. Van Bambeke & P.M. Tulkens. 2001. Macrolides: pharmacokinetics and pharmacodynamics. *Int. J. Antimicrob. Agents* 18 Suppl 1:17-23.
- S. Carryn, H. Chanteux, C. Seral, M-P Mingeot-Leclercq, F. Van Bambeke, & P.M. Tulkens. 2003. Intracellular pharmacodynamics of antibiotics. *Inf. Dis. Clin. N. Am.* 17:615-634.
- F. Van Bambeke, J.M. Michot, J. Van Eldere & P.M. Tulkens. 2005. Quinolones in 2005: an update. *Clin. Microbiol. Infect.* 11:256-280.

I.1. Cellular pharmacokinetics of antibiotics: what does that mean ?

I.1.a. Cellular versus general pharmacokinetics

General pharmacokinetics examine the absorption, distribution, metabolisation and elimination of drugs in the body. Cellular pharmacokinetics are centered on the evaluation of penetration, distribution, degradation, and efflux of drugs in individual cells. These processes bear many similarities as depicted in figure 1.

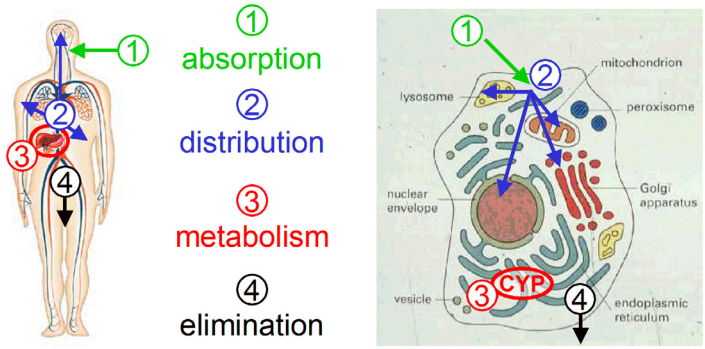


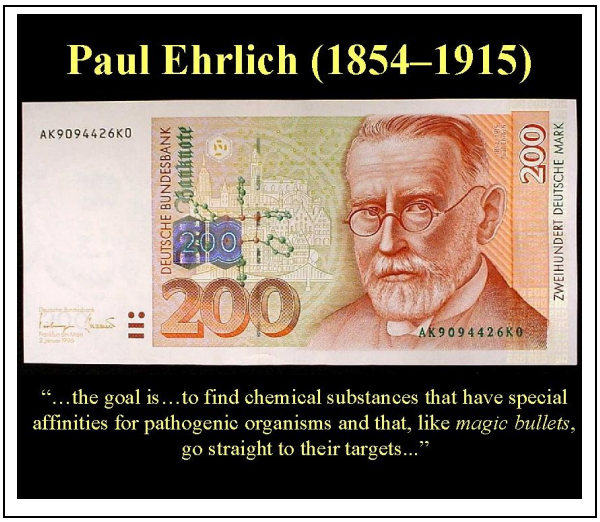
Figure 1: parallelism between general and cellular pharmacokinetics regarding the processes of absorption (penetration), distribution, metabolism, and elimination (efflux).

Analyzing the successive steps of the journey of a drug in the body or in the cell would indeed show that:

- its absorption is largely dependent upon its capacity to pass across membranes, to be taken up by pinocytosis, or to be subject to active inwards transport;
- its distribution in the various compartments of the body or within the cell essentially relates to its ability to diffuse or to be transported through membranes,
- its metabolisation and elimination is largely regulated by the metabolic and outward transport capabilities of the cells.

Cellular and general pharmacokinetics are actually closely related, since the cellular disposition of a drug (involving its accumulation, enzymatic modification or transport) often governs its general fate in the body. More importantly, cellular pharmacokinetics also provide clues, and even essential elements, for understanding drug activity and toxicity in target tissues. Thus, cellular models appear most useful for unraveling the molecular mechanisms involved in drug disposition and activity, or in drug interactions related to competition for transport or metabolism.

I.1.b. Antibiotics and eukaryotic cells



"Corpora non agunt nisi fixata", as said by Paul Ehrlich at the beginning of the XXth century, has been one of the most foresighted statements, not only for chemotherapy, but also for many areas of modern biological research. When dealing with antibiotics, one would be tempted to limit the scope of Ehrlich's principle to prokaryotic organisms, since these are their obvious targets as far as their primary pharmacological effect is concerned. However, the same concept applies to eukaryotic cells, and, in that way, to both cellular and general pharmacokinetics. For antibiotics, indeed, reaching the target also means to have access to the site of infection, and within this site, to the offending organism. At the same time, antibiotics should be kept away from sites where they could exert untoward or toxic reactions.

Thus, the "magic bullets" need to find their way to their targets and, as much as possible, only to them. Interaction between antibiotics and eukaryotic cells can, in this context, be seen at three levels :

- First, and as explained above, pharmacokinetics allow to define critical parameters regulating the capacity of antibiotics of reaching their target and exerting their pharmacological activity. This could be very critical, for example when dealing with localized infections, such as those in the central nervous system (Van Bambeke and Tulkens, 2001 for review) or other poorly accessible compartments.
- Second, several bacteria are able to survive and multiply in various subcellular compartments, causing intracellular infections. In some cases, intracellular location is an obligate element in the infection process, bacteria being unable to multiply extracellularly (as observed for *Chlamydia,* for instance [Rockey *et al.*, 2002]). But most often, bacteria are facultative or opportunistic intracellular pathogens, which means that they can multiply both extra- and intracellularly. In this case, intracellular foci are thought to play a major role either in the dissemination of the infection (as proposed for *Listeria monocytogenes* [Portnoy *et al.*, 2002]) or in its persistent or recurrent character (as suggested for *Staphylococcus aureus* [Ellington *et al.*, 2003]). In this context, studying cellular pharmacokinetics makes very good sense, and goes beyond general

pharmacokinetics, since it tells us whether a given antibiotic may or may not gain access to the infected compartment in adequate concentration.

- Third, the accumulation of antibiotics in non-target organs (with respect to their antibacterial activity) may cause toxicological manifestations. These can be related either to the capacity of antibiotics to interact with eukaryotic homologues of their bacterial target (as observed for example for antibiotics acting on topoisomerases like quinolones [Van Bambeke *et al.*, 2005a for review]), or with other targets (as is the case for aminoglycosides, which bind to acidic phospholipids within the lysosomes of kidney cells, and thereby cause cell damage and organ dysfunction [Mingeot-Leclercq and Tulkens, 1999 for review]).

I.2. Pharmacokinetic/pharmacodynamic properties of antibiotics

Pharmacodynamics is usually defined as the study of biochemical and physiological effects of drugs and of their mechanisms of action (Ross and Kenakin, 2001). For antibiotics, this discipline has been equated for many years to the study of their mode of action, as evidenced by microbiological and molecular biology approaches. However, this has left open large gaps of knowledge between pharmacokinetics and efficacy, since it does not address the relationship between concentration and antimicrobial effects. In addition, most conventional microbiological techniques characterizing antibiotics use parameters such as minimal inhibitory concentration (MIC) or minimal bactericidal concentration (MBC), but largely ignore the influence of variation in the concentration as well as that of time. Accordingly, a new discipline has emerged, which will be referred to as **pharmacokinetics/pharmacodynamics (PK/PD) of antibiotics**. It has been defined (Amsden *et al.*, 2000) as the '*discipline that strives to understand the relationships between drug concentrations and effects, both desirable (e.g. bacterial killing) and undesirable (e.g. side effects)*'. This concept is illustrated in figure 2.

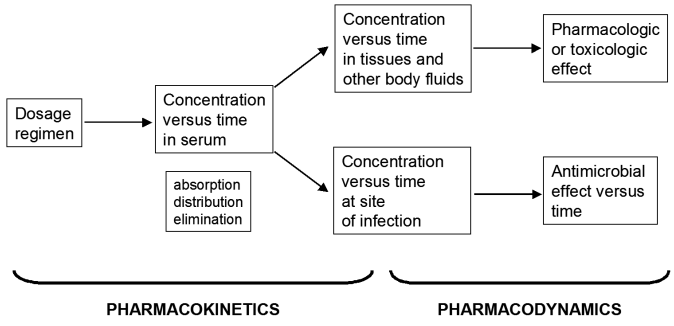


Figure 2: overview of the interaction of pharmacokinetics and pharmacodynamics for anti-infective agents.

(Craig, 1998)

Our thesis will be centered on the study of the **cellular** pharmacokinetics and pharmacodynamics of antibiotics.

Research on general pharmacokinetics and pharmacodynamics of antibiotics against extracellular organisms, however, has taught us many critical concepts that will be used throughout our work. We will therefore first briefly review them.

I.2.a. Current concepts in extracellular PK/PD parameters of antibiotics

Reaching a given concentration in a test tube or in the body is not a sufficient criterion to guarantee antibiotic efficacy. This is due to marked differences in their apparent dependence to concentration and time.

Over the last 15 years, three key pharmacokinetic-pharmacodynamic parameters (see for reviews [Craig, 2003; Andes *et al.*, 2004; Andes and Craig, 2005]) have been defined, which appear critical to predict antibiotic activity against extracellular infections in conditions mimicking their practical use in the clinics (see figure 3 for illustration).

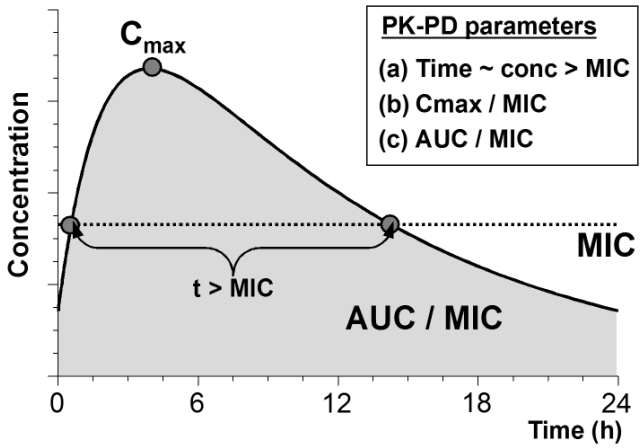


Figure 3:

pharmacokinetic - pharmacodynamic measures correlated with efficacy against extracellular infections.

(a) time (t) during which the concentration remains above the MIC of the antibiotic against the pathogen

(b) ratio between, the Cmax of the antibiotic reached in the serum and the MIC

(c) ratio between the 24-h area under the concentration-time curve ($AUC_{24\,h}$) and the MIC.

The first parameter links effects to time and is critically dependent on the drug half-life. The second one relates effects to concentration and is primarily dependent upon the dose and the volume of distribution. The third one combines both types of effects, since it corresponds to the total amount of drug to which bacteria are exposed over 24 h, and is related to the overall dose and the clearance.

The present status of research has led to the categorization of three main classes of antibiotics, as shown in table 1.

Table 1:
classification of antibiotics according to their pharmacodynamic profile (as determined against extracellular infections)

adapted from (Craig, 2003)

PK-PD profile	PK-PD parameter	Antibiotic classes
time-dependent, minimal persistent effects	Time > MIC	β-lactams
concentration-dependent, highly bactericidal, prolonged persistent effects	Peak/MIC (AUC/MIC)	aminoglycosides fluoroquinolones daptomycin metronidazole ketolides
time-dependent, little influence of concentration, prolonged persistent effects	AUC/MIC	glycopeptides tetracyclines macrolides streptogramins lincosamides oxazolidinones

These are:

(a) those for which there is little gain of activity once above the MIC and which show no persistent effects. They will need to remain in the contact of their target as long as possible; their efficacy will be best predicted by the "time above MIC" PK-PD criterion.

(b) those with concentration-dependent bactericidal activity and prolonged persistent effects (post-antibiotic effects). Their efficacy will be best predicted by the Peak/MIC ratio for killing, and by the $AUC_{24\,h}$/MIC ratio for overall efficacy.

(c) those for which activity is little influenced by concentration once above a critical threshold, but which show marked persistent effects. They will display activity in proportion of the total antibiotic exposure, which can be correlated to the $AUC_{24\,h}$/MIC ratio.

I.2.b. Integration of PK/PD parameters in global pharmacological assessment of antibiotics

The concepts presented above may come as a surprise, since they seem to imply that some classes of antibiotics (β-lactams, e.g.) would not obey the classical law of mass action, which is central to the basic concept of drug-target interaction. This is because most studies dealing with antibiotics use concentrations that are above the MIC for obvious microbiological reasons (concentrations below the MIC are considered as ineffective and susceptible to trigger the emergence of resistance. Of note is also the fact that the MIC may, for some antibiotics towards sensitive organisms, be several fold lower that the Cmin observed in the serum of patients).

However, when antimicrobial effects are examined over a wider range of concentrations, clear-cut dose-response effects can be observed, as illustrated in figure 4 for ampicillin (as typical β-lactam) and gentamicin (as typical aminoglycoside) towards *Staphylococcus aureus.*

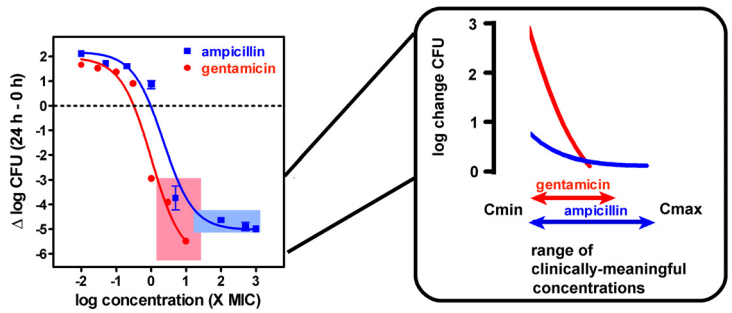


Figure 4 :

concentration-effect relationship for antibiotic activity against *S. aureus*.
The graph shows the reduction in CFU after 24 h incubation of *S. aureus* in the presence of antibiotic concentrations ranging from 0.01 X their MIC to 1000 X MIC.
The last experimental point for gentamicin is at the limit of detection.

For each drug, the amplitude of the effect over the zone of clinically-meaningful concentrations (ranging from trough [Cmin] to peak [Cmax]) is highlighted by a box, with limits of concentrations set at

- 1 and 80 mg/L for ampicillin (as observed for a daily dose of 4 g IV divided in 4 administrations; MIC: 0.07 mg/L).
- 1 and 18 mg/L for gentamicin (as observed for a daily dose of 6 mg/kg IV given once daily; MIC: 0.5 mg/L)

The right inset is a zoom on that zone of concentrations, evidencing that gentamicin activity is much more influenced by its concentration than that of ampicillin over the range of clinically-meaningful concentrations.

data from (Barcia-Macay *et al.*, 2005; Lemaire *et al.*, 2005)

The figure also shows that the relationship can be very different when limiting the observation to the Cmin-Cmax range, which is clinically-meaningful. Thus, as shown in the inset, ampicillin activity is poorly influenced by concentration within this range (justifying its categorization as concentration-independent antibiotic). In contrast, gentamicin activity is markedly influenced by concentration within the same range (justifying its categorization as concentration-dependent antibiotic).

I.2.c. Intracellular pharmacodynamics: what do we know ?

When switching to intracellular infections, the problem gains in complexity. We indeed need not only to consider the influence of drug concentration or time of exposure at the site of infection and the chemotherapeutic response, but also to examine a series of other parameters that will specifically modulate the response in the intracellular environment. These are presented in a pictorial fashion in figure 5.

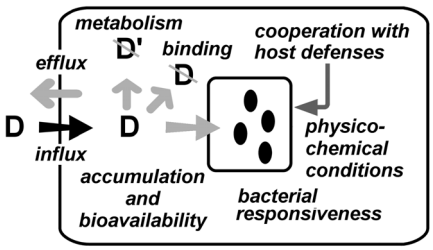


Figure 5: **Factors affecting the intracellular activity of antibiotics**. The balance between influx and efflux, metabolism and binding will determine the intracellular concentration of free active drug. The latter must, however still be able to reach its target (the box is only intended to show that such access may be prevented but does not imply that all bacteria are in membrane-bounded structures).

Activity will then be influenced by the state of bacterial responsiveness, the physico-chemical conditions prevailing at the site of infection, and the degree of cooperation (or hindrance) with the host defenses. As a result, the final outcome may bear only a very remote correlation with the actual extracellular drug concentration and even with the degree of cellular concentration. This, however, does not mean that cell penetration and cell concentration is irrelevant since an absence of penetration or an insufficient local concentration can never be associated with activity.

(Carryn *et al.*, 2003a)

A systematic overview of these interactions is still difficult to establish, since much research is still currently ongoing and because the underlying mechanisms are only partly deciphered.

We have nevertheless classified them according to the actor involved, using as illustrative examples data from our laboratory, as follows:

(a) the intracellular expression of activity of the antibiotic. The physiological conditions prevailing in the different intracellular compartments can have contrasting effects on antibiotic activity. In particular, the acidic pH of lysosomes can either increase (as observed for β-lactams) or decrease (as observed for aminoglycosides or macrolides) antibiotic activity (Barcia-Macay *et al.*, 2005).

(b) the bacterial responsiveness to the antibiotic, which can be modified in the intracellular environment. For example, we have recently shown that β-lactams are more active against intracellular than against extracellular *Listeria monocytogenes* (Carryn *et al.*, 2003b), potentially in relation with the changes in bacterial metabolism observed intracellularly (see [Portnoy *et al.*, 2002] for discussion). In contrast, *Staphylococcus aureus* becomes less sensitive to several antibiotics when it multiplies intracellularly (Seral *et al.*, 2003b), probably partly due to its slower rate of multiplication.

(c) the cooperation with host defenses, and with cytokines in particular. By stimulating cell defense mechanisms, cytokines can reinforce antibiotic activity. This is the case for example with γ-interferon, which increases fluoroquinolone activity against *L. monocytogenes* by stimulating macrophage production of oxidant species (Ouadrhiri *et al.*, 1999). But the same effect can also be deleterious in other cases, as illustrated by the inhibitory effect of γ-interferon on the activity of β-lactams against intracellular *L. monocytogenes*.

These examples underline why delineating the intracellular PK/PD properties of antibiotics remains an important goal in chemotherapeutic research. To put it in a nutshell, one is still lacking today sound, systematic approaches in this field of investigation. This thesis aims at bringing significant pieces of information, for helping to build innovative concepts.

I.3. Accumulation and efflux of antibiotics in eukaryotic cells: an overview

I.3.a. Getting in …: mechanisms and pharmacological consequences

I.3.a.1. How can antibiotics enter eukaryotic cells ?

To get access to the body or to the cellular medium, foreign molecules like drugs have to cross biological membranes, the role of which being precisely to protect the cells from the invasion by non-physiological molecules. Drugs, therefore, most often use non-specific routes of entry, like diffusion or endocytosis, depending on their physico-chemical properties, but sometimes take advantage of the presence of transporters that can recognize them because they share some structural similarities with endogenous molecules or nutriments (figure 6).

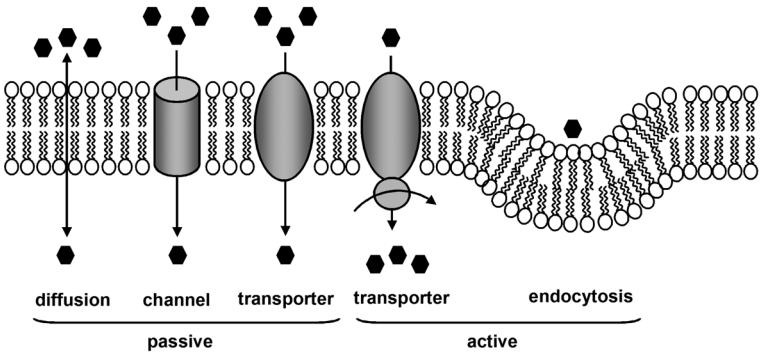


Figure 6: general mechanisms by which drugs can cross biological membranes. Passive mechanisms occur following a concentration gradient and do not need energy, while active mechanisms require energy supply.

a) Diffusion

This is the common way of crossing the membrane for molecules with a sufficiently small size (usually, molecular weight lower than 700) and lipid solubility (for review on these general concepts, see Rang *et al.*, 2003). The permeability coefficient characterizing the capacity of a molecule to freely diffuse through a lipid bilayer depends indeed primarily on the solubility in the membrane (estimated by the partition coefficient between a membrane phase and an aqueous compartment), and to a lower extent on the diffusivity, which measures the mobility of the molecules in the

membrane (expressed as a diffusion coefficient). Among the factors that drastically affect membrane permeation, the ionization status of the drug appears of prime importance: charged species have such lower lipid solubility that they are usually virtually unable to cross membranes in the absence of specific transport mechanism. The actual diffusion will thus vary according to the environmental pH (with weak bases diffusing faster at basic pH than at acidic pH and weak acids having the opposite behavior). As a result, weak bases tend to accumulate in membrane-bounded acidic compartments, whereas weak acids are excluded from these sites (see [Wilkinson, 2001] for general concepts and [de Duve *et al.*, 1974] for application to subcellular compartments).

Among antibiotics, β-lactams are thought to be able to cross the membrane by passive diffusion. They therefore gain access to the cellular medium, and their concentration equilibrates on the two sides of the membrane, resulting in an accumulation factor close to 1 (Renard *et al.*, 1987). Being weak acids, they however are thought to be largely excluded from lysosomes and related acidic vacuoles. Quinolones also probably enter most cells by simple diffusion, but, at equilibrium, are more concentrated inside that outside, for still unclear reasons (Carlier *et al.*, 1990; Michot *et al.*, 2005). Macrolides were reported as the antibiotics endowed with the highest capacity of accumulation in eukaryotic cells (this was actually true until we demonstrated that oritavancin, a new glycopeptide, accumulated much more; see the experimental studies presented in chapter II and [Van Bambeke *et al.*, 2004]). Cell-associated macrolides are largely trapped under their positively-charged, less diffusible form in lysosomes (Carlier *et al.*, 1987; see III.2.a. for further details).

b) Endocytosis

This second non-specific mechanism drives poorly diffusible molecules (too voluminous or too polar) to the lysosomal compartment. Adsorption at the cell surface, or specific interaction with surface receptors, can greatly accelerate the rate and efficacy of the uptake process (see [Pastan and Willingham, 1985] for review).

Aminoglycosides are the best characterized example of antibiotics entering cells by a double process of adsorptive and receptor-mediated endocytosis in kidney and ear cells. Thus, these very polar molecules are made of polyaminated sugars bind to the negatively-charged phospholipids of the membrane and to megalin, a protein acting as receptor for polyaminated compounds which is particularly abundant in renal proximal tubules and hair cells of the inner ear (Nagai and Takano, 2004 for review).

c) Inward transport

Inward transport of drugs is observed for molecules that present a sufficient similarity with endogenous substrates of transporters, or for prodrugs specifically designed for being recognized by such transporters (Han and Amidon, 2000). Yet, substrate specificity of transporters is often quite broad, so that apparently structurally-unrelated molecules can be recognized by a single transporter (see for example [Groneberg *et al.*, 2004]).

Active inward transport of antibiotics will be explained in details later (III.1.e.), but we can cite as typical examples the recognition of β-lactams (peptidomimetic drugs) by transporters of small peptides (Ganapathy *et al.*, 1995) or that of quinolones (bicyclic aromatic nuclei) by transporters of purines (Bounds *et al.*, 2000).

Table 2 summarizes the current view we have on the extent and mechanism of antibiotic accumulation in eukaryotic cells.

Table 2 : Influx, accumulation levels (at equilibrium), efflux and predominant subcellular localization of the main antibiotics (grouped by pharmacochemical classes).

adapted from (Carryn *et al.*, 2003a)

pharmaco-chemical class	antibiotic	influx	efflux	mechanism of entry and accumulation	accumulation level (at equilibrum)	predominant subcellular localization
β-lactams	all	fast	variable	diffusion	< 1	cytosol
macrolides	erythromycin	fast	fast	diffusion / segregation	4-10	2/3 lysosomes 1/3 cytosol
	clartithromycin roxithromycin	fast	fast	diffusion / segregation	10-20	
	azithromycin	fast	slow to very slow	diffusion / segregation	40 - 300	
	telithromycin	fast	fast to slow	diffusion / segregation	15 - 50	
fluoroquinolones	all	fast to very fast	very fast	diffusion	4-10	cytosol
aminoglycosides	all	very slow	very slow	endocytosis	2–4 (after several days)	lysosomes
lincosaminides	clindamycin	fast	fast	?	5 to 20	unknown
	lincomycin	fast	fast	?	1-4	
tetracyclines	all (?)	fast	?	?	1-4	unknown
ansamycins (rifamycins)	rifampin	fast	?	?	2-10	unknown
	rifapentin	fast	?	?	60-80	
glycopeptides	vancomycin	slow	?	endocytosis	8 (after 24 h)	lysosomes (in kidney)
	teicoplanin		?		60	unknown
	oritavancin (this work)	slow	slow	endocytosis	150 -300 (after 24 h)	lysosomal
oxazolidinones	linezolid	fast	fast		~ 1	unknown

I.3.a.2. Consequences of antibiotic accumulation in eukaryotic cells

a) Intracellular activity

Based on the simple observation of their large accumulation in eukaryotic cells, macrolides, quinolones, tetracyclines and ansamycins have been usually considered as potentially active against intracellular bacteria (Tulkens, 1991; Nix *et al.*, 1991a; Nix *et al.*, 1991b). They have, indeed, been successfully used to treat a variety of infections caused by both obligate and facultative intracellular organisms (see e.g. recent reviews by [Mulazimoglu *et al.*, 2005; Van Bambeke *et al.*, 2005a] for macrolides and quinolones). However, and as mentioned before, we observed that their intracellular activity was always lower than anticipated on the basis of their accumulation levels (Carryn *et al.*, 2003a).

More surprisingly, β-lactams or aminoglycosides, which show only poor intracellular activity in conventional *in vitro* models, are active in the clinics, as demonstrated by the fact that β-lactams are effective in the treatment of listeriosis and that aminoglycosides have been successfully used for decades for the treatment of tuberculosis (see [Hof, 2004; Fitzgerald and Haas, 2005] for recent reviews). For β-lactams, *in vitro* models actually rarely take into account the fact that the serum levels of these antibiotics *in vivo* can be quite high, so that their cellular concentration eventually reaches sufficient levels to act on intracellular bacteria. For aminoglycosides, *in vitro* models do not consider that the cellular accumulation of these drugs, although slow, may become sufficient upon prolonged treatment.

This set of observations illustrate that accumulation *per se* is not necessarily indicative of efficacy and that determination of the intracellular activity at a fixed time point and at a given extracellular concentration does not necessarily provide sufficient information. Thus, although an antibiotic must be present intracellularly to exert its activity, we simply cannot equate accumulation and activity. Determining which parameter may need to be taken into account in this context has been one of our goals in this thesis. We have addressed this question with quinolones and the new glycopeptide oritavancin on the one hand, and macrolides, on the other hand. These drugs were chosen because of their contrasting behavior with respect to the influence of concentration on their activity as determined extracellularly.

b) Cellular toxicity

The toxicological consequences of antibiotic accumulation in eukaryotic cells have been best characterized for aminoglycosides. These drugs accumulate mainly in ear and kidney cells, which express megalin in large amounts and possess a brush

border membrane particularly rich in acidic phospholipids (Nagai and Takano, 2004). This explains why these two organs constitute the primary targets for their toxicity (Mingeot-Leclercq and Tulkens, 1999; Nakashima *et al.*, 2000), with major alterations consisting in lysosomal phospholipidosis and cell death by apoptosis (Lang and Liu, 1997; El Mouedden *et al.*, 2000). The deciphering of the mechanism of cellular uptake for aminoglycosides has led to the evaluation of different strategies aimed at reducing the amount of drug accumulated by target cells. Among them, the once-a-day scheme of administration, which replaces multiple, low peak levels by a large one over 24 hours (reducing thereby global uptake by saturating the transport) has proven particularly useful and is now applied worldwide (Barclay *et al.*, 1999; Gilbert, 2005).

The question of potential toxicity is particularly pertinent for antibiotics with high accumulation levels, as is the case for macrolides, and azithromycin in particular. Morphological and biochemical studies in models of cultured cells have indeed shown that this drug induces a large accumulation of polar lipids in the lysosomal compartment, upon incubation with concentrations that could be observed in the serum of patients receiving prolonged therapy (Van Bambeke *et al.*, 1998). We actually saw similar lesions in white blood cells of HIV-positive patients treated for long time with azithromycin for *Mycobacterium avium* and *complex* infection. In this thesis, we have examined the effects induced by oritavancin, in relationship to its exceptional level of accumulation (Van Bambeke *et al.*, 2005b).

I.3.b. Getting out …mechanisms and pharmacological consequences

I.3.b.1. How can antibiotics escape from eukaryotic cells ?

Efflux mechanisms may (or may not) be the mirror images of influx mechanisms.

Drugs accumulating by endocytosis can escape from cells by regurgitation. Since this process usually concerns only a fraction of the amount accumulated in lysosomes (Pastan and Willingham, 1985), drug efflux will usually be slow and often incomplete. This is well exemplified by aminoglycosides, for which the rate of efflux from kidneys is exceedingly slow (Fabre *et al.*, 1978).

For drugs crossing membrane by simple passive diffusion, transmembrane movement can theoretically occur at a same rate in both directions (out-in or in-out), the direction of the movement being determined by the concentration gradient only. This is, actually, rarely observed, since specific efflux mechanisms or poorly reversible binding to intracellular constituents will considerably modify the overall picture and

make efflux faster or slower than influx. In this thesis, we have concentrated on efflux mechanisms.

Efflux proteins are present in the pericellular membrane of all organisms (from prokaryotic to eukaryotic cells) and appear as a mean of basic and general protection against the invasion of the cells by diffusible, potentially toxic molecules (Van Bambeke *et al.*, 2000). These proteins have usually a broad substrate specificity, and can therefore transport unrelated molecules, based essentially on physicochemical criteria (like amphiphilicity or presence of ionisable functions). These parameters actually also determine their capacity to diffuse inside the cells, a property that is often looked for in pharmacochemical research to obtain compounds with satisfactory bioavailability and diffusibility within the body.

As reviewed in Van Bambeke *et al.*, 2000, many antibiotic classes have been shown to be transported by efflux pumps (among which β-lactams, macrolides, and quinolones have been most widely studied). The consequences of this efflux in terms of resistance, pharmacokinetics, pharmacodynamics and toxicity begin now to be fully acknowledged (Van Bambeke *et al.*, 2003a; Van Bambeke *et al.*, 2003b), and will be discussed in more details later.

I.3.b.2. Consequences of efflux from eukaryotic cells

a) Reduction in activity

As evoked before, antibiotic accumulation in eukaryotic cells is of importance for their capacity to exert intracellular activity, especially for molecules presenting a concentration-dependent pharmacodynamic profile. In this context, active efflux appears as one of the factors that may contribute to reduce antibiotic intracellular concentration and, hence, activity. The observation that inhibitors of efflux pumps increase intracellular activity in infected macrophages overexpressing multidrug transporters (Nichterlein *et al.*, 1998; Heremans *et al.*, 2005) is therefore not surprising. More interestingly, the same concept is applicable to wild-type cells, characterized by a basal level of expression of efflux proteins (Rudin *et al.*, 1992; Seral *et al.*, 2003a), indicating that intracellular activity of many antibiotics may be suboptimal due to this constitutive phenomenon. We will therefore further develop these concepts here by evaluating how efflux pumps modify antibiotic activity localized in different subcellular compartments.

b) Modulation of pharmacokinetics

Active transporters are also expressed at the surface of the epithelia such as lung, intestine, kidney, or liver. Their localization being polarized, they regulate the transfer of drugs from the blood to the elimination fluid, or, conversely, from the external medium to the blood, participating by this way in the elimination and absorption processes (Van Bambeke *et al.*, 2003b). Typical examples include the active transporters involved in the renal elimination of β-lactams (Sakurai *et al.*, 2004) or in the hepatic secretion of quinolones (Sasabe *et al.*, 2004). They are also involved in the gatekeeper role of the blood-brain barrier by limiting the access of foreign substances to the central nervous system (Kusuhara and Sugiyama, 2004).

c) New mechanisms of drug interactions

An expending field of investigations concerns the implications of active efflux in drug interactions. On the basis of the large substrate specificity of efflux pumps, the probability for observing competition for transport between drugs is high. This phenomenon has been exploited in the past for reversing resistance to anticancer agents by co-administering drugs inhibiting their efflux from resistant tumor cells (Rogan *et al.*, 1984). This approach has been abandoned nowadays, because too high concentrations of these inhibitors were needed, which caused unacceptable unwanted effects (Kerr *et al.*, 1986). This strategy remains however useful in *in vitro* models, and the medical interest continues to stimulate the search for molecules with a higher specificity of action (Thomas and Coley, 2003).

Competition can also occur in cells expressing transporters at a basal level (as is the case at the surface of the diverse epithelia in our organism). This phenomenon has been early described for β-lactams with probenecid (Barza and Weinstein, 1976), in kidney or for digoxin in intestine, when co-administered with quinidine and itraconazole (Sachs *et al.*, 1993; Su and Huang, 1996).

d) Cooperation with other mechanisms of drug elimination

Cooperation between physical elimination (efflux) and chemical elimination (metabolism) was proposed in the early 90's, when Ishikawa suggested to consider active efflux as a "Phase III" type of elimination of xenobiotics (Ishikawa, 1992). This concept is now well accepted, and efflux is considered critical not only for the secretion of metabolites in the bile, but also for the limited absorption of drugs in the intestine (Christians, 2004). One of the bases of this cooperation is that efflux tends

to affect more specifically amphiphilic compounds, and that the Phase I and Phase II of the metabolism of xenobiotics precisely result in conferring a more amphiphilic character to many xenobiotics. Thus, the interplay between metabolism and efflux needs to be taken into account when establishing a profile of drug interactions (see [Niemi *et al.*, 2003] for an example with rifampicin). In the future, *in vitro* drug interaction testing, currently made with liver microsomes, will probably be replaced by more complex systems of cultured cells, in which transport and metabolism can be examined simultaneously (Benet *et al.*, 2004).

I.4. Aim of this work

This short introduction has highlighted the main links between accumulation and efflux of antibiotics in eukaryotic cells, and their capacity to exert activity against intracellular bacteria or to cause toxicity. The many uncertainties in this area were a challenge that appeared worthwhile to me.

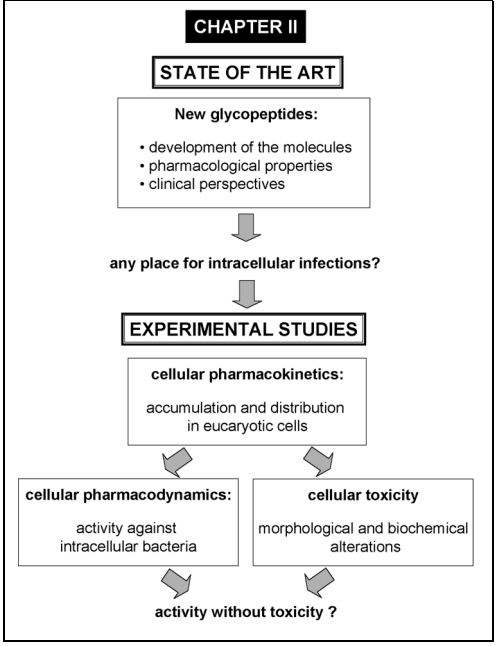
"Pharmacokinetics, pharmacodynamics and toxicodynamics of antibiotics in eukaryotic cells: modulation by efflux transporters and consequences for antibacterial activity and cellular toxicity" was indeed the perhaps ambitious but highly motivating title of the research program submitted to the *Fonds National de la Recherche Scientifique* in 2000 when applying for a position of *Chercheur Qualifié.*

In this Thesis, I have selected two main topics developed over the last five years in this general context, to illustrate relationships between cellular pharmacokinetics and cellular activity and/or toxicity, namely (i) drug accumulation and its consequences for activity and toxicity, and (ii) drug efflux and its consequences for the handling of antibiotics by cells and modulation of their intracellular activity.

The first topic (outline shown in figure 7) uses a new generation of antibiotics, namely the **semi-synthetic glycopeptides**, as drug material. These molecules were developed as substituents of vancomycin, to act not only against Methicillin-Resistant *S. aureus* (MRSA), but also against vancomycin-resistant enterococci (Nicas *et al.*, 1996). They have also shown activity against the recently isolated strains of vancomycin-intermediate (VISA) and -resistant (VRSA) *S. aureus* (Aeschlimann *et al.*, 2000; Bozdogan *et al.*, 2003). They may therefore become a weapon of last chance for treating infections by such organisms, which are resistant to most of the currently available drugs. Our interest for these new glycopeptides was triggered by an unanticipated observation. Unlike vancomycin, indeed, these antibiotics are highly bactericidal and show a markedly concentration-dependent activity against *S. aureus.* We also know that *S. aureus* is able to survive in eukaryotic cells, a property which is associated with the recurrent character of many staphylococcal infections like osteomyelitis, endocarditis, keratitis, or skin infections in humans, and mastitis in animals (see [Lowy, 2000] for review).

We have therefore undertaken to evaluate the capacity of oritavancin, the lead compound in this class (Cooper *et al.*, 1996), to accumulate in phagocytes. Having demonstrated an exceptional level of accumulation, we studied its mechanism, in relation with the subcellular localization of the drug. We then examined whether the cell-associated drug was active against intracellular bacteria. In parallel, and based on our previous experience with azithromycin, we undertook to evaluate the potential toxicological consequences of this accumulation.

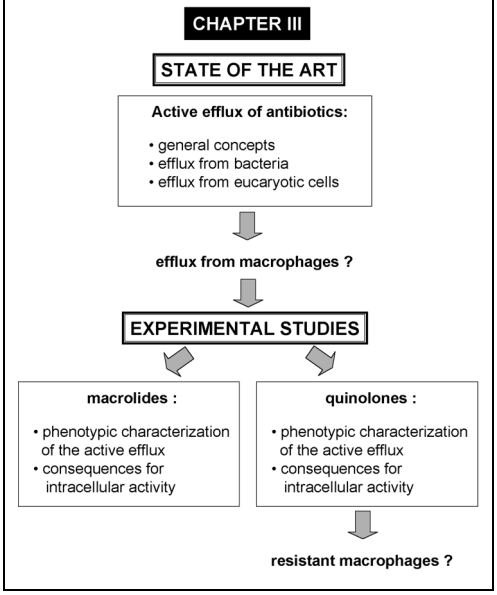
Figure 7:
outline of our studies on glycopeptides



The **second topic** is dedicated to the study of the phenomenon of **active efflux** in macrophages (outline is shown in figure 8), using macrolides and quinolones, two classes of antibiotics for which cellular accumulation had been studied previously in our laboratory (Carlier *et al.*, 1987; Carlier *et al.*, 1990).

Whereas active efflux of antibiotics had been well recognized as playing a role in resistance in bacteria, less is known on its occurrence and impact in cells involved in the defense against infections. Macrolides and quinolones are widely used in the treatment of intracellular infections. Studying their efflux from phagocytes was therefore of obvious interest. We first characterized the transporters involved in this efflux at a phenotypic level and then evaluated its consequences for intracellular activity. We are currently further developing this work by using macrophages resistant to quinolones (by overexpression of efflux transporters).

Figure 8:
outline of our studies on active efflux



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II. Accumulation of antibiotics in eucaryotic cells: consequences for activity and toxicity.

The example of new glycopeptides

Based on the following publications

Reviews:

- F. Van Bambeke, Y. Van Laethem, P. Courvalin, & P.M. Tulkens. 2004. Glycopeptide antibiotics: from conventional molecules to new derivatives. Pharmacological properties and clinical use. *Drugs*. 64:913-936.
- F. Van Bambeke. 2004. Glycopeptides in clinical development: pharmacological profile and clinical perspectives. *Cur. Op. Pharmacol.* 4:471-478.

Primary papers:

- F. Van Bambeke, S. Carryn, C. Seral, H. Chanteux, D. Tyteca, M.P. Mingeot-Leclercq & P.M. Tulkens. 2004. Cellular pharmacokinetics and pharmacodynamics of the glycopeptide antibiotic oritavancin (LY333328) in a model of J774 mouse macrophages. *Antimicrob. Agents Chemother*. 48:2853-2860.
- F. Van Bambeke, J. Saffran, M.P. Mingeot-Leclercq & P.M. Tulkens. 2005. Mixed lipid storage disorder induced in macrophages and fibroblasts by oritavancin (LY333328), a new glycopeptide antibiotic with exceptional cellular accumulation. *Antimicrob. Agents Chemother*. In press.

II.1. Review on new glycopeptides

Glycopeptide antibiotics were introduced in the clinics fifty years ago with vancomycin as the only agent for almost 30 years. Together with teicoplanin, which was launched in Europe in the mid 80's, these two molecules remain the only members in this class available for human use so far, but related derivatives have been used widely as food additive for livestock. The emergence and spreading of resistance in enterococci and staphylococci towards vancomycin has, however, stimulated active research for new glycopeptides over the last 10 years, leading to the production of a series of semi-synthetic derivatives.

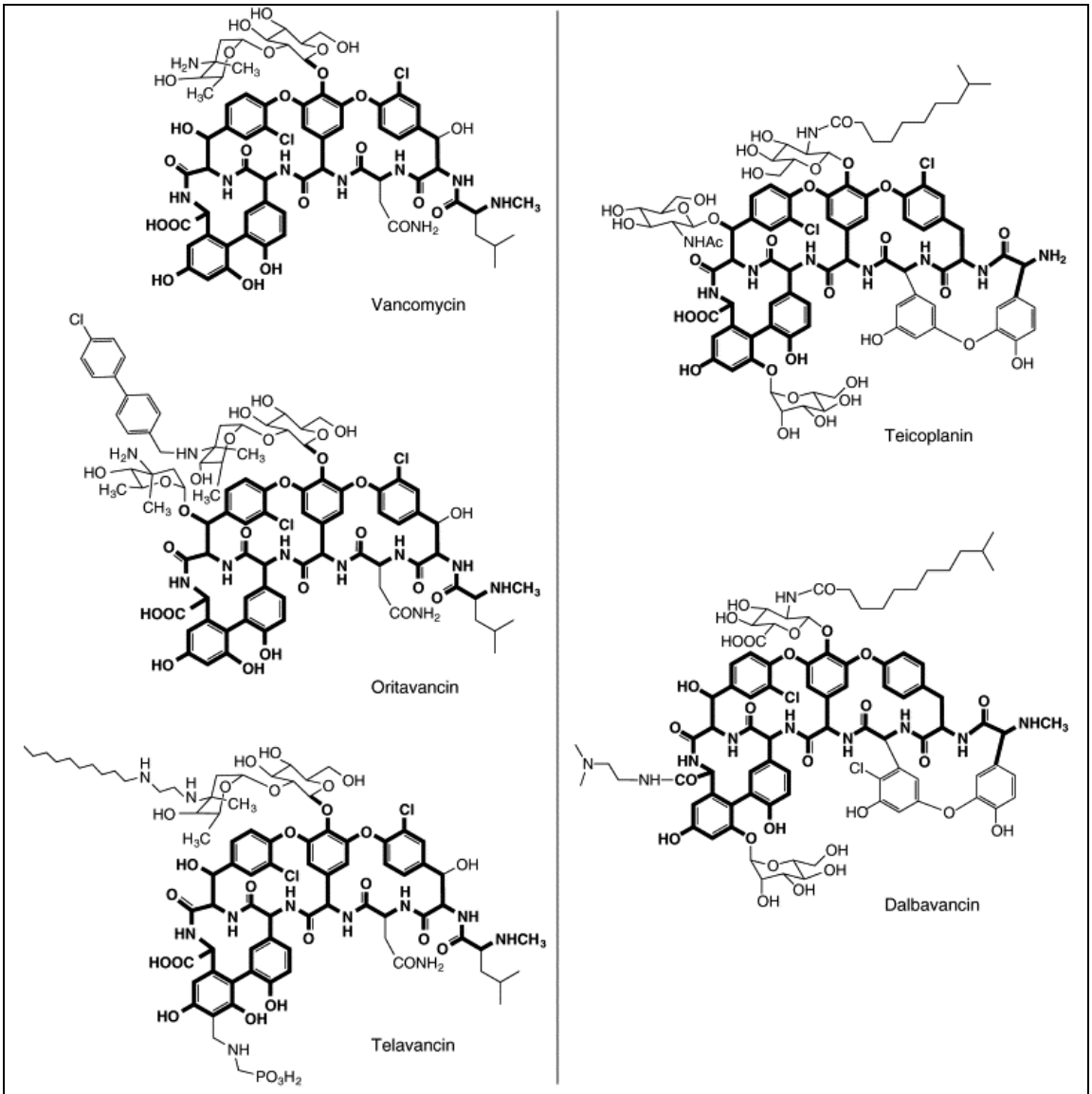


Figure 1: chemical structure of vancomycin, teicoplanin, and their semi-synthetic derivatives currently in clinical development. Left: derivatives of vancomycin; right, derivatives of teicoplanin**.** The core common to these molecules is shown in bold characters.

(Van Bambeke, 2004)

Starting from vancomycin and moving through a short description of the various compounds obtained so far, we will review the bases that have guided the selection of oritavancin, telavancin, and dalbavancin as new glycopeptides for clinical development (the chemical structure of these products is presented in figure 1). We will also discuss their advantages and potential role in our therapeutic armament.

II.1.a. Vancomycin and teicoplanin, the starting point of the story

II.1.a.1. Mechanism of action

Biochemical studies indicate that glycopeptides inhibit the late stages of peptidoglycan synthesis (Reynolds, 1989). The biosynthetic pathway of this polymer involves three steps: (1) the synthesis of cytosolic precursors made of pentapeptides fixed on a disaccharide; (2) the coupling of these precursors with a lipid carrier and the transfer of the resulting amphiphilic molecule to the outer surface of the membrane; (3) the reticulation between individual precursors by transpeptidation and transglycosylation reactions, accompanied by the release of the lipid carrier and its recycling to the inner face of the membrane.

Bacteria incubated with vancomycin accumulate cytosolic precursors (Reynolds, 1989), suggesting that glycopeptides interfere with the assembly of peptidoglycan, and in particular, with transglycosylation reactions (see next pages; figure 4). At the molecular level, the primary target of vancomycin was shown to be the D-Ala-D-Ala terminus of the precursors. Molecular modeling and experimental studies (Williams and Waltho, 1988; Reynolds, 1989; Arthur *et al.*, 1996; Loll and Axelsen, 2000) indicate that vancomycin forms a stoechiometric complex with the D-Ala- D-Ala dipeptide via the formation of five hydrogen bounds with the peptidic backbone of the glycopeptide (Figures 2 and 6). The formation of this complex prevents the transpeptidation reactions by steric hindrance.

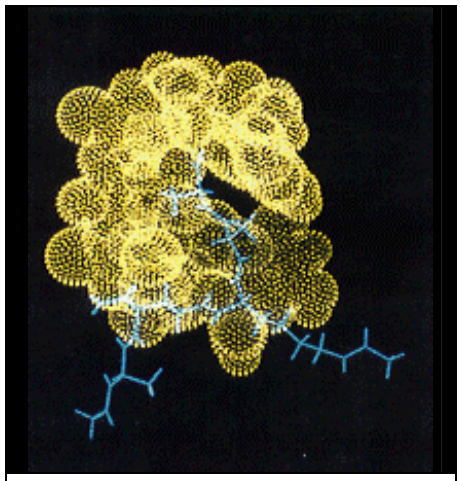


Figure 2: space-filling model showing the bulky glycopeptide molecule bound to the D-Ala-D-Ala ending precursor. Formation of this complex is thought to inhibit transglycosylases by steric hindrance, and Penicillin Binding proteins (PBPs) by directly preventing interactions with the carboxy-terminal D-Ala residues.

(Arthur *et al.*, 1996)

The tightness of the interaction between the glycopeptide and the D-Ala-D-Ala motif can be enhanced by two mechanisms (figure 3):

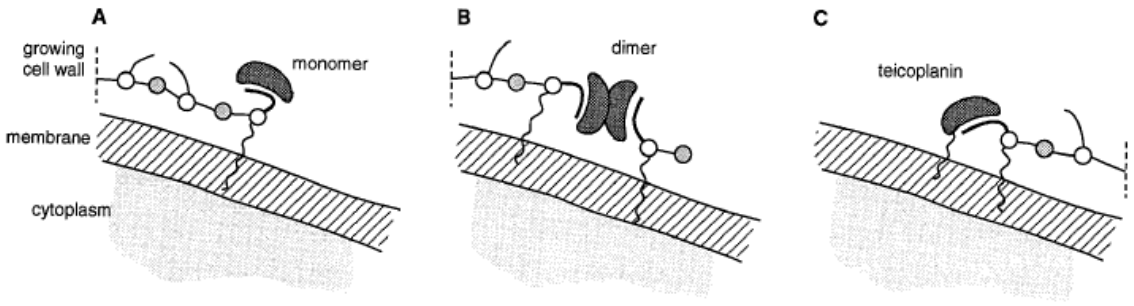


Figure 3: Intramolecular binding of dimeric antibiotics and teicoplanin to cell wall subunits, illustrating enhancement through the chelate effect. The growing cell wall is attached to a C_{55} lipid, which anchors it to the cytoplasmic membrane while elongation by transglycosylation occurs. Cell wall repeat units are composed of N-acetylmuramate-N-acetylglucosamide disaccharide which carries a pentapeptide terminating in -peptidyl-D-Ala-D-Ala (bold line). (A) the binding of two monomeric glycopeptides requires two biomolecular steps. (B) in contrast, the binding of a glycopeptide dimer is enhanced because the second binding event is essentially intramolecular. (C) teicoplanin, anchored to the membrane by the undecanoyl substituent, binds to the cell wall in an analogous intramolecular manner.

(Beauregard *et al.*, 1995)

1) the formation of homodimers between glycopeptide molecules, which has been demonstrated for glycopeptide of the vancomycin group, such as vancomycin itself (Schafer *et al.*, 1996), eremomycin (Groves *et al.*, 1994), or chloroeremomycin (Shiozawa *et al.*, 2002). Organization of molecules in dimers confers a structural rigidity that locks the binding pocket into the correct conformation and may allow for a cooperative binding to the ligand (Mackay *et al.*, 1994a; Mackay *et al.*, 1994b; Beauregard *et al.*, 1995; Schafer *et al.*, 1996; Williams *et al.*, 1998). These dimers are formed by hydrogen bonds between the glycopeptide aglycone, which are maintained in the rigid conformational state favorable for ligand binding by the sugar residues on the glycopeptide molecule (Kaplan *et al.*, 2001).
2) The anchoring of the antibiotic in the membrane, as proposed for glycopeptides carrying a lipophilic tail, such as teicoplanin derivatives (Beauregard *et al.*, 1995). Lipid moieties actually are a common determinant in several antibiotics having as target membrane-associated steps of peptidoglycan synthesis, such as ramoplanin, moenomycin, and tunicamycin. This suggests that such moieties help to maintain the drug close to their target. The importance of hydrophobic determinants has been further emphasized by the observation that vancomycin derivatives may inhibit transglycosylation without binding to D-Ala-D-Ala (Ge *et al.*, 1999; Printsevskaya *et al.*, 2003).

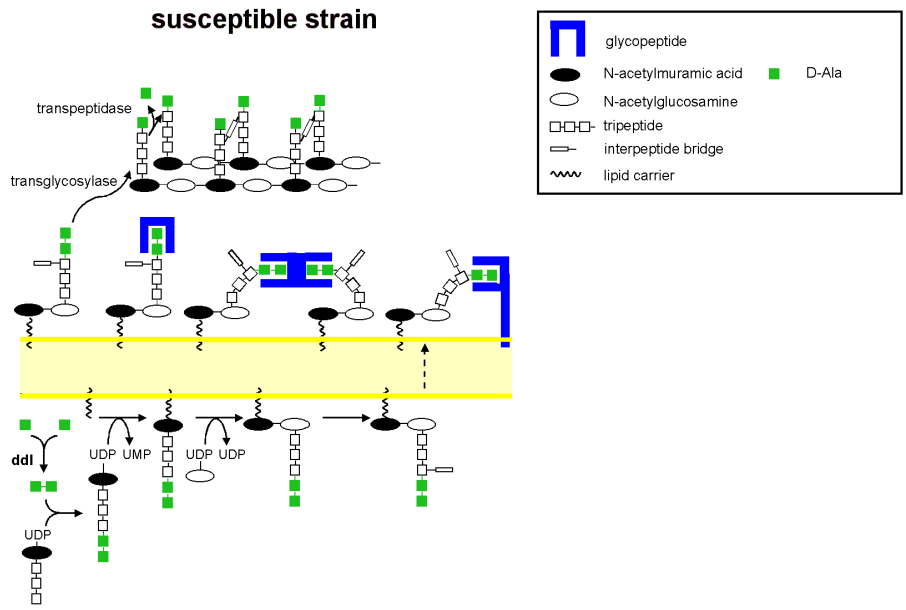


Figure 4: Peptidoglycan synthesis in glycopeptide-susceptible bacteria. Peptidoglycan precursors are ending by D-Ala-D-Ala termini synthesized by D-Ala:D-Ala ligases (ddl). Glycopeptides interact with D-Ala-D-Ala termini of pentapeptides (through five hydrogen bonds) at the cell surface, preventing transglycosylation reactions and therefore peptidoglycan reticulation. The figure also illustrates how dimerization of glycopeptide molecules or anchoring in the membrane by a hydrophobic tail may increase the tightness of this interaction. Hydrophobic derivatives may also inhibit the transglycosylation step without binding to peptidoglycan precursors.

(Van Bambeke *et al.*, 2004b)

II.1.a.2. Resistance: mechanisms and importance

The American National Committee for Clinical Laboratory Standards (NCCLS) has set up breakpoints for susceptibility and resistance of ≤ 4 and ≥ 16 µg/ml for vancomycin and ≤ 8 and ≥ 32 µg/ml for teicoplanin (see table 1 for the main characteristics of the resistant strains). Over the years, two organisms of medical interest, namely the enterococci and *S. aureus*, have developed a different, ingenuous resistance mechanism.

a) Resistance in enterococci

Resistance of enterococci to vancomycin was first reported in 1988 (Leclercq *et al.*, 1988; Uttley *et al.*, 1988). The situation has evolved quite rapidly in the USA, where resistance in blood isolates reached 13 % in 1995 and 26 % in 2000 (figure 5a; Bonten *et al.*, 2001). Colonization by resistant strains is common in critically ill and immuno-suppressed patients as well as in patients hospitalized in wards with high antibiotic use (figure 5b). It is probably the main route for spread of these strains, but currently, outpatients are also often colonized and may, therefore, also represent another important source of contamination (Kenner *et al.*, 2003). This alarming

observation has stimulated drastic measures in terms of hygiene, restriction in antibiotic use, and surveillance to try to control or even reverse this situation (Bonten *et al.*, 2001).

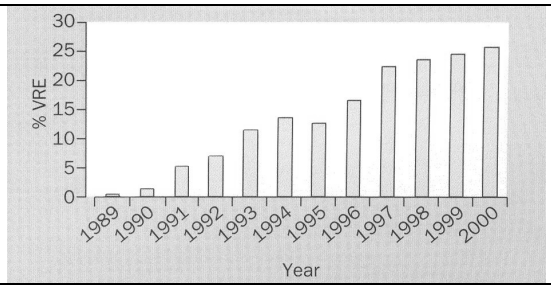


Figure 5a: Prevalence of vancomycin resistance among enterococci in nosocomial infections. In intensive-care patients in the USA.

(Bonten *et al.*, 2001)

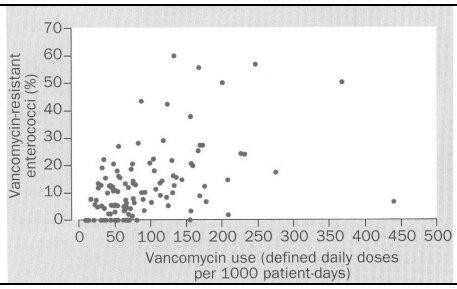


Figure 5b: Correlation of the rate of parenteral use of vancomycin and the prevalence of vancomycin-resistant entero-cocci in 126 intensive care units.

(Bonten *et al.*, 2001)

In sharp contrast, resistance of enterococci in Europe is minimal (3 %) and only observed in nosocomial infections (Schouten *et al.*, 2000); outbreaks have been reported but remain sporadic. Carriage of resistant enterococci in animals, however, is frequent in Europe and is thought to be due to the massive use of avoparcin, a glycopeptide antibiotic used as growth promoter in animal feeding (van den Bogaard *et al.*, 1997; van den Braak N. *et al.*, 1998) but which has the same mode of action as vancomycin and therefore shares cross-resistance. The ban of avoparcin use instituted by European Union countries has caused in most cases a fall in the prevalence of resistance in both animals and humans (Klare *et al.*, 1999; van den Bogaard and Stobberingh, 2000; Bonten *et al.*, 2001; Aarestrup *et al.*, 2001).

The mechanism of resistance in enterococci relies on synthesis of peptidoglycan by an alternative pathway, which produces precursors ending in D-Ala-D-Lac or D-Ala-D-Ser instead of D-Ala-D-Ala, and eliminates concomitantly precursors ending in D-Ala-D-Ala (Arthur *et al.*, 1996; Gholizadeh and Courvalin, 2000; Reynolds and Courvalin, 2005 for reviews). The replacement of D-Ala by D-Lac suppresses one of the five hydrogen bonds between the glycopeptides and their target, which contributes to a 1,000-fold decrease in the binding affinity (figure 6; Bugg *et al.*, 1991; Arthur *et al.*, 1996). The substitution in D-Ser causes a conformational change, which also reduces vancomycin affinity, although not as markedly as with D-Lac (Reynolds and Courvalin, 2005).

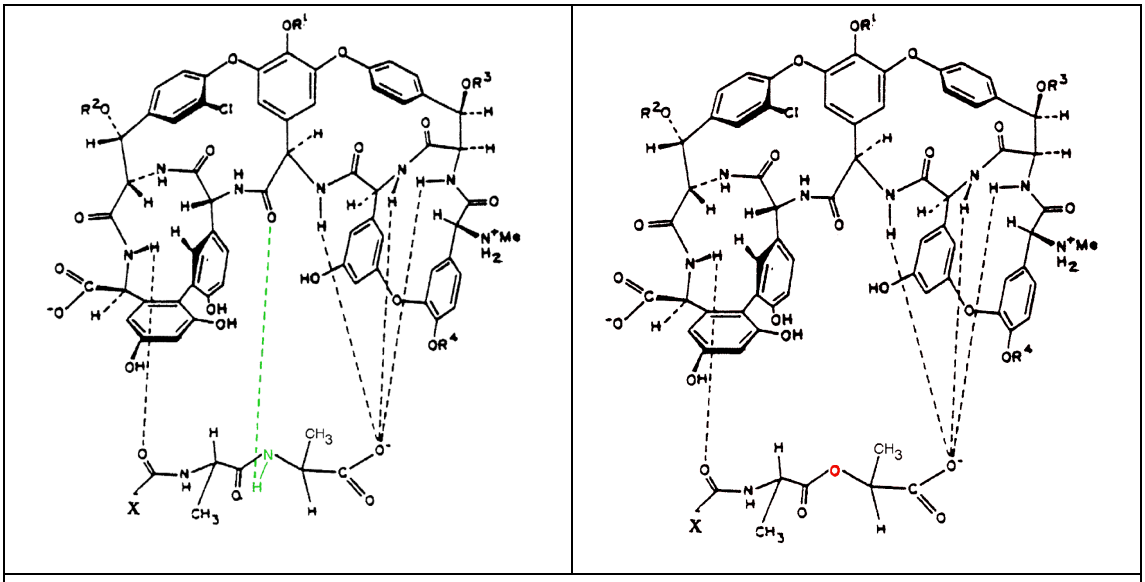


Figure 6: binding of a glycopeptide to a D-Ala-D-Ala (left) or D-Ala-D-Lac (right) extremity of peptidoglycan precursor. In the presence of D-Ala-D-Ala ending precursors, this binding involves the formation of 5 hydrogen interactions (dashed lines). In the presence of D-Ala-D-Lac ending precursors, one critical hydrogen bound is not formed due to the replacement of the peptidic bond by and ester bond.

adapted from (Arthur *et al.*, 1996)

To be phenotypically detectable and significant, resistance requires the coordinated action of several enzymes, which constitute the "letters" of what P. Courvalin called the "Van alphabet" (figure 7).

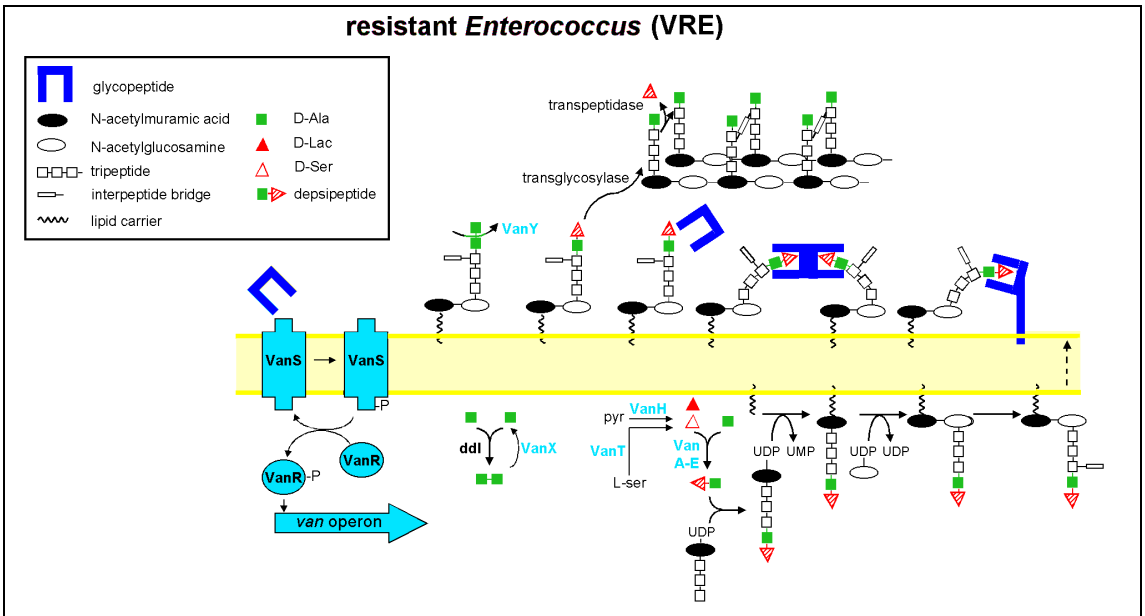


Figure 7: Peptidoglycan synthesis in glycopeptide – resistant enterococci. The presence of glycopeptides activates the signal transducing system VanS (sensor) – VanR (regulator), which allows transcription of the resistance genes. These include enzymes involved in the hydrolysis of D-Ala-D-Ala termini (VanX and VanY) or in the synthesis of precursors ending by either D-Ala-D-Lac (VanH and VanA, VanB or Van D) or D-Ala-D-Ser (VanT and VanC, VanE, or VanG), characterized by a lower affinity for glycopeptides.

(Van Bambeke *et al.*, 2004b)

Thus, the bacteria need to synthesize D-Lac (VanH) or D-Ser (VanT), and D-Ala-D-Lac or D-Ala-D-Ser (VanA, B, D or C, E, G), and to degrade D-Ala-D-Ala (VanX) or to remove D-Ala from growing precursors (VanY) or both (VanXY). Moreover, a two component regulatory system (VanS-VanR) is responsible for the inducible character of resistance by either vancomycin (VanB, C, E, G phenotype) or vancomycin and teicoplanin (VanA phenotype) (Arthur *et al.*, 1996; Perichon *et al.*, 1997; Fines *et al.*, 1999; Gholizadeh and Courvalin, 2000). In the VanD genotype, production of precursors ending in D-Ala-D-Lac becomes constitutive due to the dysfunctioning of the regulatory system as a consequence of mutations in the *vanS* or *vanR* genes (Depardieu *et al.*, 2004). The resistance proteins are encoded by genes physically grouped in operons (figure 8) that are located on plasmids or in the chromosome, and can been easily transferred, even between different species (Arthur *et al.*, 1993; Quintiliani, Jr. and Courvalin, 1994; Arthur *et al.*, 1996).

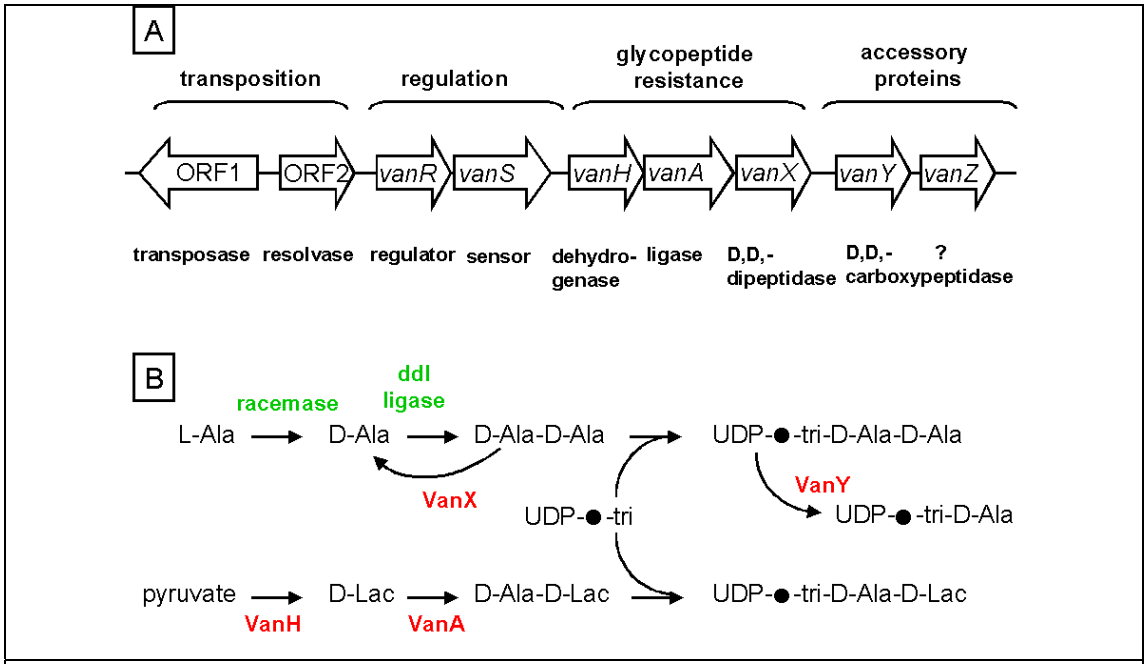


Figure 8: VanA type glycopeptide resistance. (A) Tn1546. The open arrows indicate genes and sense of transcription. (B) Schematic representation of the synthesis of peptidoglycan precursors in a VanA-type strain after induction with a glycopeptide. ddl=D-Ala:D-Ala ligase; • = N-acetyl muramic acid.

reproduced from (Gholizadeh and Courvalin, 2000)

Six phenotypes of resistance have been described (table 1), which differ by the genetic support, the regulation of expression and the level of resistance conferred.

An intriguing phenotype of glycopeptide dependence has also been reported, in which synthesis of the D-Ala-D-Ala-ending peptidoglycan precursors is impaired through a mutation in the host ligase. Induction of production of the resistance proteins by

glycopeptides restores peptidoglycan synthesis by allowing production of precursors ending in D-Ala-D-Lac (figure 9; Baptista *et al.*, 1997; Van Bambeke *et al.*, 1999).

Table 1: **Main characteristics of glycopeptide resistant bacteria**

(Van Bambeke *et al.*, 2004b)

Bacterial species	Type	Expression	Mechanism of resistance	MIC range of glycopeptides (mg/l)	
				vancomycin	teicoplanin
E. faecium	VanA	inducible	Modified target	64 - 1000	16 - 512
E. faecalis		(by vancomycin and teicoplanin)	(D-Ala-D-Lac)		
E. avium					
E. durans					
E. hirae					
E. mundii					
E. raffinosus					
E. gallinarum					
E. casseliflavus					
E. faecium	VanB	inducible	Modified target	4 - 1000	0.5 - 1
E. faecalis		(by vancomycin)	(D-Ala-D-Lac)		
S. bovis					
E. gallinarum	VanC	constitutive / inducible	Modified target	2 - 32	0.5 - 1
E. casseliflavus			(D-Ala-D-Ser)		
E. flavescens					
E. faecium	VanD	constitutive	Modified target	64 - 128	4 - 64
E. faecalis			(D-Ala-D-Lac)		
E. faecalis	VanE	inducible	Modified target	16	0.5
		(by vancomycin)	(D-Ala-D-Ser)		
E. faecalis	VanG	inducible	Modified target	16	0.5
		(by vancomycin)	(D-Ala-D-Ser)		
S.aureus	VISA		thickened cell wall with increased proportion of D-Ala-D-Ala termini	8	8-32
	GRSA		Stop codon in the gene of PBP4		
	VanA	inducible	Modified target		
		(by vancomycin and teicoplanin)	(D-Ala-D-Lac)		

VISA: Vancomycin Intermediate *S. aureus*; GRSA: Glycopeptide Resistant *S. aureus*

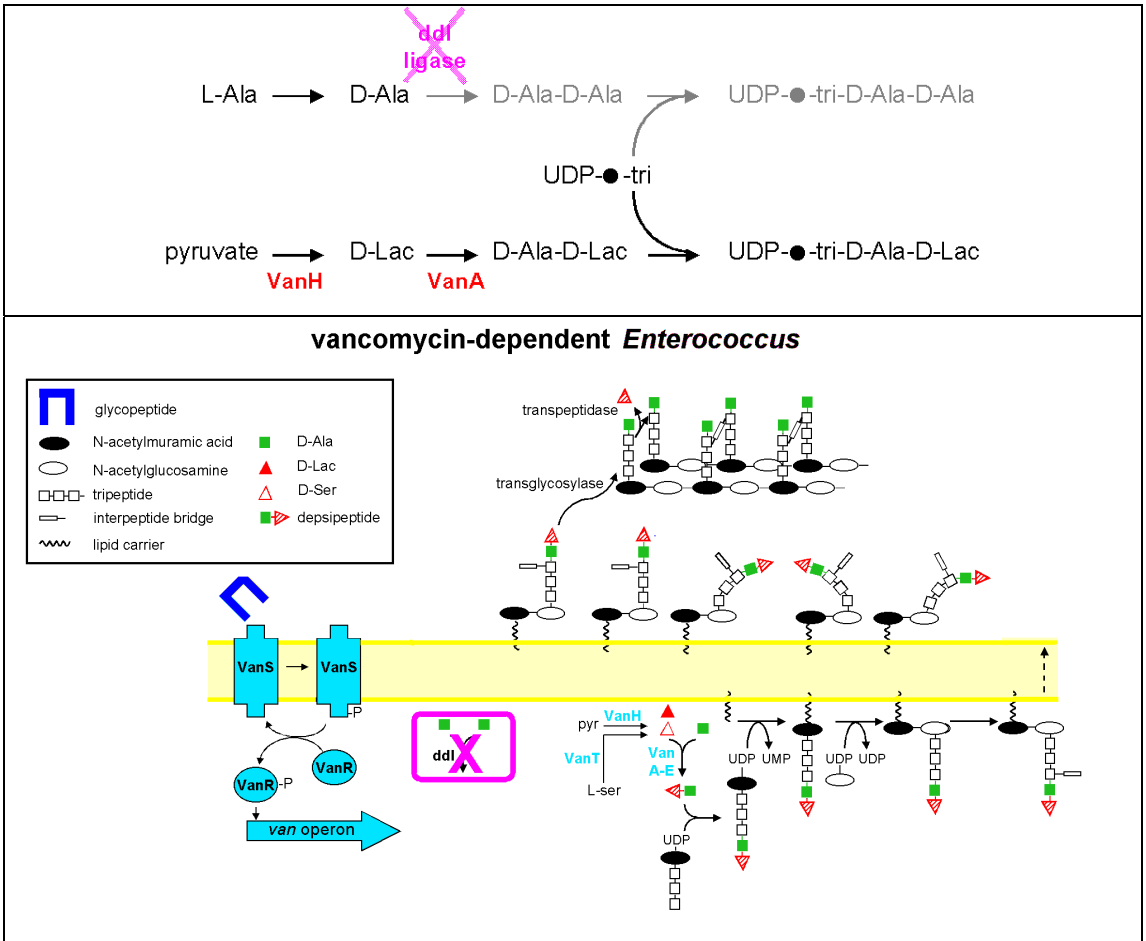


Figure 9: **Peptidoglycan synthesis in glycopeptide – dependent enterococci.** The ddl enzyme responsible for the synthesis of D-Ala-D-Ala is inactive, so that these strains rely on the induction by glycopeptides of the production of the enzymes of resistance allowing for the synthesis of precursors ending in D-Ala-D-Lac (the pathway leading to the formation of precursors ending in D-Ala-D-Ala [shown in grey in the upper panel] is no more functional).

b) Resistance in staphylococci

Resistance in *S. aureus* has first emerged under the form of strains with elevated MIC towards vancomycin [or both vancomycin and teicoplanin] which have been named Vancomycin-Intermediate *S. aureus* [VISA] or Glycopeptide-intermediate *S. aureus* [GISA]). Originally described in Japan in 1996 in methicillin-resistant *S. aureus* [MRSA] (Hiramatsu *et al.*, 1997), VISA and GISA strains have now been isolated in numerous countries, in particular from patients having received prolonged vancomycin therapy (Hiramatsu, 2001; Hamilton-Miller, 2002). These bacteria are characterized by a thickened cell wall (figure 10), production of an abundant extra cellular material of still ill characterized nature, and an impaired ability to divide (Hamilton-Miller, 1999).

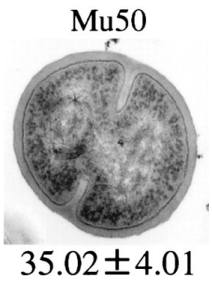


Figure 10: **Appearance in electron microscopy of a VISA strain.** The thickness of the cell wall increases to 53.3 ± 3 nm in the presence of vancomycin (mean thickness in a wild strain: 20-25 nm).

(Cui *et al.*, 2000; Cui *et al.*, 2003)

These phenotypic changes can be explained by the production of an altered peptidoglycan with an increased proportion of free D-Ala-D-Ala termini (less reticulation), which can trap vancomycin molecules and prevent their access to the target at the cytosolic membrane (figure 11). The thickened cell wall of a VISA strain may contain up to 2-4 times more D-Ala-D-Ala residues than that of a susceptible strain, and is able to bind up to 3-6 times more vancomycin molecules before peptidoglycan synthesis becomes impaired (Hiramatsu, 2001). This mechanism implies also a reduced cross-linkage of peptidoglycan. The latter has been suggested to result from a decreased activity of PBPs (Sieradzki and Tomasz, 1999), or from an alteration of murein precursors (Cui *et al.*, 2000). Yet, multiple, additive, but not clearly identified mutations, are probably necessary to obtain resistance. VISA and GISA also need to import a larger amount of precursors than normal strains, which compromises their fitness in antibiotic-free environment. This explains why VISA and GISA tend to loose their resistance when relieved from vancomycin pressure, giving rise to the so-called hetero-VISA phenotype (Hiramatsu *et al.*, 2002).

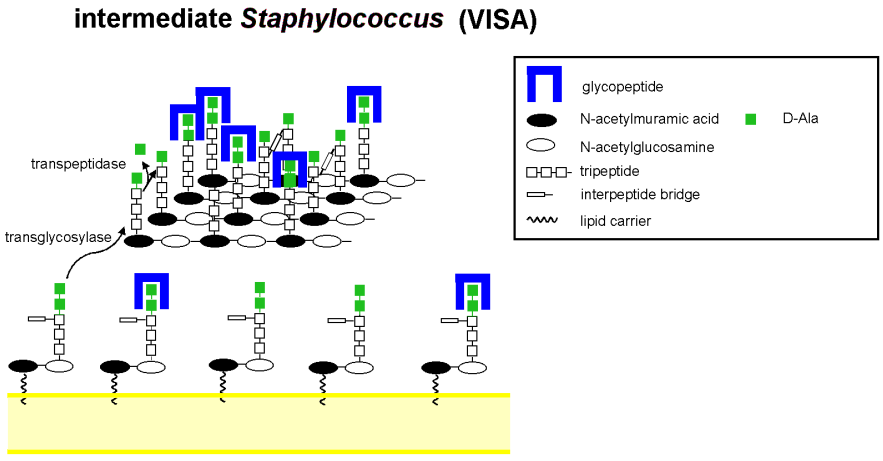


Figure 11: Peptidoglycan synthesis in glycopeptide-intermediate staphylococci (so called VISA or GISA strains). More murein monomers are supplied and incorporated in the peptidoglycan, in which more free D-Ala-D-Ala residues remain present because of a decreased level of reticulation. More glycopeptide molecules are therefore trapped in the multiple layers of the ticker cell wall but less reach their target at the cell surface .

(Van Bambeke *et al.*, 2004b)

Moreover, a glycopeptide-tolerant phenotype has been observed in clinical isolates of MRSA, in which MICs of glycopeptides are not affected but MBCs are considerably increased (May *et al.*, 1998). In a still more frightening fashion, three methicillin-resistant *S. aureus* with high levels of resistance to vancomycin and teicoplanin have now been reported in different hospital institutions in the USA (Centers for Disease Control and Prevention, 2002a; Centers for Disease Control and Prevention, 2002b; Centers for Disease Control and Prevention, 2004). These strains harbor the *vanA* gene cluster, indicating that they had acquired the corresponding set of genes from enterococci (Weigel *et al.*, 2003; Tenover *et al.*, 2004). Disturbingly, one of these strains was isolated from a patient who was not exposed to vancomycin, but to other antibiotics, which may have provided sufficient selective pressure to promote colonization by VRE and MRSA, and horizontal gene transfer (Whitener *et al.*, 2004). The level of resistance acquired also markedly differs from one strain to another, probably in relation to the stability of the genetic element carrying the resistance genes (Perichon and Courvalin, 2004). Should such strains spread in hospitals, treatment options would rapidly become very limited (Johnson and Woodford, 2002).

II.1.a.3. Pharmacological properties

The spectrum of activity of glycopeptides covers essentially the Gram-positive organisms and a few anaerobes, and their activity on Gram-negative organisms is most often marginal (table 2). Vancomycin and teicoplanin have a similar intrinsic activity, except against streptococci and coagulase negative staphylococci, which are more susceptible to teicoplanin.

The key pharmacokinetic properties of glycopeptides are summarized in table 3. The most striking difference between vancomycin and teicoplanin is their capacity to bind serum proteins, which is much higher for teicoplanin than for vancomycin. This explains the prolonged half-life of teicoplanin in the organism. High protein binding, however, needs also to be taken into account for activity predictions (see table 4), since only the free serum fraction is directly active. Teicoplanin, perhaps because of its lipophilic character, is characterized by a higher volume of distribution than vancomycin, allowing to reach therapeutic concentrations in fat, muscles (including pericardium and myocardium) and, to some extent, in bone and cartilage, but not in the CNS (Wilson, 2000).

In vitro pharmacodynamic models show that vancomycin exhibits a time-dependent bactericidal activity against most Gram-positive organisms. Vancomycin

is, however, essentially bacteriostatic against enterococci (Lowdin *et al.*, 1998), but may become bactericidal when combined with an aminoglycoside (Fantin and Carbon, 1990; Houlihan *et al.*, 2000). Vancomycin activity is adversely affected by a large inoculum but not by acidic pH. The drug also demonstrates faster killing rates against actively growing organisms, which can be easily understood on the basis of its mechanism of action (Lamp *et al.*, 1992). Finally, glycopeptides produce persistent effects (post-antibiotic effect lasting 1 to 6 h [Drabu and Blakemore, 1991; Lowdin *et al.*, 1998]). While the dosage recommendations for glycopeptides insisted on low doses because of fear of toxicity, the recent developments in the pharmacodynamics have allowed coming with more rational bases. The first models suggested that the free serum concentration of glycopeptides needs to remain above the MIC of the infecting organism for a prolonged period and therefore concluded that activity was primarily driven by the so-called “time above MIC” parameter (Peetermans *et al.*, 1990; Chambers and Kennedy, 1990; see also general introduction I.2.). More recent and thorough studies have indicated, however, that the parameter which best predicts efficacy is the AUC/MIC ratio (Aeschlimann *et al.*, 2000), as usually found for most antibiotics exhibiting time-dependent killing and significant post-antibiotic effect (Craig, 2001). However, in non-neutropenic animals, the ratio of the free serum peak concentration to the MIC (Cmax-free/MIC) also plays a determinant role in efficacy (Knudsen *et al.*, 2000). This Cmax-free/MIC ratio needs to reach at least 5-6 for vancomycin and 2-3 for teicoplanin (note that the determination of the peak and trough levels for these drugs, which are determined in routine in hospital laboratories, can be used for calculation of both Cmax/MIC and AUC/MIC ratios). As a consequence, glycopeptides are probably best given by discrete administrations with a total daily dose sufficiently large to match with the MIC of the offending organism. A recent study comparing vancomycin 30 mg/kg once daily versus two administrations for the treatment of patients with bacteriemia or arthritis did not show significant difference between the two groups in terms of clinical response (> 92 % in both cases [Cohen *et al.*, 2002]; no endocarditis case, however, was included in this study). On a pharmacodynamic basis, there is therefore little reason to administer vancomycin by continuous infusion (James *et al.*, 1996; Klepser *et al.*, 1998) even though other parameters such as more sustained levels in given tissues, or cost containment and ease of administration have been advocated (Wysocki *et al.*, 2001; Byl *et al.*, 2003); note that the stability and compatibility of vancomycin with other drugs in relation to its use by continuous infusion need to be critically assessed (Basma *et al.*, 2004).

Table 2: In vitro activity (MIC $_{90}$) of glycopeptides against selected target Gram-positive bacteria

(Van Bambeke, 2004*)

Bacterial species	Resistance status	MIC $_{90}$ (mg/L)				
		vancomycin	oritavancin	telavancin	teicoplanin	dalbavancin
S. aureus	MSSA	1 to 2 [a]	1	0.5	1 to 4 [a]	0.06 to 0.125
	MRSA	1 to 4 [a]	1 to 2 [a]	0.5 to 1 [a]	2 to 8 [a]	0.06 to 0.25
	VISA	8	1 - 8	2		2
	GRSA	> 128	0.5	2		
S. epidermidis	MSSE	1 to 2 [a]	2	0.5	4 to 8 [a]	0.25
	MRSE	2 to 4 [a]	1	1	8 to 16 [a]	0.25
S. pneumoniae	PenS	0.5	0.008	0.016	0.06 to 0.125 [a]	0.03 to 0.06
	PenR	0.25[b] to 0.5 [a]	< 0.002*		0.03 [b]	0.03
Enterococcus spp	VanS	1		0.5	0.5 to 1 [a]	0.12
	VanA	> 128 to > 256 [a]	1 to 4 [a]	4 to 8 [a]	> 32 to > 128 [a]	> 128
	VanB	128	0.125		2	1

* Constructed based on data from (Candiani *et al.*, 1999; Judice and Pace, 2003; Pace *et al.*, 2003; King *et al.*, 2004; Streit *et al.*, 2004); see also Van Bambeke *et al.*, 2004 for more details on the activity of vancomycin, oritavancin, teicoplanin, dalbavancin)

[a] range based on MIC_{90} values reported in different studies

[b] MIC $_{50}$ (no MIC_{90} data provided in this study)

Table 3: pharmacokinetic parameters for glycopeptides at doses pertinent of their use in humans (or the foreseen doses for molecules in development)

(Van Bambeke, 2004*)

Parameter (units)	Glycopeptide and dosage				
	Vancomycin (15 mg/kg)	**Oritavancin (3 mg/kg)**	**Telavancin (7.5 mg/kg)**	**Teicoplanin (6 mg/kg)**	**Dalbavancin (15 mg/kg)**
Cmax (mg/L)	20-50	31	89	43	312
Vd (L/kg)	0.3		0.1	0.9-1.6	0.11
Prot. binding (%)	10-55	90	90-93	90	98
Terminal half-life (h)	4-8	360	7	83-168	149
AUC (mg.h/L)	260	152	600	550	27103

* based on references (Wilson, 2000; Feketi, 2000; Barriere *et al.*, 2003; Leighton *et al.*, 2004; Van Bambeke *et al.*, 2004)

Table 4: pharmacodynamic breakpoints[a] for glycopeptides at doses pertinent of their use in humans (or the foreseen doses for molecules in development)

(Van Bambeke, 2004)

Parameter (units)	Glycopeptide and dosage				
	Vancomycin (15 mg/kg)	**Oritavancin (3 mg/kg)**	**Telavancin (7.5 mg/kg)**	**Teicoplanin (6 mg/kg)**	**Dalbavancin (15 mg/kg)**
PD Breakpoint based on (free AUC)/MIC ratio [a]	2 (15 mg/kg twice-daily)	0.1 (3 mg/kg) 0.3 (10 mg/kg)	0.5	0.4 (6 mg/kg) 0.8 (12 mg/kg)	4
PD Breakpoint based on (free Cmax)/MIC ratio [a]		0.3 (3 mg/kg) 1 (10 mg/kg)	1		0.6

[a] This breakpoint corresponds to the higher MIC for which a free AUC/MIC ratio of 125 or a free Cmax/MIC of 10 (for concentration-dependent drugs) can be reached based on a conventional daily dose (note that for new glycopeptides, these values may be underestimated based on the fact that the presence of serum proteins do probably not fully impair their activity)

II.1.a.4. Clinical use: pros and cons

Originally introduced in the clinics as an agent active against β-lactamase producing *S. aureus*, vancomycin remained largely unused because of development of less toxic alternatives, namely the β-lactamase-resistant penicillins (methicillin, isoxazolyl penicillins) and cephalosporins, as well as the introduction of β-lactamase inhibitors like clavulanic acid (mainly in Europe) or sulbactam, which have become highly popular when combined with ampicillin or amoxicillin (mostly against Gram-negative bacteria, however). Yet, orally administered vancomycin became popular for treatment of *Clostridium difficile* associated diarrhea and colitis (Cheung and DiPiro, 1986). The pandemic of nosocomial methicillin-resistant *S. aureus* (MRSA) infections which started in the mid 70's, and the fact that these strains were not only resistant to all β-lactams but often also to aminoglycosides, macrolides, lincosamides, and fluoroquinolones (Livermore, 2000), heralded the come back of vancomycin for systemic use. Alternatives such as fusidic acid, rifampicin and cotrimoxazole have indeed been poorly studied and did not stand in face of the extensive data and the everyday experience available with glycopeptides (Michel and Gutmann, 1997). Moreover, easy selection of resistant mutants (especially for rifampicin and fusidic acid when used alone) or toxicity problems has further limited their use. As we have seen, however, resistance developed in enterococci almost in parallel with the increase in vancomycin use in the USA (figure 5 and Centers for Disease Control and Prevention, 1993; Kirst *et al.*, 1998; Fridkin *et al.*, 2001). This led health authorities and concerned clinicians to try to restrict glycopeptide usage in order to maintain the activity of these useful agents for situations where alternatives were not usable (see Centers for Disease Control and Prevention, 1993; Shojania *et al.*, 1998; Goeckner *et al.*, 1998; Hamilton *et al.*, 2000; Kumana *et al.*, 2001). The introduction of quinupristin/dalfopristin and linezolid in the late 90's has not really changed this situation since both drugs are expensive, have rare but potentially worrying side effects (hematological toxicity for long course linezolid treatments, thrombophlebitis, arthralgias and/or myalgias, and drug interactions for quinupristin/dalfopristin), and are already facing emergence of resistance (Rubinstein *et al.*, 1999; Olsen *et al.*, 2001; Linden, 2002; Gerson *et al.*, 2002; Eliopoulos, 2003; Nilius, 2003). In particular, linezolid-resistant strains are now increasingly reported, not only in enterococci but also in *S. aureus*, which could become a clinically significant problem in the future (Meka and Gold, 2004).

Table 5: Appropriate clinical use of glycopeptides, based on the recommendations from HICPAC for vancomycin and on pharmacodynamic considerations (Table 4 and [Gruneberg *et al.*, 1999])

(Van Bambeke *et al.*, 2004b)

1. Situations in which the use of glycopeptides is appropriate or acceptable:
treatment : - Serious infections caused by β-lactam-resistant gram-positive microorganisms. - Infections caused by gram-positive microorganisms in patients who have serious allergies to β-lactams. - Antibiotic-associated colitis failing to respond to metronidazole therapy or potentially life-threatening. **prophylaxis** : - Endocarditis following certain procedures in patients at high risk for endocarditis. - Major surgical procedures involving implantation of prosthetic materials or devices at institutions that have a high rate of infections caused by MRSA or methicillin-resistant *S. epidermidis*.
2. Situations in which the use of glycopeptides should be discouraged:
- Routine surgical prophylaxis other than in a patient who has a life-threatening allergy to β-lactams. - Empiric antimicrobial therapy for a febrile neutropenic patient, unless initial evidence indicates that the patient has an infection caused by gram-positive microorganisms and the prevalence of infections caused by MRSA in the hospital is substantial. - Treatment in response to a single blood culture positive for coagulase-negative *Staphylococcus*, if other blood cultures taken during the same time frame are negative - Continued empiric use for presumed infections in patients whose cultures are negative for β-lactam-resistant gram-positive microorganisms.
3. Appropriate dosages [a,b]:
Vancomycin: 15 mg/kg twice-a-day Teicoplanin: 6-12 mg/kg once-a-day (after 3 loading doses of 6 mg/kg every 12 h)

[a] higher IV doses are needed for treating peritonitis in patients undergoing peritoneal dialysis (Manley *et al.*, 2001; Stamatiadis *et al.*, 2003)

[b] dosage reduction required in patients with renal insufficiency (Wilson, 2000; Feketi, 2000).

The recommendations presented in table 5 were issued in 1995 by the Hospital Infection Control Practices Advisory Committee in the USA (HICPAC; Centers for Disease Control and Prevention, 1995), and have been adopted, with adaptations, in most countries (see [Gordts *et al.*, 2000] for Belgium). They have been further extended to special populations (see [Nourse *et al.*, 2000] for children). In a nutshell, and outside the field of bacteriologically documented MRSA infections, empirical therapy with glycopeptides should be limited to severe infections in immunocompromised patients (i.e. burn patients, clinical sepsis in ICU ...) when local epidemiological data show a high percentage of MRSA, and/or to life-threatening infections associated to the presence of foreign bodies (often infected with methicillin-resistant coagulase negative staphylococci, i.e. indwelling percutaneous catheters, prosthetic cardiac valves,...). Indeed, animal models and clinical studies have shown that vancomycin is less bactericidal than isoxazolyl penicillins against methicillin-sensitive staphylococci (causing prolonged periods of fever and persistence of positive blood cultures). This should preclude its use (and, despite less data, the use of teicoplanin) when such strains are involved, especially in life-threatening infections (endocarditis,…). Therefore, their indiscriminate use in non-documented infections [or

infections caused by Gram-positive strains susceptible to other common agents] should be discouraged with the exception of true β-lactam allergy. Out of these severe infections by multi-resistant staphylococci, the main indications for vancomycin are therefore restricted to : (i) serious diphteroid infections (when the strain is penicillin resistant or the patient had IgE mediated allergy to β-lactams), (ii) penicillin-resistant *S. pneumoniae* infections of the CNS in combination with cefotaxime or ceftriaxone, and (iii) life-threatening or failing-to-respond to metronidazole antibiotic-associated colitis (only oral vancomycin; low fecal concentrations by IV route and few data with teicoplanin). Routine prophylaxis with glycopeptides should also be prohibited (Centers for Disease Control and Prevention, 1995; Gordts *et al.*, 2000), and restricted to prevention of bacterial endocarditis in penicillin/ampicillin allergic patients at risk, undergoing gastrointestinal/genitourinary procedures (plus gentamicin), or dental procedures (vancomycin alone). In selected surgical wards with a high incidence of methicillin-resistant staphylococci infections, a single dose before surgery may be used in prosthetic surgery, but one should always try to solve the underlying fundamental hygiene problem. Teicoplanin could be used in most of the same indications, with the exception of the CNS infection, because of the poor penetration of the drug in the infected compartment. Its main advantages are (i) the possibility to administer it by the intramuscular route with a once-a-day schedule (thanks to its longer half-live) and (ii) a lower incidence of side effects. Several indications, however, are less documented than for vancomycin, and there are no solid, large, comparative studies between the two drugs. In severe infections (as in *S. aureus* endocarditis), teicoplanin needs to be given at a dose of at least 12 mg/kg/day once a day, after a minimum of 3 to 4 loading doses (each given every 12 hours).

Safety issues also need to be taken into account and may limit glycopeptide use in given populations. Vancomycin has been notorious for early toxicity related to impurities and to histamine release (causing the so-called “Red man syndrome”). These adverse effects have been markedly reduced through better purification procedures and by giving the drug as a slow infusion over at least 1 h (Farber and Moellering, Jr., 1983; Wang *et al.*, 1988; Sivagnanam and Deleu, 2003). With these precautions, vancomycin is considered as safe, causing only relatively mild or self-limiting general toxicity (Sorrell and Collignon, 1985; Elting *et al.*, 1998). The dose should, however, be corrected in case of renal insufficiency. The main side effects are phlebitis at the site of injection as well as nephrotoxicity and ototoxicity, the latter remaining the most problematic because it can be irreversible (Feketi, 2000). Both nephro- and ototoxicity are aggravated by the concomitant use of aminoglycosides or

other drugs toxic for these organs (Elting *et al.*, 1998). Infrequent toxic manifestations include neutropenia, thrombocytopenia, fever, bullous dermatosis, necrotizing cutaneous vasculitis, and toxic epidermal necrolysis (Rocha *et al.*, 2002). Teicoplanin has been claimed to be less toxic than vancomycin (Wood, 1996). A difficulty, however, relates to the fact that the doses of teicoplanin have been kept increasing over time, and that most comparative studies with vancomycin were done with teicoplanin doses (typically 6 mg/kg or less) that are now considered as insufficient (table 5). Further studies are therefore needed to evaluate toxicity of teicoplanin in the current conditions of use.

II.1.b. New developments in the glycopeptide area

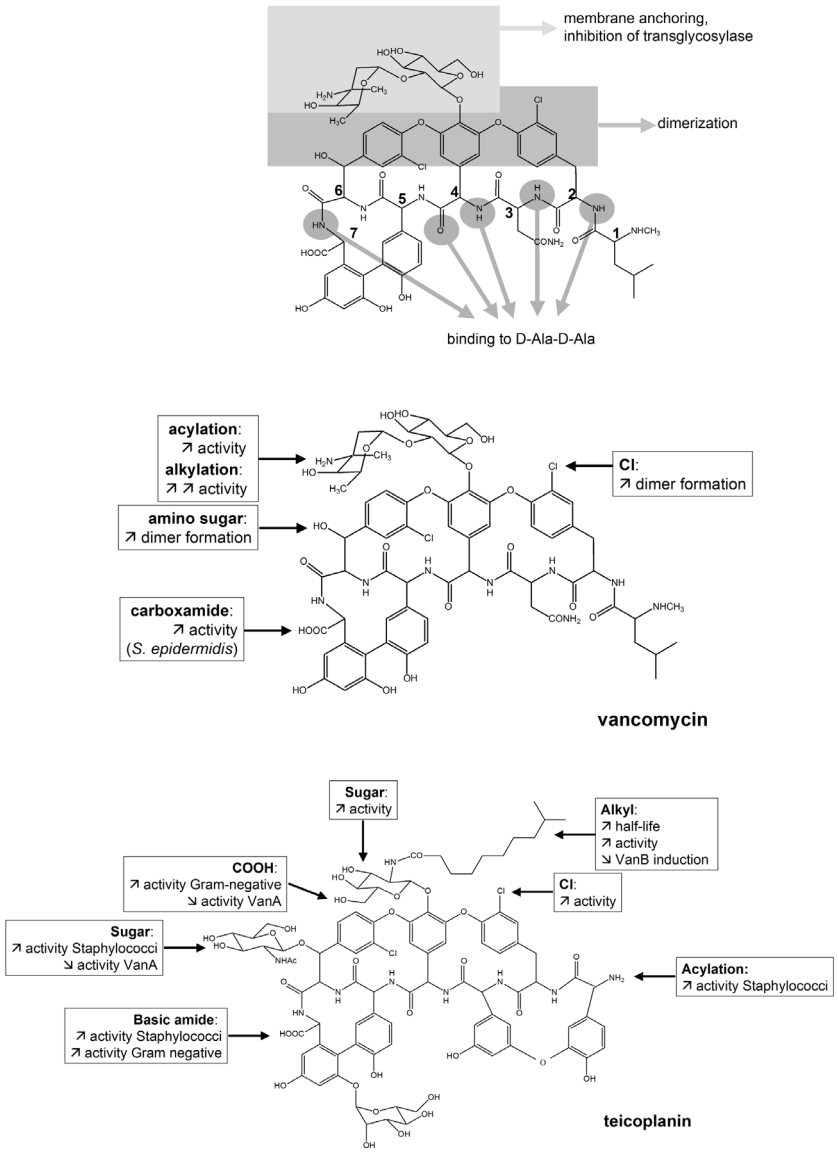


Figure 12:

Structure – activity relationship of glycopeptides derived from vancomycin and teicoplanin.

Upper panel: current view of the parts of the molecules involved in the various modes of action proposed for glycolpeptides.
Other panels : most salient structure - activity relationships that can be drawn for vancomycin (middle) or teicoplanin (bottom) derivatives.

(Van Bambeke *et al.*, 2004b)

Given the potential interest of glycopeptides in severe infections, considerable effort has been made to obtain new derivatives with improved pharmacological properties and activity against resistant strains.

Because of a highly complex structure, which makes total synthesis of glycopeptides very challenging (Boger, 2001), most of the new molecules obtained so far are semi-synthetic derivatives of existing, natural glycopeptides.

Structure-activity relationships are presented in figure 12, based on our current understanding of the molecular mode of interaction between glycopeptides and their pharmacological target. Modifications can be subdivided into three main categories, each of them concerning distinct domains of the molecule and affecting the general mode of action of vancomycin in various ways. Other recent strategies have explored the formation of hybrid or dimerized molecules, or the synthesis of inhibitors of the resistance mechanisms to be administered in combination with unmodified glycopeptides.

II.1.b.1. Semi-synthetic modification of natural glycopeptides

a) Modification of the binding pocket

Binding studies have shown that 5 sites on the peptidic backbone of the drug are involved in the interaction with the D-Ala-D-Ala termini (figures 6 and 12). Efforts have been made to change these aminoacid residues, with the aim to generate compounds with increased affinity for both dipeptide and depsipeptide termini. These have included the trimming or the enlargement of the heptapeptide backbone, the epimerization of the C3 chiral center (in teicoplanin only), or the substitution of aminoacids 1 and 3 (Malabarba *et al.*, 1997). Unfortunately, most of these modifications have resulted in a reduction of antibacterial activity. The possibility of obtaining active compounds by this type of approach remains nevertheless open, based on the observation that substitution of the Asn at position 3 by a hydrophobic amino acid enhances the affinity of vancomycin for D-Ala-D-Lac precursors (Axelsen and Li, 1998).

b) Modification / addition of functional groups

A few functional groups can be modified out of the binding pocket, causing essentially changes in physicochemical properties. These studies have generated useful knowledge in structure-activity relationship and have allowed proposing changes in the natural compounds.

b.1) Modification of vancomycin

As stated earlier, the affinity of vancomycin for its target is increased by its capacity to dimerize. This process is facilitated by the addition of an aminosugar to residue 6 and by the presence of a chlorine atom in meta on the aromatic substituent of residue 2 (Mackay *et al.*, 1994b; Malabarba *et al.*, 1997; Kaplan *et al.*, 2001). Other modifications at the extremities of the peptidic backbone have also brought significant pieces of information. Thus, replacement of the free carboxylate terminus by a carboxamide increases the activity on *S. epidermidis*, whereas D-aminoacids at the N-terminus appear more active than their corresponding L-isomers (Malabarba *et al.*, 1997).

b.2) Modification of teicoplanin

As for vancomycin, structure-activity studies of teicoplanin have examined the influence of substituting the extremities of the peptidic backbone. At the C-terminus, substitution by a basic, positively-charged amide considerably increases activity on staphylococci and also confers a moderate activity on Gram-negative bacteria, because of improved ability to cross their outer membrane (Hancock and Farmer, 1993). Esterification with hydrophobic alcohols also leads to a marginal activity on Gram-negative. Similarly, basic amides of the A40,926 derivative of teicoplanin (which lacks the N-acetylglucosamine sugar, figure 16) show increased activity against staphylococci (particularly coagulase-negative) and streptococci. The most active compounds also show moderate activity against VanA enterococci (Kenny *et al.*, 1995; Goldstein *et al.*, 1995). At the N-terminus, acylation or alkylation causes either a slight increase or no modification in activity. Another important modification in the teicoplanin backbone is the introduction of chlorine atoms, which increase both target affinity and antibacterial activity (Malabarba *et al.*, 1997).

c) Alteration of sugars

Despite their location apparently far from the binding pocket, sugars play a determinant role in glycopeptide activity since aglycones of both vancomycin and teicoplanin are systematically less active than their parent compounds (Nagarajan, 1993; Malabarba *et al.*, 1997). Substituting these sugars can actually have a considerable impact on glycopeptide pharmacological properties.

c.1) Modification of vancomycin

Beside the beneficial addition of a 4-epi-vancosamine on the benzylic function of residue 6 (Cooper *et al.*, 1996), substitution of the vancosamine sugar by hydrophobic substituents proved mostly useful, probably by somehow mimicking the lipophilic side chain of teicoplanin derivatives. N-alkyl derivatives were found to be more active than N-acyl products, in particular against enterococci, streptococci, and staphylococci (Malabarba *et al.*, 1997). When combined with the additional 4-epi-vancosamine (as in LY264826; see later, figure 13), an alkyl side chain confers to the molecule an unexpectedly rapid, concentration-dependent bactericidal activity, including against VanA- and VanB-type resistant enterococci (Nicas *et al.*, 1996; Rodriguez *et al.*, 1998; Allen and Nicas, 2003). The activity of these hydrophobic derivatives on resistant strains has been proposed to result from the combination of a facilitation of the binding to the peptidoglycan precursors due to their anchoring in the membrane (Mackay *et al.*, 1994a; Beauregard *et al.*, 1995), and of a direct inhibition of transglycosylases (Printsevskaya *et al.*, 2002; Printsevskaya *et al.*, 2003). Recent studies also suggest that some of these derivatives may act as inhibitors not only of cell wall synthesis but also of phospholipid synthesis (Debabov *et al.*, 2002). Acidic and hydrophilic substituents have also been added on the resorcinol in alpha of the free carboxylate of lipid analogs of vancomycin, with favorable consequences on distribution and safety profile, without negative effect on activity against MRSA or VRE (Leadbetter *et al.*, 2002).

c.2) Modification of teicoplanin

The impact of sugars on the activity is emphasized by the fact that the removal of the N-acylglucosamine reduces activity, in particular against streptococci and enterococci, whereas that of the N-acetylglucosamine decreases the activity against staphylococci, but may confer activity on Gram-negative as well as on VanA-type enterococci. This is especially true for derivatives having also a basic amide substitution of the C-terminus (Malabarba *et al.*, 1997).

Substituents on sugars also play important roles. As mentioned above, lipophilic substituents are of major interest for vancomycin activity. Teicoplanin contains naturally a lipophilic side chain that is responsible for its prolonged half-life. This side chain is, however, also important for activity, since deacylated derivatives are only weak antibiotics (Malabarba *et al.*, 1997). The acyl chain has been proposed not only to favor the anchoring in the membrane, and therefore the interaction with the target

(Beauregard *et al.*, 1995), but also to circumvent VanB induction (Dong *et al.*, 2002), explaining teicoplanin activity against resistant strains of the VanB-type. On the other hand, the replacement of the N-acetylglucosamine by a N-acylglucuronic acid (as in A40926, see figure 16) has a detrimental effect on the activity against VanA enterococci but improves the anti Gram-negative activity (Malabarba *et al.*, 1997).

II.1.b.2. Hybrid and multivalent glycopeptides

Based on the current knowledge on the mode of action of glycopeptides, more innovative strategies have been explored to optimize activity against both susceptible and resistant strains. First, considering the potential cooperative binding occurring in multivalent drugs, as well as the enhanced activity of glycopeptides capable of self-association, dimers of vancomycin have been prepared using different linkers and various linkage positions (Nicolaou *et al.*, 2001; Griffin *et al.*, 2002). Improved activity has been observed against Gram-positive organisms, including GISA and VRE strains, opening promising lines of investigations in this area. Second, taking into account the possibility of a dual target for glycopeptides, namely the dipeptide termini of peptidoglycan precursors, and the enzymes involved in the transglycosylation step, vancomycin derivatives have been prepared where the aglycone is separated from the sugars by linkers, with the aim to correctly position each part of the molecule (Sun *et al.*, 2001). This approach could now be extended to other glycopeptides in which both the aglycone and the carbohydrate moieties would be individually optimized for interaction with their own target, so as to generate very active compounds.

II.1.b.3. Inhibitors of resistance mechanisms

In the same way as clavulanic acid or sulbactam have been designed to restore the activity of β-lactams towards β-lactamase-producing organisms, inhibitors of glycopeptide resistance mechanisms could be considered to specifically circumvent the mechanisms of resistance developed by enterococci (which, as we have seen, may also apply to the recently described highly-resistant *S. aureus* strains). This goal has been achieved by designing small molecules such as ε-aminopentanoylated prolinol (Chiosis and Boneca, 2001), which are able to selectively cleave the ester linkage of the D-Ala-D-Lac depsipeptide of resistant bacteria. The concomitant administration of this compound and vancomycin increases the activity of the

glycopeptide *in vitro* against VanA-type enterococci, without affecting its activity against susceptible strains. Activity remains, however, globally low, probably because VanX hydrolyses the D-Ala-D-Ala dipeptide, so that there is no pentapeptide left for binding glycopeptides. These inhibitors will, therefore, need to be combined with inhibitors of the VanX D-D-peptidase (Wu and Walsh, 1995; Yang *et al.*, 2000). They have been obtained with inhibitory potencies in the micromolar range, but microbiological data are still lacking to demonstrate the interest of combining these inhibitors with vancomycin.

II.1.c. Properties of the ideal glycopeptide

Based on examination of the limitations of the current molecules and also on the promising properties of some of the semi-synthetic derivatives described above, we delineate in table 6 the main desirable characteristics expected from new glycopeptides for future clinical development.

Three molecules, oritavancin, telavancin, and dalbavancin, fulfill these conditions in various ways and are presently in Phase II/III.

Table 6 : Properties of an ideal glycopeptide
(Van Bambeke *et al.*, 2004b)

Microbiological properties:
High intrinsic activity against Gram-positive organisms, including MRSA, VRE, GRSA

Pharmacodynamic properties:
Rapid and concentration-dependent bactericidal activity

Pharmacokinetic properties:
- AUC and Cmax for free fraction adequate to cover MIC of target pathogens
- Prolonged half-life (daily administration)
- High diffusibility in tissues, including in the central nervous system

Safety profile:
Lower incidence of side effects than current molecules

II.1.d. Glycopeptides undergoing clinical evaluation

II.1.d.1. Oritavancin

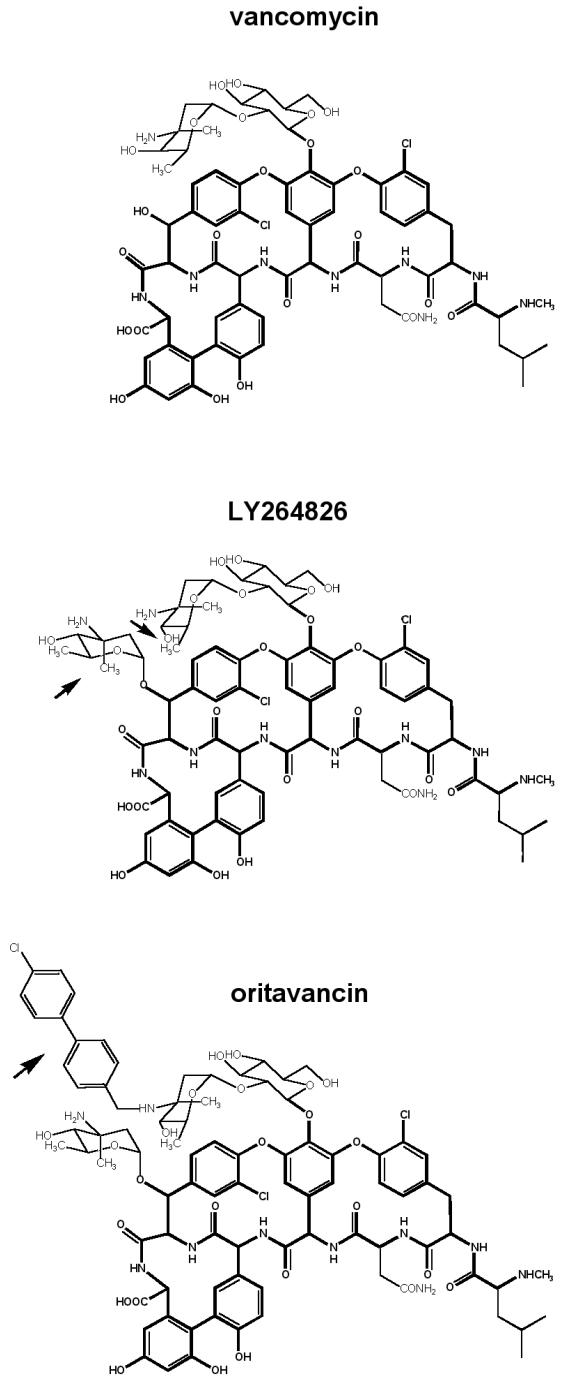


Figure 13: From vancomycin (upper panel) to oritavancin (lower panel). Middle panel: natural derivative of vancomycin used for the synthesis of oritavancin. The part of the molecule that is common to all glycopeptides appears in bold. The arrows point to the differences between molecules.

(Van Bambeke *et al.*, 2004b)

Oritavancin (LY333328) was discovered at Eli Lilly but was then further developed by InterMune (see figure 13 for chemical structure). This molecule was obtained by reductive alkylation with 4'chloro-biphenyl-carboxaldehyde of A82846B (chloroeremomycin; LY 264826), a natural glycopeptide that differs from vancomycin by the addition of a 4 epi-vancosamine sugar and the replacement of the vancosamine of the disaccharide moiety by an epi-vancosamine (Cooper *et al.*, 1996). The design of this drug was based on the previous observations that chloroeremomycin was more potent than vancomycin due to its stronger tendency to self-associate (Williams and Waltho, 1988) and that the substitution of the vancosaminyl moiety of vancomycin by a lipophilic side chain resulted in enhanced activity and restored in some cases activity towards vancomycin-resistant enterococci (Nagarajan, 1991; Nagarajan, 1993).

a) Microbiological and pharmacological properties

a.1) Spectrum of activity and resistance

Oritavancin activity (table 2) is similar to that of vancomycin against staphylococci, with MICs ranging between 0.03 and 8 mg/L; no differences are seen between methicillin-susceptible and resistant stains. Against enterococci, oritavancin is more active than vancomycin or teicoplanin, with MICs consistently lower than 1 mg/L. Remarkably, it is as active against glycopeptide-resistant strains as against glycopeptide-susceptible isolates. Oritavancin is also very potent against pneumococci (both penicillin-susceptible and resistant). It shows low MICs against other Gram-positive species including other streptococci, *Listeria*, *Clostridium*, and corynebacteria (Biavasco *et al.*, 1997), but is not active against Gram-negative bacteria, including *H. influenzae*. Resistance of *S. aureus* to oritavancin has so far not been described in clinical isolates (Garcia-Garrote *et al.*, 1998; Zeckel *et al.*, 2000; Sanchez-Silos *et al.*, 2001), including VISA strains. In contrast, enterococci with reduced susceptibility to oritavancin (MIC = 16 mg/L) have been obtained *in vitro*, using strains harboring the *vanA* or the *vanB* gene clusters. Three mechanisms seem to operate [Arthur *et al.*, 1999], namely (i) the complete elimination of D-Ala-ending precursors by overexpression of the *vanA* gene cluster or by reduced expression of the host D Ala:D-Ala ligase, (ii) mutations in the $VanS_B$ sensor of the *vanB* cluster (which can occur in clinical isolates [Aslangul *et al.*, 1997]) or (iii) the expression of VanZ, a protein encoded by a gene present in the resistance transposon but the precise function of which is still unknown. A clinical strain of oritavancin-dependent *E. faecalis* has also been isolated but the underlying mechanism should be different from that conferring dependence to vancomycin and teicoplanin, since the growth of this strain is not restored in the presence of D-Ala-D-Ala precursors (Wilson *et al.*, 1998).

a.2) Pharmacodynamic properties in relation with the mode of action.

Oritavancin is bactericidal in vitro, with MBCs only 1 to 8-fold higher than the corresponding MICs against most of the covered microorganisms (Biavasco *et al.*, 1997). Killing curve experiments have revealed a striking difference between vancomycin and oritavancin concerning the rate of killing and its concentration-dependent character (figure 14).

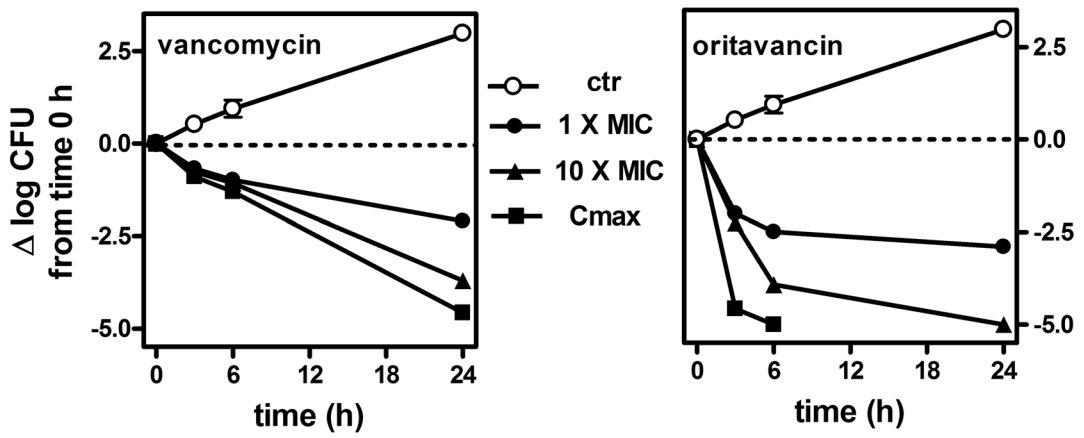


Figure 14: Comparison between the bactericidal activity of vancomycin and oritavancin against a glycopeptide-susceptible strain of *S. aureus*. The drugs are compared at concentrations corresponding to their respective MICs (1 mg/L for vancomycin; 0.25 mg/L for oritavancin), 10 X this MIC and the Cmax reached in the serum of patients receiving conventional therapies (50 mg/L for vancomycin; 25 mg/L for oritavancin). The figure shows that the activity of vancomycin develops on a time-dependent fashion, and is concentration–dependent in the 1-10 X MIC range of concentrations. In contrast, oritavancin is concentration-dependent in the whole range of concentrations and displays a very rapid bactericidal effect at high concentrations (- 5 log is the limit of detection).

adapted from (Barcia-Macay *et al.*, 2005)

Oritavancin shows indeed a very rapid and highly concentration-dependent bactericidal activity (3-log reduction in bacterial counts after 1 to 8 hours) in conditions where vancomycin requires at least 8 to 24 hours to reach the same effect (Biavasco *et al.*, 1997; Coyle and Rybak, 2001). Oritavancin however acts more slowly against vancomycin-resistant enterococci (Mercier *et al.*, 1997). These properties suggest that oritavancin could have another mode of action than conventional glycopeptides. The current view is that oritavancin combines the advantages of a high capacity to dimerize and to interact with the membrane, and therefore may cooperatively bind to peptidoglycan residues of both susceptible and resistant strains. It may also directly perturb the membrane properties and inhibit transglycosylation reactions (Allen and Nicas, 2003).

Oritavancin also displays a concentration-dependent post-antibiotic effect, increasing from ~ 2 h at 1 X MIC to 4-8 h at 4 X MIC against MRSA and VRE, respectively (Mercier *et al.*, 1997). As for vancomycin, the combination of oritavancin with gentamicin is synergistic (Mercier *et al.*, 1997; Hershberger *et al.*, 1999; Zelenitsky *et al.*, 1999). The combination with ampicillin enhances the bactericidal activity of oritavancin, without being truly synergistic. It also prolongs its postantibiotic effect against vancomycin resistant enterococci from 18 to 23 h at 10 X its MIC (Baltch *et al.*, 1998).

Oritavancin activity is negatively affected by large inocula (Mercier *et al.*, 1997), but not by acid pH or by the growth phase of the bacteria (Mercier *et al.*, 2002). However, as pointed out by these authors, activity might be slightly reduced against vancomycin-resistant enterococci in stationary growth phase, as observed in infective endocarditis or in acidic foci of infection.

a.3) Pharmacokinetic profile and relationship with pharmacodynamic properties

The pharmacokinetic properties of oritavancin are presented in table 3 in comparison with those of the other glycopeptides. Like teicoplanin, oritavancin is characterized by a high protein binding to albumin, which explains its prolonged retention in mammals, but also reduces the rate and the extent of its bactericidal activity and shortens the post-antibiotic effect (Baltch *et al.*, 1998; Zhanel *et al.*, 1998). This could be of importance when serum levels become close to the MIC of the infecting organism. Further studies should address PK/PD relationships for these drugs, but we may anticipate, on the basis of its concentration-dependent bactericidal activity *in vitro*, that high serum levels or AUC relative to MIC will correlate with optimal efficacy. Preliminary pharmacodynamic studies in a neutropenic mouse thigh model of *Staphylococcus aureus* infection suggest indeed that the parameter which best predicts oritavancin efficacy is the ratio between the free Cmax concentration and the MIC of the offending organism (free Cmax/MIC; Boylan *et al.*, 2003). In this context, its concentration in blister fluid is well above the MIC of *S. aureus,* rationalizing its potential use for skin and soft tissue infections (Fetterly *et al.*, 2005).

a.4) Models of infection

The promising *in vitro* activity of oritavancin has prompted to evaluate its activity in models of difficult-to-treat infections. As anticipated, oritavancin given once daily (q24h) is as effective as vancomycin given every 8 h (q8h) against methicillin-resistant *S. aureus* rabbit endocarditis (Kaatz *et al.*, 1998), and is bactericidal against *S. pneumoniae* rabbit meningitis (Gerber *et al.*, 2001; Cabellos *et al.*, 2003), even though the drug penetration in the CSF reaches only 5 % of the serum level (Gerber *et al.*, 2001). Oritavancin activity has also been studied in models of infections by glycopeptide-resistant enterococci. While oritavancin was effective in a model of rat central venous catheter infection (Rupp *et al.*, 2001), its activity was more limited in a rabbit endocarditis model, causing a reduction in the number of bacteria in the

vegetations, but failing to sterilize them (Saleh-Mghir *et al.*, 1999). Increasing serum levels to values as high as 80 mg/L did not provide significant improvement and did not prevent the selection of resistant mutants, probably due to the heterogeneous distribution of the drug in the vegetations. Yet, combination with gentamicin proved synergistic and bactericidal, and prevented the emergence of resistant mutants (Lefort *et al.*, 2000). Taken together, these data suggests a definite clinical interest for this drug, provided it can reach the infected compartment at sufficiently high concentration. Efficacy against infections occurring in less accessible compartments as well as against intracellular organisms needs therefore to be examined in the light of the tissue distribution of the drug *in vivo*.

b) Clinical studies

Oritavancin is currently in Phase III development (Loutit, 2002), with two studies in progress published as abstracts only. The first study (double-blind, randomized) is focused on complicated skin and soft tissue infections caused by Gram-positive including MRSA and shows an equivalent clinical success in an intent-to-treat analysis for both arms (oritavancin 1.5 or 3 mg/kg once daily for 3 to 7 days *vs* vancomycin 15 mg/kg twice daily for 3 to 7 days followed by oral cephalexin for up to 10-14 days [Wasilewski *et al.*, 2001]). The second study is a phase II, open-label, randomized trial comparing oritavancin (5 to 10 mg/kg once daily for 10 to 14 days) with vancomycin (15 mg/kg twice daily) or a β-lactam for 10 to 14 days in patients with *S. aureus*-associated bacteraemia (Loutit *et al.*, 2004). Oritavancin was as effective as comparators, with higher clinical and bacteriological success in the 10 mg/kg cohort.

c) Safety profile

The only published data available so far are those of the Phase I studies, in which oritavancin was well-tolerated (Barrett, 2001). A recent study enrolling 11 healthy volunteers mentioned however asymptomatic transaminase elevation in 5 of them, calling for more careful toxicological evaluation (Bhavnani *et al.*, 2004).

II.1.d.2. Telavancin

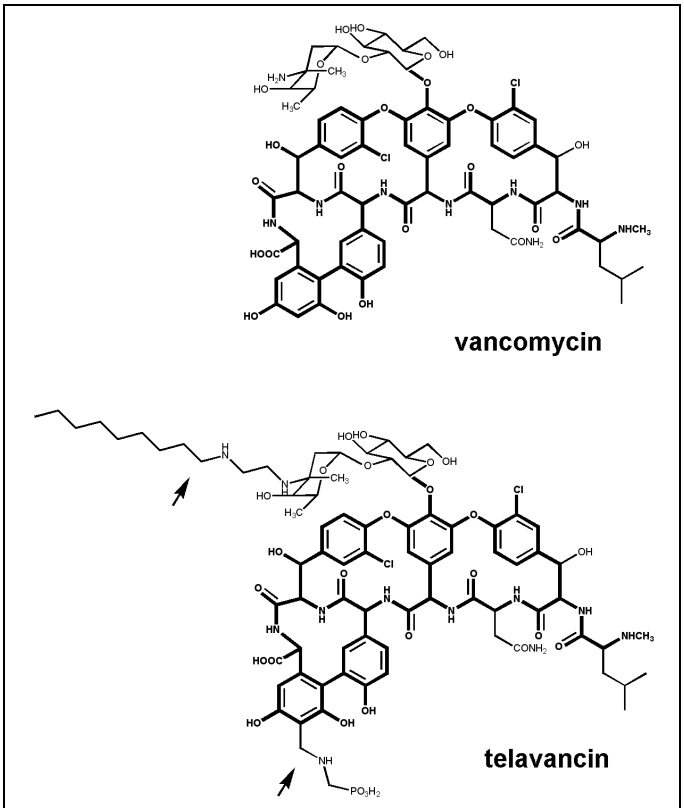


Figure 15: From vancomycin (upper panel) to telavancin. (lower panel). The part of the molecule that is common to all glycopeptides appears in bold. The arrows point to the differences between molecules.

As oritavancin, telavancin (TD-6424) is a semi-synthetic derivative of vancomycin (figure 15), possessing a hydrophobic side chain on the vancosamine sugar (decylaminoethyl) and a (phosphonomethyl)aminomethyl substituent on the cyclic peptidic core (Leadbetter *et al.*, 2004). The length of the hydrophobic side chain was chosen to reach a compromise between optimized activity against MRSA (8 to 10 C) and VanA enterococci (12 to 16 C). On the other hand, the (phosphonomethyl)-aminomethyl substituent is rather hydrophilic, counterbalancing the hydrophobicity of the side chain so as to globally obtain an amphiphilic molecule. Pharmacological studies suggest that the enhanced activity of telavancin on *S. pneumoniae, S. aureus* (to a lower extent), and staphylococci or enterococci harboring the *vanA* gene cluster (table 2) results from a complex mechanism of action, which involves a perturbation of lipid synthesis (Debabov *et al.*, 2003) and of membrane permeability (Debabov *et al.*, 2004; Higgins *et al.*, 2005). In contrast to oritavancin, however, no experimental evidence of increased binding to the cell wall precursors or direct inhibition of transglycosylase activity has been found (Judice and Pace, 2003).

a) Microbiological and pharmacological properties

a.1) Spectrum of activity and resistance

Globally, the spectrum and activity of telavancin are close to that of oritavancin, with a slightly improved activity against *S. epidermidis* but a lower activity against enterococci harboring the *vanA* resitance gene cluster (table 2).

a.2) Pharmacodynamic properties in relation with the mode of action.

Pharmacodynamic properties include a prolonged post-antibiotic effect, and a concentration-dependent bactericidal activity (Debabov *et al.*, 2003), so that we would propose to calculate the pharmacodynamic breakpoint on the basis of the free Cmax/MIC ratio, as done for oritavancin (table 4). The activity of the drug in vitro is not modified by the presence of serum for staphylococci, but is reduced for enterococci, including strains resistant to vancomycin (Kaniga *et al.*, 2003).

a.3) Pharmacokinetic profile and relationship with pharmacodynamic properties

The polar substituent added on the resorcinol moiety improves the distribution of the molecule in the body and counterbalances the prolonging effect of the lipophilic side chain on the half-life, which comes now to a 7 h value, still compatible with a once-daily administration (table 3; [Hedge *et al.*, 2003; Shaw *et al.*, 2005]).

a.4) Models of infection

Telavancin proved uniformly efficacious in models of infection in immunosuppressed or immunocompetent animals (endocarditis by MRSA or VISA, in which telavancin proved superior to vancomycin [Basuino *et al.*, 2003]; meningitis using a strain of penicillin-resistant *S. pneumoniae*, in which telavancin was significantly more effective compared to a standard regimen [combination of ceftriaxone and vancomycin] (Theravance, data on file; www.theravance.com).

b) Clinical studies

Telavancin is currently in Phase II/III for the treatment of complicated skin and soft tissue infections. The results from exploratory Phase II clinical studies, comparing telavancin with standard therapy in 167 patients with complicated Gram-positive skin and skin structure infections have been recently published and showed that the efficacy of telavancin was similar to standard therapy, but offered an advantage in the subset of patients infected by MRSA, with cure rates of 82% for patients treated with telavancin versus 69% for standard therapy (Stryjewski *et al.*, 2004).

c) Safety profile

In the same phase II study, the rate of adverse events and discontinuations for adverse effects were similar in the telavancin-treated and standard therapy groups, confirming safety data collected in Phase I trials (Shaw *et al.*, 2005).

II.1.d.3. Dalbavancin

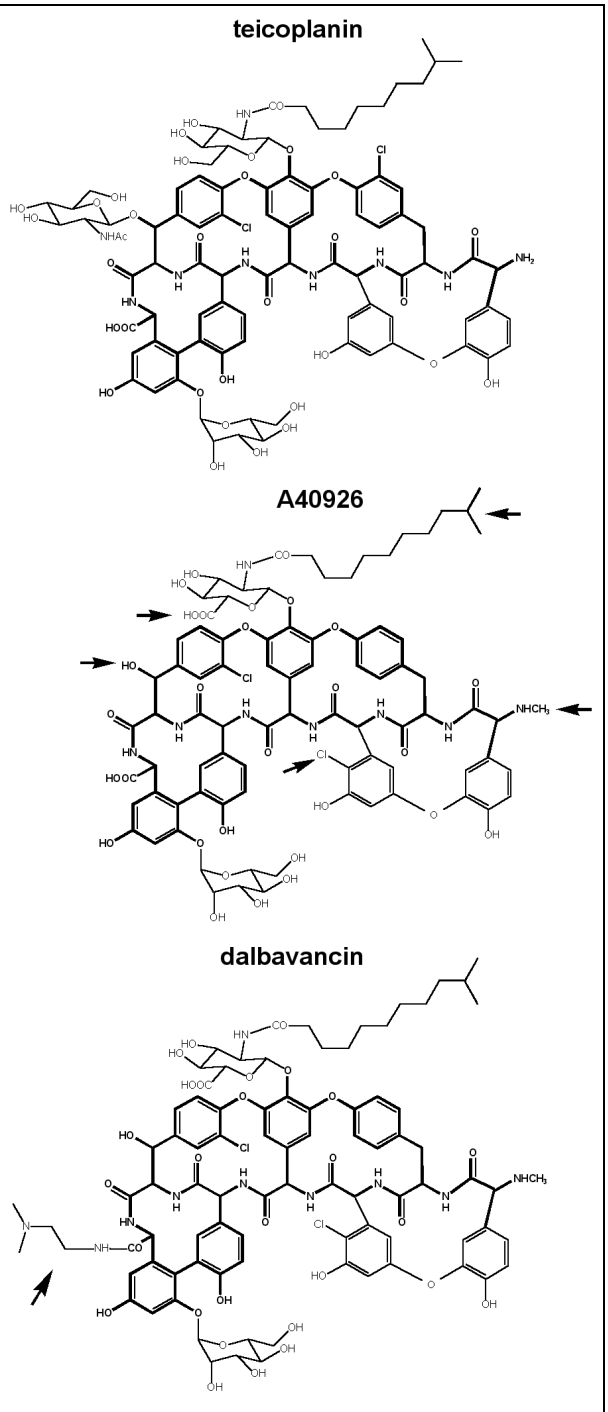


Figure 16: From teicoplanin (upper panel) to dalbavancin. (lower panel). Middle panel: natural derivative of teicoplanin used for the synthesis of dalbavancin. The part of the molecule that is common to all glycopeptides appears in bold. The arrows point to the differences between molecules.

(Van Bambeke *et al.*, 2004b)

Dalbavancin (BI397) is a semi-synthetic derivative from the natural glycopeptide A40926 (see figure 16 for structure). It was discovered by Biosearch and outlicenced for North America to Versicor. Both companies have now merged to create Vicuron Pharmaceuticals, and continue to develop the product. A40926 differs from teicoplanin by the absence of the acetyl-glucosamine in the benzylic position, the replacement of the acyl-glucosamine in position 4 by an acylaminoglucuronic acid, the length of the fatty acid chain, the position of one chlorine atom, and the terminal methylaminogroup (Malabarba *et al.*, 1994). In dalbavancin, a 3,3-dimethylaminopropylamide replaces the peptide carboxy group of A40926 (Malabarba *et al.*, 1995). SAR of teicoplanin indicated an improvement in activity by derivatization of its carboxy group while an improved activity against VanA-type enterococci is expected to occur when removing the acetylglucosamine (Malabarba and Ciabatti, 2001). The most active compound in the series was actually the 6β-decarboxy-6β-hydroxymethyl amide of A40926 (Malabarba *et al.*, 1995), but development of this compound was halted because of its poor tolerability in animals (causing side effects typical of histamine release which have been ascribed to

its basic character (Malabarba and Ciabatti, 2001). Unfortunately, most data on dalbavancin have only been published as abstracts or as short quotations in review papers. This makes difficult an in-depth comparison of dalbavancin with oritavancin.

a) Microbiological and pharmacological properties

a.1) Spectrum of activity and resistance

In general (table 2), dalbavancin is more potent than vancomycin, teicoplanin and, to some extent, oritavancin against staphylococci (with MICs ranging from < 0.03 to 1 mg/L for susceptible and methicillin-resistant strains, respectively) and *S. pyogenes* (MIC in the < 0.002 – 0.06 mg/L range) (Candiani *et al.*, 1999; Hackbarth *et al.*, 1999; Jones *et al.*, 2001). It is also very active against *S. pneumoniae*, with MIC 2 to 4-fold higher than those of oritavancin. It is as active as oritavancin or teicoplanin against vancomycin-susceptible enterococci, but does not offer the same advantage as oritavancin against glycopeptide-resistant strains. Thus, the MIC distribution range towards VanB- and VanA-type strains include more susceptible strains but the MIC_{50} remain only marginally (4-fold) lower than those of teicoplanin (Candiani *et al.*, 1999). This drug, therefore, represents a significant improvement, except against glycopeptide-resistant enterococci (Malabarba and Ciabatti, 2001; Guay, 2004).
Subpopulations of staphylococci having acquired low level of resistance (2- to 4-fold increase in MIC) have been selected upon serial passage at sub-MIC of dalbavancin (Lopez *et al.*, 2003). In vivo selection of such mutants may however be more difficult since trough levels of dalbavancin are above the current MIC of the targeted microorganisms.

a.2) Pharmacodynamic properties in relation with the mode of action.

Like oritavancin, dalbavancin is bactericidal, with MBC/MIC ratios close to 1, even in the presence of 30 % serum (Steiert and Schmitz, 2002). It is synergic with ampicillin, including against VanA-type enterococci and glycopeptide-intermediate *S. aureus*.

a.3) Pharmacokinetic profile and relationship with pharmacodynamic properties

The pharmacokinetic profile of dalbavancin is compared with that of the other glycopeptides in table 3. Phase I studies have used higher doses, providing higher

AUC values. However, when corrected for the free fraction, the AUC/MIC and serum levels/MIC ratios of both drugs are in the same order of magnitude as those of other glycopeptides. Plasma levels of dalbavancin still exceeds the MBC values for staphylococci one week after the administration of a single dose of 1000 mg but free serum levels are close to the MIC (Steiert and Schmitz, 2002; Dowell *et al.*, 2002).

a.4) Models of infection

The activity of dalbavancin has been assessed in models of infections by glycopeptide-susceptible Gram-positive organisms and by GISA. Preliminary reports indicate a reduction in bacterial load after a single dose administration in models of penicillin-resistant *S. pneumoniae* pneumonia or of MRSA rat pouch infection (Malabarba and Ciabatti, 2001; Candiani et al., 2001). Its activity is superior to that of conventional glycopeptides in models of endocarditis due to MRSA or GISA, and of acute septicemia caused by staphylococci, streptococci, or enterococci (Steiert and Schmitz, 2002; Lefort *et al.*, 2004).

b) Clinical studies

Phase II/III clinical trials are ongoing to evaluate the efficacy and safety of dalbavancin for the treatment of complicated skin and soft tissue infections caused by Gram-positive organisms or the use of dalbavancin in a once-a-week mode of administration for other Gram-positive hospital infections (Steiert and Schmitz, 2002). In a first report of a phase II study (Seltzer *et al.*, 2003), the administration of two doses (1000 mg on day 1 and 500 mg on day 8) was found as effective as comparators (7 to 21 days with clindamycin, ceftriaxone, vancomycin, or cefazolin) for the treatment of deep skin and soft tissue infections caused by MSSA or MRSA.

c) Safety profile

Preclinical studies in rats and dogs show that dalbavancin is well tolerated upon intravenous bolus administrations at doses several times higher than those expected to be used in humans (Malabarba and Ciabatti, 2001). Phase I or phase II clinical trials have not reported major side effects in the range of concentrations where dalbavancin is effective (Steiert and Schmitz, 2002; Seltzer *et al.*, 2003). No dosage adjustments are necessary in case of mild renal insufficiency (Dowell *et al.*, 2003).

II.1.e. New glycopeptides in the clinics: for which indications ?

The improved pharmacological properties of new glycopeptides make them very potent agents, with potentially large clinical indications. However, their use should be as restricted as that of conventional glycopeptides if one wishes to avoid the rapid emergence of resistance.

The advantages of oritavancin that should prove to be useful in clinics include its fast rate of bacterial killing, its spectrum of activity, which covers the VRE, Methicillin-resistant staphylococci, and to some extent the GISA strains, its activity against intracellular forms of enterococci and staphylococci (see experimental studies section), and its prolonged half-life. The profile of telavancin would probably be close to that of oritavancin, but the less abundant literature available so far (in particular, in animal models of infection) calls to more caution concerning the prediction of its potential clinical interest. For dalbavancin, the main improvement seems to be pharmacokinetic profile, which should allow a once-a-week administration, but the prolonged retention of this drug in then organism also raises questions regarding its safety profile that still need to be answered (Guay, 2004). Microbiological properties of dalbavancin are less favorable than those of oritavancin, since VRE are not covered.

On these bases, and also in view of the results from animal models, oritavancin may become a drug of interest for infections caused by VRE, while both oritavancin and dalbavancin could be considered for severe infections by multiresistant organisms, like methicillin-resistant staphylococci or *S. pneumoniae*. In the latter case, however, other alternatives such as fluoroquinolones (moxifloxacin, gatifloxacin, or gemifloxacin) should be examined first. In some countries, however, resistance to fluoroquinolones has already reached alarming levels (15 % in South East Asia [Hong Kong, South Corea; Felmingham *et al.*, 2002]). There are also situations such as CNS infections in which fluoroquinolones are poorly efficient (Vandecasteele *et al.*, 2001; Buckingham *et al.*, 2002). New glycopeptides could, therefore, prove very useful in these "niche" but important indications. Finally their activity against GISA strains appears limited (table 2), which could deter from recommending them in these situations. However, synergy has been demonstrated for dalbavancin when combined with β-lactams (Hackbarth *et al.*, 1999). Encouraging results have also been obtained in animal models (Lefort *et al.*, 2004), which should stimulate further testing this molecule against GISA infections.

Reasonable indications could therefore include (i) nosocomial septicemia or infections of deep organs caused by MRSA or VRE (oritavancin only), (ii) severe and recurrent skin and soft tissue infections by MRSA (in relation with an activity against both extra- and intracellular bacteria [demonstrated so far only for oritavancin]), and (iii) endocarditis due to resistant organisms (but probably in combination with an aminoglycoside for oritavancin against VRE, based on disappointing animal data [Lefort *et al.*, 2000]). The usefulness of oritavancin in nosocomial CNS infections caused by MRSA, resistant *S. pneumoniae*, or VRE may be suggested on the basis of its activity in animal models. No corresponding data do exist for dalbavancin, for which penetration is CSF is low. The potential use of oritavancin in humans in this indication needs, however, to be assessed, as well as of the influence of corticosteroids on the CNS penetration of these new glycopeptides (influence of corticoids was found minor for vancomycin).
Globally speaking, however, clinical studies or additional animal data are needed to confirm the interest of new glycopeptides in these indications, since the proposals made above rely almost exclusively on an analysis of the pharmacological properties of these drugs and on the results obtained in animal models of infection.

Oritavancin, telavancin and dalbavancin offer thus considerable interests over vancomycin and teicoplanin, essentially in terms of pharmacokinetics and pharmacodynamic properties, as well as of activity against resistant strains (except for dalbavancin). Whether these properties can translate in improved clinical efficacy awaits completion of clinical studies. Should these molecules soon become approved drugs, we must remember that rational and prudent use is the best, if not the only way to protect antibiotics against rapid emergence of resistance and to promise them to a long life.

II.2. Experimental studies: accumulation of oritavancin in eukaryotic cells and consequences for activity and toxicity

On the bases developed in the introduction, oritavancin can be considered on a physico-chemical point of view as amphipathic molecule with three protonable amines. We have seen that these confer to this antibiotic new pharmacodynamic and pharmacokinetic properties.

First, it possesses a rapid, concentration-dependent bactericidal activity against most Gram-positive organisms, whether susceptible or resistant to vancomycin, associated to a prolonged post-antibiotic effect. These characteristics largely plead for an interest of this molecule in difficult-to-treat infections by *S. aureus*. *S. aureus* is indeed one of the most common pathogenic bacteria, both in humans and animals. Despite the large range of antibiotic classes displaying anti-staphylococcal activity *in vitro*, these infections remain difficult to eradicate, often giving rise to chronic or relapsing diseases. There is now growing evidence that the capacity of *S. aureus* to survive intracellulary plays a critical role in this persistence. Antibiotic treatments of staphylococcal infections should therefore be optimized not only towards extracellular, but also intracellular forms of *S. aureus.* Studies aimed at evaluating the cellular pharmacokinetics and pharmacodynamics of oritavancin would be worthwhile in this context.

Second, oritavancin shows a very prolonged terminal half-life (150 – 300 h versus 6 h for vancomycin) and high protein-binding (> 85 % versus 10-55 % for vancomycin). The persistence of oritavancin in the organism is believed to result from this large protein binding, by analogy with teicoplanin (Nicas *et al.*, 1997), but may also suggest the existence of deep storage compartments for the drug in the body. In this sense, the behavior of oritavancin may remind that of other antibiotics endowed with a cationic amphiphilic character, such as azithromycin, which has a prolonged half-life and retention in tissues (Foulds *et al.*, 1990), and highly accumulates in eucaryotic cells (Carlier *et al.*, 1994).

Accordingly, we have thus studied the cellular pharmacokinetics of oritavancin in a model of cultured cells, and then evaluated whether its cellular accumulation would be associated with intracellular activity and/or toxicity.

II.2.a. Cellular pharmacokinetics

The cellular pharmacokinetics and disposition of oritavancin in cultured cells was studied following the approaches used previously to study the intracellular fate of other cationic antibiotics such as aminoglycosides (Tulkens and Trouet, 1978), macrolides (Carlier *et al.*, 1994) and basic derivatives of β-lactam antibiotics (Renard *et al.*, 1987; Chanteux *et al.*, 2003b). The mechanism of accumulation was studied using as comparators chloroquine and azithromycin, which accumulate in lysosomes by diffusion and segregation of their protonated form (de Duve *et al.*, 1974; Wibo and Poole, 1974; Carlier *et al.*, 1994, and with horseradish peroxidase and small-sized latex beads as markers of endocytosis (Tyteca *et al.*, 2002; Rejman *et al.*, 2003). The experimental procedures corresponding to the data presented hereunder are detailed in our publication (Van Bambeke *et al.*, 2004a).

II.2.a.1. Description of the cellular pharmacokinetics of oritavancin

a) Kinetics of accumulation and efflux of oritavancin

The uptake and efflux of oritavancin were examined in J774 macrophages as a function of time at a fixed extracellular concentration (25 mg/L). Figure 17 shows that accumulation at 37°C proceeded according to a one phase exponential association process, reaching near-saturation after approx. 20 h, and a maximal cellular concentration of about 46 µg/mg protein (corresponding to an apparent accumulation ratio of 370).

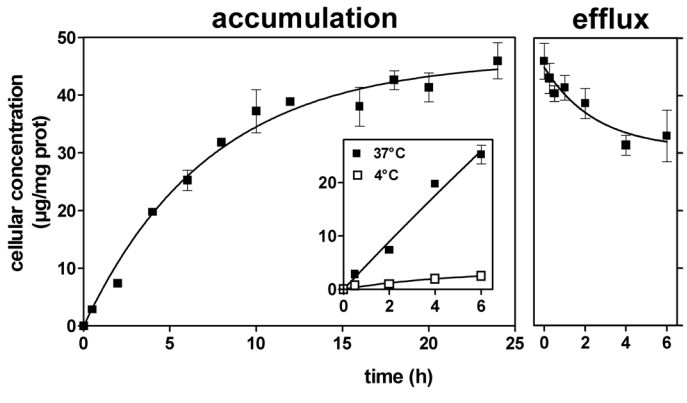


Figure 17: Kinetics of accumulation and efflux of oritavancin in J774 macrophages incubated with an extracellular concentration of 25 mg/L at 37°C in medium supplemented by 10 % FCS (complete culture medium). Results are given as arithmetic means ± standard deviation (n=3) of the cellular concentrations recorded (µg oritavancin per mg cell proteins; the apparent accumulation ratio for each value of extracellular concentration can be calculated by multiplying the figures of the ordinate by 200 (assuming a cell volume of 5 µl/mg protein). Left panel: influx (regression parameters [one phase exponential association]: $R^2 = 0.986$, $k = 0.138 \pm 0.014$ h^{-1}, plateau = 46.2 ± 3.8). Inset in left panel: influx kinetics over the first 6 h and comparison between cells incubated at 37 and 4°C. Right panel: efflux from cells incubated with oritavancin for 24 h (regression parameters [one phase exponential decay]: $R^2 = 0.911$, $k = 0.401 \pm 0.252$ h^{-1}; plateau = 30.7 ± 3.6).

(Van Bambeke *et al.*, 2004a)

Figure 17 also shows that oritavancin uptake was considerably slower at 4°C (less than 5 % of the value at 37°C). Efflux of oritavancin was then examined in cells incubated for 24 h at 37°C with 25 mg/L of drug and then transferred to oritavancin-free medium.

Release proceeded according to a one phase exponential decay ($t_{1/2}$ approx 1.73 h), but was largely incomplete, with an apparent plateau value reached at a drug apparent cellular concentration of about 2/3 of the original value (more prolonged efflux experiments could not be performed because of loss of cell viability).

In sharp contrast to oritavancin, vancomycin accumulated only very slowly in J774 macrophages and reached an apparent cellular to extracellular concentration ratio of only 8.0 ± 5.4 after 24 h incubation (not shown in the figure).

To establish whether the extensive accumulation of oritavancin was specific to J774 macrophages, these experiments were repeated with 4 other cell lines, 3 of which are not of macrophage type.

Accumulation levels observed after 2 h incubation are presented in table 7. THP-1 macrophages and fibroblasts accumulated oritavancin to essentially the same extent as J774 macrophages.

Accumulation by the epithelial cell lines LLC-PK1 and Caco-2 was lower but nevertheless quite important.

Table 7: Oritavancin accumulation (Cc/Ce) in different cell types incubated during 2 h at 37°C with 25 mg/L of the drug in a medium containing 10 % FCS

(Van Bambeke *et al.*, 2004a)

Cell type	Cc/Ce (no of determinations)
J774 mouse macrophages	66.4 ± 11.8 (12)
THP-1 human monocytes	84.3 ± 7.0 (9)
Rat embryo fibroblasts	72.4 ± 9.4 (6)
LLC-PK1 pig kidney proximal tubular cells	37.8 ± 6.4 (3)
Caco-2 human colorectal cells	13.8 ± 0.4 (3)

Because oritavancin is known to bind to serum proteins, we examined in details its accumulation (2 h incubation at increasing extracellular concentrations) in the absence and in the presence of calf serum (10 and 20 %). Figure 18 shows that the uptake in cells incubated with the complete culture medium (10 % FCS) for 2 h did not proceed linearly with the extracellular concentration, but rather on an apparent cooperative fashion. This was not observed with cells incubated in serum-free medium, for which uptake was quasi-linearly related to extracellular concentration and was therefore considerably higher in the 10-50 mg/L range. Conversely, increasing

the FCS concentration to 20 % made the uptake process less effective in the range of concentrations investigated.

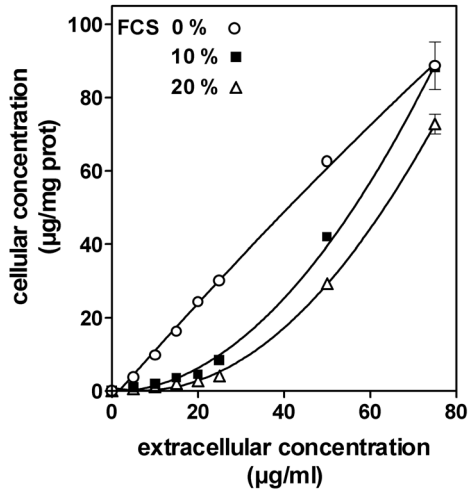


Figure 18: Accumulation of oritavancin in J774 macrophages incubated for 2 h at 37°C with increasing extracellular concentrations of oritavancin in serum-free medium or in medium supplemented with 10 or 20 % FCS. Results are given as arithmetic means ± standard deviation (n=3) of the cellular concentrations recorded (µg oritavancin per mg cell proteins; the apparent accumulation ratio for each value of extracellular concentration can be calculated by multiplying the figures of the ordinate by 200 (based on a cell volume of 5 µl/mg protein).

(Van Bambeke *et al.*, 2004a)

b) Subcellular distribution of oritavancin

In preliminary experiments, we observed that cell-associated oritavancin was recovered for about 80 % in the granular (MLP) fraction with only 5 % in the cell supernatant and the remaining in the nuclei/unbroken cells fraction. The MLP fraction was therefore analyzed by isopycnic centrifugation to determine the distribution of oritavancin among the organelles present in that fraction (mainly mitochondria, lysosomes, endoplasmic reticulum, and plasma membrane). Figure 19 (left diagrams) shows the distribution of marker enzymes of lysosomes (N-acetyl-β-glucosaminidase), mitochondria (cytochrome *c*-oxidase) and plasma/endoplasmic reticulum membranes (inosine 5'-diphosphatase) in control cells. These profiles are well distinct from one another (cathepsin B was also assayed in these experiments and showed a profile similar to that of N-acetyl-β-glucosaminidase). Figure 19 (right diagrams) shows the results of three experiments in which cells were incubated for 2 h either with oritavancin (20 mg/L), chloroquine (20 mg/L), or HRP (2 g/L). Considering cells incubated with oritavancin first, it clearly appears that the drug and N-acetyl-β-glucosaminidase show an essentially common behavior in all fractions with a density lower than 1.15. Chloroquine, a known lysosomotropic agent, also showed a large overlap of distribution with that of N-acetyl-β-glucosaminidase, but the latter equilibrated at a somewhat lower density than in control cells. HRP was also co-distributed with N acetyl-β-glucosaminidase, with however, a slight but detectable shift of density of the latter enzyme towards higher densities.

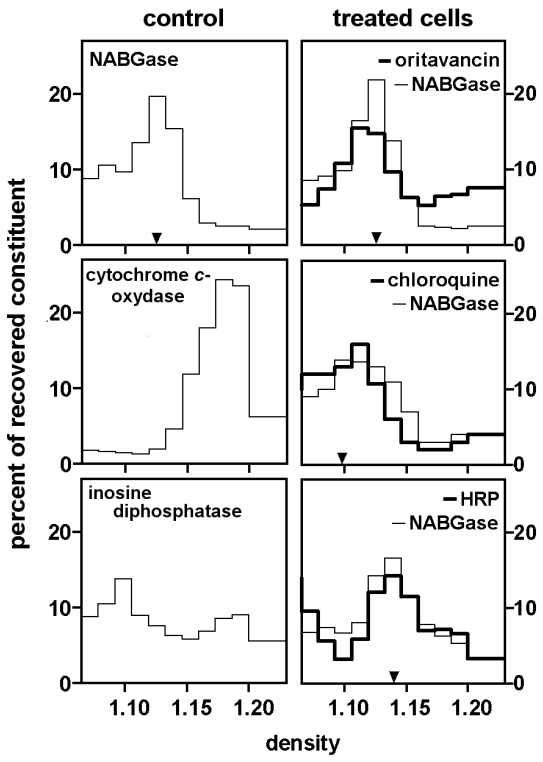


Figure 19: Density distribution of marker enzymes (N-acetyl-β-glucosaminidase [lysosomes], cytochrome *c*-oxidase [mitochondria]), and inosine 5'-diphosphatase [plasma and endoplasmic reticulum membranes]), **drugs, and HRP** after isopycnic centrifugation of a granule fractions (MLP fraction) prepared from homogenates of J774 cells in linear sucrose gradients. The ordinate shows the percentage of each constituent recovered in each fraction.

Left panels: distribution of marker enzymes (NABGase = N-acetyl-β-glucosaminidase) in control macrophages.

Right panels: distribution pattern of NABGase (thin lines), oritavancin, chloroquine, or HRP (horseradish peroxidase) (tick lines) in cells incubated during 2 h with oritavancin (20 mg/L), chloroquine (20 mg/L), or HRP (2 g/L), respectively. The vertical arrowheads point to the median density of the NABGase distribution in both control (top left panel) and treated cells (all three right panels).

(Van Bambeke *et al.*, 2004a)

II.2.a.2. Mechanism of the lysosomal accumulation of oritavancin

Having established that oritavancin accumulated by J774 macrophages was primarily associated with lysosomes, we investigated the mechanisms of its uptake by means of short-term kinetics and pharmacological approaches. Figure 20 compares the kinetics of oritavancin accumulation with those of HRP and small-sized latex beads (taken as tracers of fluid phase and adsorptive pinocytosis, respectively [Steinman *et al.*, 1974; Tyteca *et al.*, 2002; Rejman *et al.*, 2003]) on the one hand, and of chloroquine and azithromycin (taken as examples of molecules diffusing through membranes and accumulating in lysosomes by proton trapping [de Duve *et al.*, 1974; Wibo and Poole, 1974; Carlier *et al.*, 1994]) on the other hand. Oritavancin, HRP, and small-sized latex beads accumulated on a linear fashion over time. Clearance rates of oritavancin and small-sized latex beads were quite similar (~ 150 and ~ 120 µl x mg $prot^{-1}$ x h^{-1}) and considerably higher than that of HRP (~ 0.7 µl x mg $prot^{-1}$ x h^{-1}). In contrast, uptake kinetics of chloroquine and azithromycin over the first 4 h were not linear but followed both a one phase exponential association process with similar time constants (approx. 1 h^{-1}).

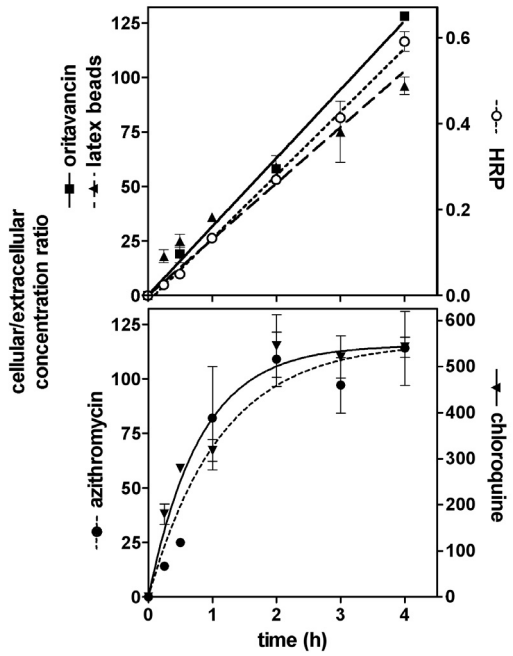


Figure 20: Kinetics of accumulation over a 4 h period of oritavancin (extracellular concentration, 25 mg/L), 0.1 µm latex beads (extracellular concentration, 230 x 10^{12} beads/L) and HRP (extracellular concentration, 2 g/L; *upper panel*) and of chloroquine (extracellular concentration, 12 mg/L) and azithromycin (extracellular concentration, 25 mg/L; *lower panel*) in J774 macrophages incubated at 37°C in medium supplemented with 10 % FCS. Results are given as arithmetic means ± standard deviation (n=3) of the apparent cell accumulation levels (ratio between the apparent cellular and the extracellular concentrations). The data were fitted to a linear equation (oritavancin, latex beads, HRP) or a one phase exponential association (chroroquine, azithromycin). Regression parameters: oritavancin, R^2 = 0.995, slope = 31.42 ± 1.43 h^{-1} ; latex beads, R^2 = 0.929, slope = 25.73 ± 1.65 h^{-1}; HRP, R^2 = 0.997, slope = 0.15 ± 0.01 h^{-1}; azithromycin, R^2 = 0.941, k = 0.89 ± 0.27 h^{-1}, plateau = 116.6 ± 12.8; chloroquine, R^2 =0.966 , k = 1.24 ± 0.23 h^{-1} , plateau = 546.8 ± 31.7 .

(Van Bambeke *et al.*, 2004a)

We next examined the influence of the extracellular pH and of monensin on the accumulation of oritavancin in comparison with chloroquine. Acid pH is known to depress the cellular accumulation of weak organic bases that accumulate in lysosomes by diffusion/segregation of their protonated form, due to the corresponding change in the lysosome-extracellular pH gradient (de Duve *et al.*, 1974). In this context, the accumulations of oritavancin (25 mg/L) and chloroquine (20 mg/L) were examined after 2 h incubation at pH 6 and 8 in comparison with pH 7.4, yielding values of 128 ± 7.5 % and 86.5 ± 5.3 % for oritavancin, and 18.1 ± 1.5 % and 140.4 ± 10.7 % for chloroquine. Monensin is a proton ionophore that (i) collapses transmembrane gradients (Tartakoff, 1983), suppressing almost completely the accumulation of weak organic bases (Tyteca *et al.*, 2002), and (ii) partly defeats pinocytosis by stimulating the regurgitation of the content of early endosomes (Cupers *et al.*, 1994). Cells were preincubated for 1 h with 20 µM monensin and then exposed to oritavancin (25 mg/L), HRP (2 g/L), or chloroquine (20 mg/L) for 2 h in the continuing presence of monensin. Oritavancin and HRP accumulated to 28.5 ± 2.0 % and 29.9 ± 2.7 % of control values (no monensin treatment; this decrease in HRP uptake is similar to that observed previously in J774 macrophages [Tyteca *et al.*, 2002]), whereas the accumulation of chloroquine was reduced to 4.8 ± 0.6 %.

II.2.a.3. Conclusions : oritavancin, a new type of cell-associated antibiotic ?

Accumulation and subcellular disposition of aminoglycosides, macrolides and fluoroquinolones have been extensively studied in eukaryotic cells, yielding useful pharmacodynamic and toxicodynamic information (Tulkens and Trouet, 1978; Carlier *et al.*, 1990; Carlier *et al.*, 1994; Garcia *et al.*, 2000; Seral *et al.*, 2003b). In contrast, little is known so far concerning glycopeptide antibiotics, including vancomycin (Van der Auwera *et al.*, 1988; Beauchamp *et al.*, 1992). The present study has focused on oritavancin, a semi-synthetic derivative of vancomycin, and shows that it reaches an exceptionally large cellular accumulation both in macrophages and fibroblasts, in sharp contrast with its parent compound vancomycin. Most of the cell-associated oritavancin must be truly intracellular, since (i) accumulation is drastically reduced at 4°C (a condition which should not prevent the drug binding at the membrane as shown for gentamicin [Decorti *et al.*, 1999] or pivaloyl- or phthalimidomethyl esters of ampicillin [Chanteux *et al.*, 2003b]) and (ii) the drug distributes in the same fractions as lysosomes upon isopycnic centrifugation of the granular cell fraction, with no or only a minor proportion found in the soluble fraction.

Accumulation of exogenous compounds in lysosomes has been shown to result from two main mechanisms, namely endocytosis and diffusion/segregation (de Duve *et al.*, 1974; Steinberg, 1994). The former mechanism is demonstrated for many non-permeant molecules, and can be distinguished between fluid-phase and adsorptive endocytosis, depending on the capacity of the substance to bind or not to the pericellular membrane (Silverstein *et al.*, 1977; Mukherjee *et al.*, 1997). Fluid phase pinocytosis is exemplified here by HRP, which has been widely used and validated as a typical marker of this cellular activity in J774 macrophages (Tyteca *et al.*, 2002). Receptor-mediated or adsorptive endocytosis is typically observed in J774 macrophages for substances such as transferrin or IgG (which have specific receptors) or small-sized latex beads (which bind unspecifically at the cell surface [Tyteca *et al.*, 2002]). The second mechanism is observed with amphiphilic, cationic molecules that are able to diffuse through membranes under their unprotonated form but get sequestered in lysosomes as protonated molecules because of the acid pH created therein through the activity of an ATP-dependent proton pump (Ohkuma and Poole, 1978). This mechanism is exemplified here by azithromycin and by chloroquine, which have been extensively studied in this context (Wibo and Poole, 1974; Carlier *et al.*, 1994; Tyteca *et al.*, 2002).

The data we presented here strongly suggest that oritavancin accumulates in J774 macrophages via the endocytic route since (i) its kinetics of uptake over the 4 first hours follows the same model than that of HRP or small-sized latex beads and is clearly distinct from that of azithromycin and chloroquine; (ii) its accumulation is only partially impaired by monensin, and to the same extent as that of HRP, while monensin almost entirely suppresses the accumulation of chloroquine (this study) and that of azithromycin (Tyteca *et al.*, 2002). The fact that the rate of uptake of oritavancin is similar to that of latex beads and much higher than that of HRP suggests the occurrence of binding sites at the cell surface allowing oritavancin to enter by receptor mediated/adsorptive endocytosis. No binding sites, however, were evidenced here in the fractionation studies. This may result from elution of the membrane-bound oritavancin during the washing of the cells and the homogenization/fractionation procedures. In this context, the defeating effect exerted by serum on accumulation at low drug concentrations may indicate a competition between serum proteins and cell membrane binding sites. Conversely, the increased accumulation observed at acidic pH could reflect a higher binding of the cationic oritavancin to the cell membrane, which is globally negatively-charged.

Most of our studies were performed using culture medium containing 10 % calf serum, because this is required for optimal growth and long-term maintenance of most eucaryotic cells, including J774 macrophages. Oritavancin, however, is known to tightly bind to serum proteins, but such binding is difficult to study because of a tendency of the molecule to stick to various materials such as dialysis membranes, filters, etc… that need to be used for performing these experiments (T. Nicas, personal communication). Studies using the dextran-charcoal method, however, suggest that the protein binding of oritavancin in human serums spiked with total drug concentrations of up to 91 mg/L is about 85 to 90 % (Rowe and Brown, 2001). Since the mean peak serum concentrations measured in ongoing clinical trials is 31 mg/L (table 3 and [Van Bambeke *et al.*, 2004b]), we may estimate that the free serum concentration in humans will be around 3-5 mg/L. Data from figure 18 shows that cells exposed to 5 mg/L of oritavancin in the absence of serum display a drug cellular concentration of about 4 µg/mg protein. A similar level is obtained for cells exposed to 15-25 mg/L of oritavancin in the presence of 10 % calf serum. Thus, our standard conditions may actually reproduce what could be observed in humans in terms of free drug exposure.

In a larger context, the unusual cellular pharmacokinetic properties of oritavancin open interesting perspectives both in terms of structure-activity relationships and of potential uses of this molecule in clinics. Regarding structure-activity relationships, it is indeed possible that the lipophilic character of oritavancin is not only what causes its fast uptake and large retention (as suggested by the present study), but also its rapid bactericidal effect (Allen *et al.*, 1996; Williams, 1996). It is interesting to note, in this context, that the few available reports on teicoplanin, another glycopeptide with lipophilic properties and high protein binding, also point to a marked accumulation of this antibiotic (Maderazo *et al.*, 1988; Carlone *et al.*, 1989). Teicoplanin, however, is not more bactericidal than vancomycin. Accumulation and efflux studies and microbiological evaluations using glycopeptide derivatives with systematic variations in their lipophilic/hydrophilic balance would be interesting to perform in this context. Concerning the potential use of oritavancin in the clinics, the highly cell-associated character of the drug warrants further studies aimed at evaluating its activity against intracellular infections.

II.2.b. Cellular pharmacodynamics

In an attempt to correlate accumulation, disposition and expression of activity, we have thus assessed the intracellular activity of oritavancin as compared to that of vancomycin against phagolysosomal (*Staphylococcus aureus*) and cytosolic (*Listeria monocytogenes*) organisms (figure 21), using previously established models of macrophages (Chanteux *et al.*, 2003a; Seral *et al.*, 2003a; Seral *et al.*, 2003c; Barcia-Macay *et al.*, 2005).

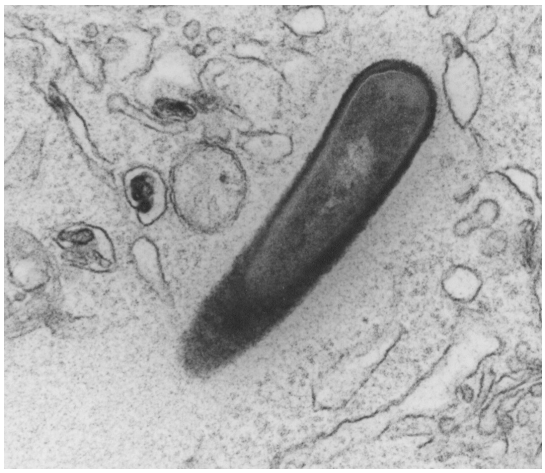
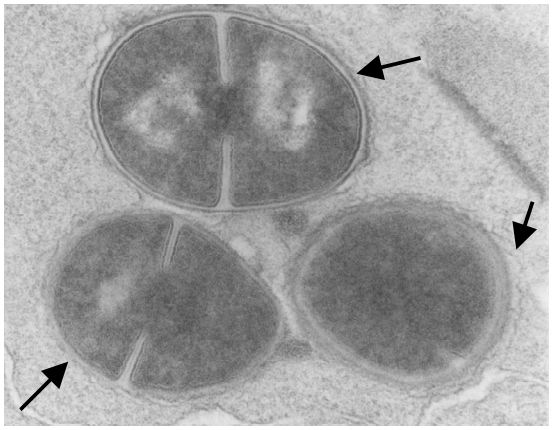
***L. monocytogenes* infected cells**	***S. aureus* infected cells**
	

Figure 21: demonstration in electron microscopy of the subcellular localization of *L. monocytogenes* and *S. aureus* in infected macrophages.

L. monocytogenes appears moving free in the cytosol, and surrounded by actin, while *S. aureus* is confined in vacuoles surrounded by a membrane (highlighted by arrows).

II.2.b.1. Intracellular activity of oritavancin against phagolysosomal (*S. aureus*) and cytosolic (*L. monocytogenes*) bacteria

The MIC and MBC of oritavancin in comparison with vancomycin against the strain of *S. aureus* and *L. monocytogenes* used in this study are shown in table 8. They illustrate (i) the bactericidal character of glycopeptides against *S. aureus* (MBC/MIC ratio = 4), and (ii) the high sensitivity of *L. monocytogenes* to oritavancin. In contrast, vancomycin was not bactericidal against *L. monocytogenes*. Since *S. aureus* is thought to thrive in phagolysosomes which are acidic vacuoles, MIC were also measured at pH 5.0, but were not different from those measured at pH 7.4.

Table 8: MIC and MBC of oritavancin and vancomycin against the bacterial strains used in this study					
Antibiotic	***S. aureus***			***L. monocytogenes***	
	MIC (µg/ml)		**MBC (µg/ml)**	**MIC (µg/ml)**	**MBC (µg/ml)**
	pH 7.4	**pH 5.0**			
Oritavancin	0.25	0.25	1	0.02	2
Vancomycin	1	1	4	1	> 64

In our experiments, oritavancin and vancomycin were usually used at extracellular concentration of 25 mg/L (as in our pharmacokinetic studies), and 50 mg/L, respectively, the latter mimicking the maximal serum peak level (Cmax) observed in patients receiving conventional doses of vancomycin (15 mg/kg).

a) Time – effects

Figure 22 shows that oritavancin exerted a marked bactericidal effect towards intracellular *S. aureus.* This effect developed rapidly, yielding an almost 4 log decrease of the post-phagocytosis within 24 h. In contrast, vancomycin was essentially static. After 5 h of incubation, neither oritavancin nor vancomycin were capable of preventing the intracellular growth of *L. monocytogenes*, which proceeded at approx. 60 % of the rate observed in controls.

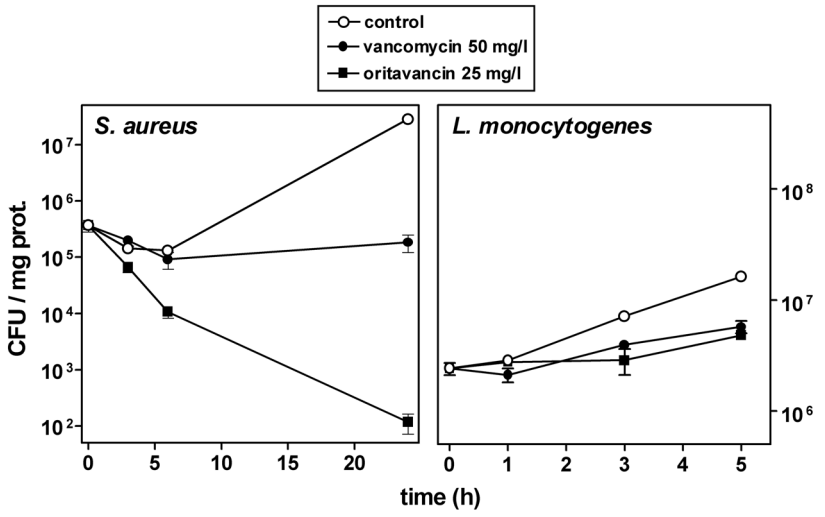


Figure 22: Variation of the number of viable (CFU) of *S. aureus* (left panel) or *L. monocytogenes* (right panel) phagocytosed by J774 macrophages upon subsequent incubation of cells in the presence of 50 mg/L vancomycin or 25 mg/L oritavancin or in control conditions (gentamicin 0.5 mg/L [left panel], no antibiotic [right panel]). Values are given as arithmetic means of CFUs per mg of protein ± standard deviation (n=3).

(Van Bambeke *et al.*, 2004a)

b) Concentration – effects

In order to better examine the potential concentration-dependence of the effects exerted by oritavancin on *S. aureus*, 24 h incubation studies were performed using a broad range of extracellular concentrations (figure 23). Considering extracellular activity first, two main observations can be made. First, and as anticipated, a bacteriostatic effect was obtained when bacteria were exposed to a concentration close to the MIC. Second, oritavancin was able to sterilize the culture (5 log decrease) upon exposure to therapeutically meaningful concentrations. Moving now to intracellular activity, oritavancin showed a clear dose-dependent effect that could be described, in first approximation, as a sigmoid relationship (see regression parameters below the figure). Some evidence of a 2 steps process with a shoulder at low multiples of the MIC can however be observed. As compared to the extracellular activity, it is noteworthy to mention that exposure to higher extracellular concentration is needed to reach an intracellular static effect (approx. 10 X MIC) and that intracellular sterilization cannot be achieve, even upon exposure at these larger extracellular concentrations.

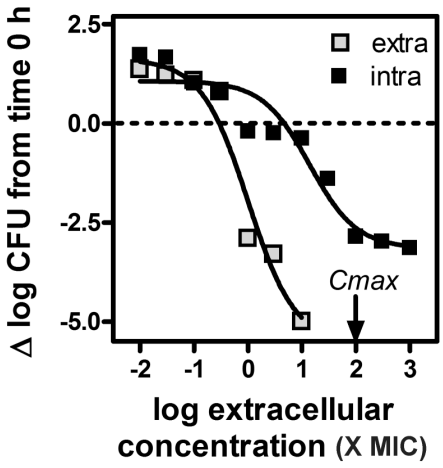


Figure 23: Change in the number of CFU (in log scale) after 24 h incubation of *S. aureus* in culture medium (grey symbols) or of *S. aureus* phagocytozed by THP-1 macrophages (closed symbols) upon exposure to increasing concentrations of oritavancin ranging from 0.05 X MIC to 1000 X MIC. The dotted line shows a static effect. – 5 log is the limit of detection.

Data are means ± SD of 3 independent experiments (when non visible, the SD bars are smaller than the symbols). All data were used to compute a sigmoid dose-response, by non linear regression (with the relevant parameters given below). The concentration corresponding to the Cmax is indicated by an arrow on the corresponding X axis.

(Barcia-Macay *et al.*, 2005)

Regression parameters for the sigmoid fit

extracellularly					**intracellularly**				
Static concentration	**EC_{50}**	**top**	**bottom**	**R^2**	**Static concentration**	**EC_{50}**	**top**	**bottom**	**R^2**
0.3	1.00	1.63	-5.55	0.956	4.8	13.8	1.07	-3.147	0.927

concentrations are expressed in X MIC; effects, as change from the initial inoculum

c) Morphological studies

In order to ascertain that the reduction in intracellular bacterial counts in our studies truly corresponded to an activity of oritavancin, we examined in electron microscopy the morphology of cell-associated *S. aureus* after 24 h incubation in control conditions or after exposure to the drug (figure 24). In control conditions, bacteria appeared darkly stained (panel A), often actively multiplying and surrounded by a tick cell wall (panels B, C). Upon exposure to oritavancin, a large number of bacteria appeared as ghosts with rarefied cytoplasmic material (D), and profiles also often showed granular material sometimes apposed on the periphery of the bacterial body (E).

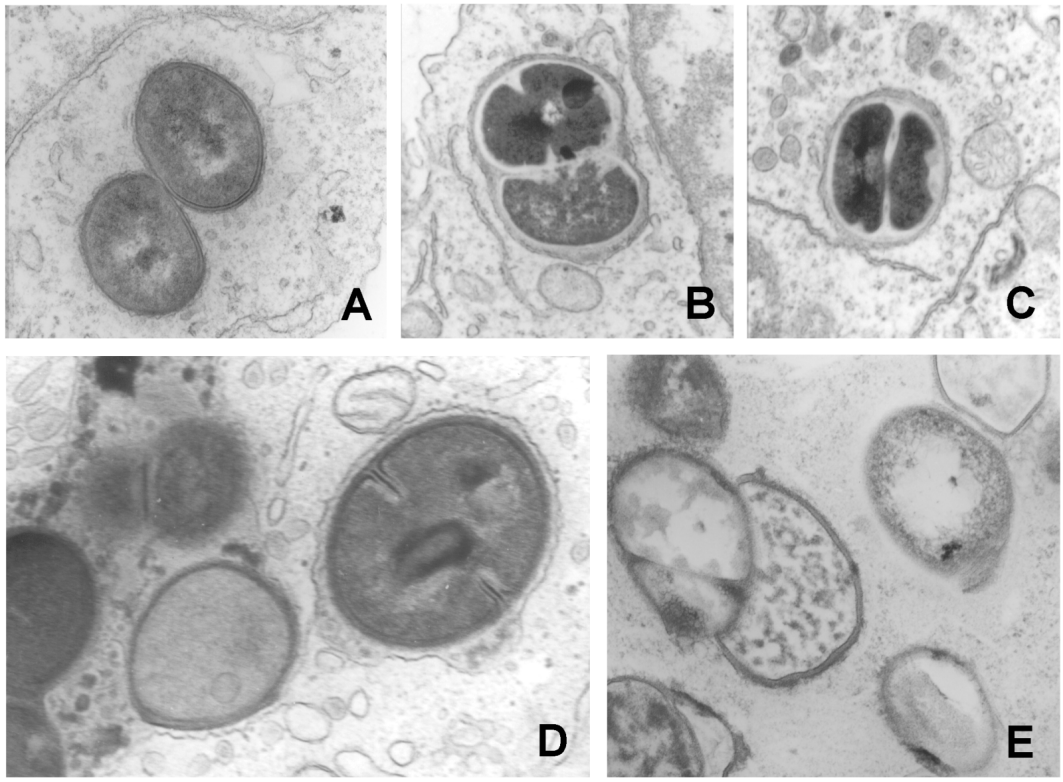


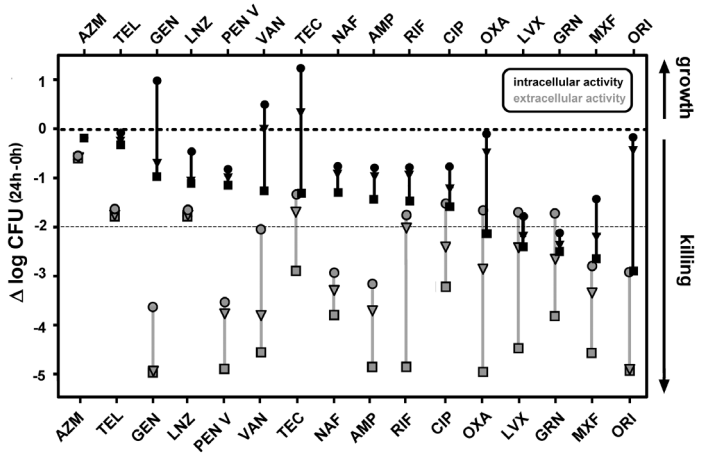
Figure 24: Electron microscopic appearance of serum-opsonised *S. aureus* in THP-1 macrophages 24 h after phagocytosis. Upper row: control cells (gentamicin was added at an extracellular concentration corresponding to its MIC [0.5 mg/L] to prevent extracellular growth); lower row: cells were exposed to oritavancin at an extracellular concentration corresponding to its Cmax (25 mg/L).

(Barcia-Macay *et al.*, 2005)

II.2.b.2. Conclusion: oritavancin, a useful drug for intracellular infection

The data presented here demonstrate that oritavancin exerts a time- and markedly concentration-dependent bactericidal effect against intracellular *S. aureus.* It is noteworthy to mention that it is actually the most active drugs among all those we tested in this model (figure 25).

Figure 25: Summary of the results obtained for all antibiotics tested during a 24 h incubation in culture medium (extracellular *S. aureus*; grey lines and symbols) or in THP-1 macrophages (intracellular *S. aureus*; black lines and symbols).



The ordinate shows the change in CFU in log scale. Each antibiotic was tested at a concentration corresponding to its MIC (circle), 10 X its MIC (triangles) and its Cmax (squares), with each of the corresponding results aligned vertically and linked with a bar to better illustrate the spread (concentration-dependency) of the results over that range (antibiotics for which symbols cannot be distinguished from one another are those for which concentration-dependency was minimal).

(Barcia-Macay *et al.*, 2005)

We must, however, note that the intraphagocytic activity of oritavancin is considerably lower that can be anticipated on the basis of its accumulation. A defeating effect of the acidic environment or of the slower rate of bacterial multiplication can probably be excluded based on the observation that MIC is not affected by pH (table 8) and that oritavancin activity is not affected by the growth phase of the bacteria (Mercier *et al.*, 2002). Other factors including an incomplete intracellular bioavailability of the drug (binding to some cellular constituents ?) could be evoked, as well as a reduced bacterial responsiveness.

On an indirect manner, the data from these pharmacodynamic studies also confirm the intracellular localization of the antibiotic. We indeed demonstrate a marked bactericidal effect against a phagolysosomal micro-organism *(S. aureus* [Seral *et al.*, 2003c]), but no or only negligible activity against a cytosolic bacteria (*L. monocytogenes* [Ouadrhiri *et al.*, 1999]), even though the latter is highly susceptible to oritavancin *in vitro*. This is in sharp contrast to fluoroquinolones, which

show activity against both types of bacteria in the same cellular model (Seral *et al.*, 2003a).

With respect to clinical applications, our data appear of interest, since they demonstrate useful activity against intracellular staphylococci in conditions of incubation (25 mg/L with 10 % calf serum) that are probably pertinent of those observed in humans taking into account the influence of protein binding on drug accumulation (see discussion on pharmacokinetic studies). Encouraging data have also been obtained by others in models of intracellular infections by MRSA or enterococci (Al Nawas and Shah, 1998; Al Nawas *et al.*, 2000). Targeted animal studies and clinical trials would therefore be most welcome in this context.

A still open question, which needs to be carefully addressed before considering a large-scale clinical use of oritavancin, concerns the potential toxicity of such a high cellular concentration of the drug. The prolonged terminal half-life of the molecule as measured in humans may indeed also suggest the existence of storage compartments, which could constitute potential targets for toxicity, even though major side effects have not been reported so far.

II.2.c. Cellular toxicity

At this stage, we have thus demonstrated that oritavancin accumulates in very large amounts in macrophages and other cultured cells, and is not homogeneously distributed in cells but is predominantly, if not exclusively, localized in lysosomes. This specific tropism is probably the reason for its high activity against *S. aureus*, which is believed to take refuge and to thrive within these organelles. However, such a huge and specific accumulation also raises issues regarding the potential for cytotoxicity of oritavancin. We therefore decided to first examine the morphology of cells exposed to clinically meaningful concentrations of oritavancin, using both phagocytic (J774 macrophages) and non-phagocytic (fibroblasts) cell lines, to detect gross alterations that the drug could induce. We also measured the cellular content in the main lipids (phospholipids and cholesterol), based on the appearance of part of the material accumulated in the cells. All the methods used in these experiments are described in details in (Van Bambeke *et al.*, 2005).

In a first series of studies, we systematically explored the conditions of incubation causing gross cell toxicity, as assessed by the release of lactate dehydrogenase in the culture medium. Using as a limit a release corresponding to twice the amount found in control cells at the beginning of the experiments (as in our previous studies of macrolide-induced phospholipidosis (Montenez *et al.*, 1999), we observed that macrophages could not be maintained for more than 24 h in the presence of 25 mg/L oritavancin, and fibroblasts, for more than 72 h with 40 mg/L. All experiments described hereunder used incubation times and drug concentrations that did not exceed these values.

II.2.c.1. Morphological studies

Examination of macrophages and fibroblasts incubated with 20 mg/L oritavancin in optic microscopy (using the plastic section method) revealed conspicuous modifications, which are illustrated in figure 26.

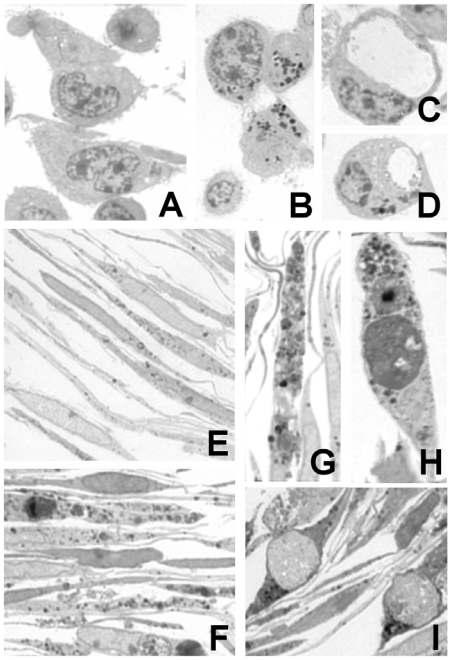


Figure 26: Appearance of macrophages and fibroblasts in the optic microscope (plastic sections technique); objective magnification: 63 X; A: control macrophages; B-D: macrophages exposed for 24 h to 20 mg/L oritavancin; E: control fibroblasts after 72 h of culture; F-I: fibroblasts exposed during 72 h to 20 mg/L oritavancin.

adapted from (Van Bambeke *et al.*, 2005)

Compared to control macrophages (A), treated cells showed an abundance of small, darkly colored vesicles in their cytosol (B). Occasionally, large, apparently empty structures were seen (C). In some cells, both types of alterations were found together (D). Dark granules were also observed in treated fibroblasts (F), in an abundance that was markedly larger than in control cells (E), and occupying an important proportion of the cytosol (G). But, most conspicuously, treated fibroblasts also showed several other types of alterations, which include giant, medium-dark colored vesicles structures (H), and other semi-translucent vesicles which were so large as to cause a deformation of the cell shape (I).

This triggered us to undertake a systematic study in the electron microscope. Typical images of the alterations seen with cells exposed to 20 mg/L oritavancin are presented in figure 27.

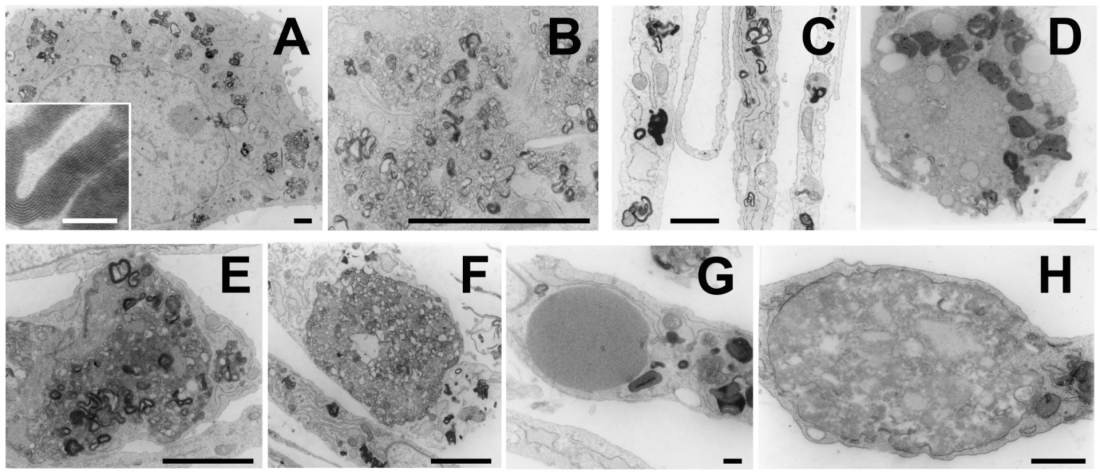


Figure 27: Appearance in electron microscopy of cultured cells exposed to oritavancin at and extracellular concentration of 20 mg/L. A-B: mouse macrophages (24 h incubation); C-H: rat embryo fibroblasts (72 h incubation). Bars : 1 µm (100 nm for the inset to panel A).

adapted from (Van Bambeke *et al.*, 2005)

The cytoplasm of macrophages (A) appeared dotted with electron-dense structures of varying size and shape that (i) were seen in vacuoles, and (ii) revealed a lamellar structure when examined at high magnification (see inset of panel A). In some cells (B), accumulation of this material became so extensive that it filled up large zones of the cytosol with a mixture of strongly electron-dense structures (corresponding to tightly apposed lamellae) and less solid elements that looked as small, closely apposed vesicles. A limiting membrane was often seen around these structures. Alterations in fibroblasts (C-H) were more diverse in appearance and shape, but always in vacuoles or membrane-bounded structures. Electron-dense, medium-sized structures could easily be seen in almost all cells that otherwise looked capable of protein synthesis as judged by the aspect and size of their rough endoplasmic cisternae (C). In some cells (D), large, irregular bodies were seen with an unstructured, electron-dense background, together with large, electron-lucent or poorly electron-dense matrix. Other cells (E) showed large clumps of vesicles with a mixture of electron-dense and electron-lucent inclusions. Sometimes, these vesicles seem to be hold together by a surrounding membrane as in a giant digestive vacuole (F). In other cells, we saw large vacuoles with a finely granular, homogenous content (G). Sometimes also, we observed cells with giant vacuoles containing a highly heterogeneous material of blurred appearance (H).

II.2.c.2. Biochemical analyses and determination of cell accumulation of oritavancin

Since part of the material accumulated by cells was structurally evocative of what is commonly observed in the tissues of patients suffering from disorders of the lysosomal catabolism of polar lipids, we examined the influence of oritavancin on the cell content in total phospholipids and cholesterol.

a) Dose-effects

For this purpose, cells were incubated with increasing concentrations of oritavancin for 1 day (macrophages) or 3 days (fibroblasts). Figure 28 (upper panels) shows (i) that phospholipids accumulated on a concentration-dependent fashion in both types of cells, reaching a content of about 150 % that in controls at the highest concentration tested (25 mg/L and 40 mg/L for macrophages and fibroblasts, respectively); (ii) that accumulation of cholesterol was even more extensive, reaching values about 3.5 times those of controls under the same conditions. The form (free

vs. esterified) under which cholesterol was accumulating was then examined and the results are shown in the middle panels of figure 28.

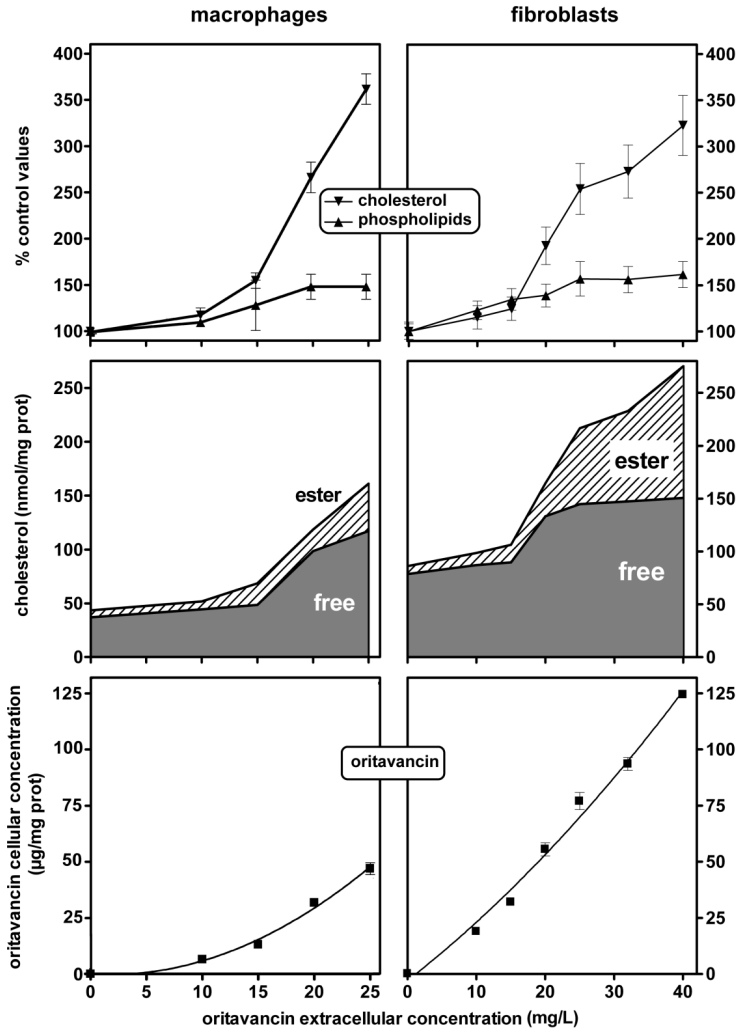


Figure 28: Accumulation of total cholesterol and phospholipids (top panel), free and esterified cholesterol (middle panels), and of oritavacin (lower panels) in mouse J774 macrophages (*left panels*; 24 hor in rat embryo fibroblasts (*right panels*; 72 h incubation) with increasing extracellular concentrations of oritavancin. All data are means ± SD of three independent experiments (error bars that are not visible are included in the symbols). Data for phospholipids and cholesterol are expressed as percentage of the original content of control cells collected at the same time (160.0 ± 5.3 nmol/mg protein for phospholipids and 40.2 ± 2.6 nmol/mg protein for cholesterol in macrophages; 259.9 ± 21.7 nmol/mg protein for phospholipids and 86.1 ± 8.8 nmol/mg protein for cholesterol for fibroblasts).

(Van Bambeke *et al.*, 2005)

In control cells, cholesterol was mostly under its free form (about 85 % of the total content). In macrophages exposed to oritavancin, the cell content in both forms increased roughly in parallel. In fibroblasts, the cell content in both forms also increased in parallel but up to an extracellular concentration of 20 mg/L only. At larger

concentrations, however, the increase concerned almost only the esterified form, which accounted for about 45 % of the total cell cholesterol content in cells exposed to 40 mg/L oritavancin. In parallel, we measured the cell accumulation of oritavancin, and the results are shown in the lower panels of figure 28. As described previously, the accumulation of oritavancin occurred on a cooperative fashion with respect to its extracellular concentration. Based on the data obtained at the largest extracellular concentration tested, we calculated that the apparent cellular concentrations of oritavancin reached values about 400-fold (macrophages) and 600-fold (fibroblasts) higher than in the extracellular medium.

b) Time-effects

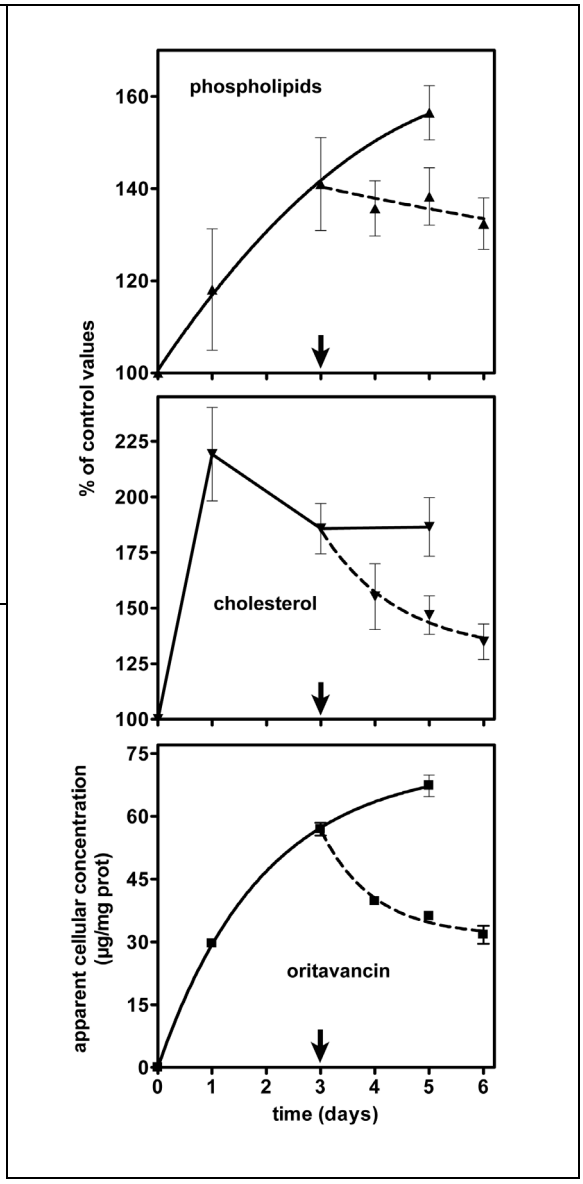


Figure 29: Kinetics of accumulation and loss of phospholipids (upper panel), total cholesterol (middle panel), oritavancin (lower panel) in fibroblasts exposed to 20 mg/L oritavancin. All cells were incubated with of 20 mg/L oritavancin for a first period of 3 days. At this time point (indicated by a vertical arrow), cultures were either maintained in the continuing presence of oritavancin (solid lines), or washed and reincubated in drug-free medium (broken lines). All data are means ± SD of three independent experiments. Data for phospholipids and cholesterol are expressed as percentage of the content of control cells at the same day (values were 221.3 ± 28.2 nmol/mg prot for phospholipids and 58.5 ± 6.7 nmol/mg prot for cholesterol at day 0, and raised during the culture to reach 252.9 ± 11.3 and 94.9 ± 4.1 nmol/mg prot at day 5).

(Van Bambeke *et al.*, 2005)

As shown in figure 29 (upper panel), the cell phospholipid content increased continuously as a function of the time so as to reach a value approx. 1.5-fold that of controls after 5 days. This accumulation was only partially reversible upon transfer of cells to drug-free medium within the time-frame of the experiment. The middle panel shows that, in contrast with phospho-lipids, the accumulation of cholesterol

was rapid, reaching twice the value observed in controls after one day of incubation only, and slightly decreased thereafter. This accumulation of cholesterol was also largely reversible when cells were transferred to drug-free medium (with only 30 % excess over controls after 3 days). The lower panels of figure 29 show that the accumulation of oritavancin proceeded continuously during the period of exposure, with however, a trend towards a plateau at the 5th day (at an apparent cellular to extracellular concentration ratio of about 700). Efflux of oritavancin from fibroblasts proceeded at a rate apparently faster than its uptake for the first 24 h, but remained thereafter largely incomplete since about half-of the cell-associated drug was still present after 3 days of efflux.

c) Correlations

The data generated by these studies were then used to examine whether constant molecular relationship could be deduced from our observations. For this purpose, all accumulation data were converted into nmol per mg protein, and for phospholipids and cholesterol, expressed as excess over control values.

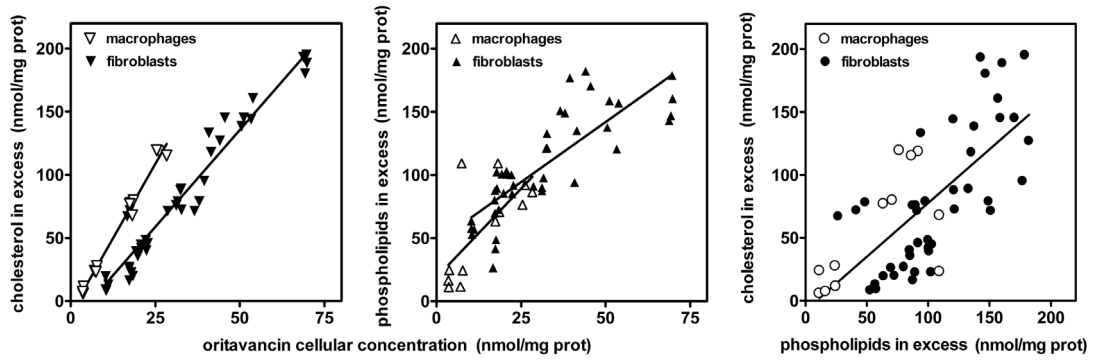


Figure 30: **Correlation between the cell accumulation in oritavancin and the excess of total cholesterol (left panel) and phospholipids (middle panel), and between the excess of cholesterol and the excess of phospholipids (right panel) in macrophages (open symbols) and fibroblasts (closed symbols) exposed to oritavancin**. The data used to construct this figure are those of figures 28 and 29, but all data are expressed in molar amounts (for cholesterol and phospholipids, the values found in the matched control cells have been subtracted). Regression parameters: cholesterol *vs* oritavancin: R^2 = 0.987, slope = 4.73 ± 0.17 in macrophages and R^2 = 0.930, slope = 3.07 ± 0.13 in fibroblasts; phospholipids *vs* oritavancin: R^2 = 0.488, slope = 2.82 ± 0.91 in macrophages and R^2 = 0.663, slope = 1.90 ± 0.21 in fibroblasts; cholesterol *vs* phospholipids: R^2 = 0.507, slope = 0.85 ± 0.11.

(Van Bambeke *et al.*, 2005)

Figure 30 shows the correlations obtained between data of phospholipids, cholesterol and oritavancin. An excellent correlation was observed between the excess of total cholesterol and the cell accumulation of oritavancin, with a slope suggesting a molar ratio of 3.1 (fibroblasts) to 4.7 (macrophages). The correlation was less significant for

phospholipids, partly due to their lack of disappearance upon drug efflux, but the slope suggested a 1.9 to 2.8 phospholipids:oritavancin molar ratio. The correlation between the molar excess of cholesterol and that of phospholipids was also only partial, but it was interesting to note that the slope of the correlation suggested a constant molar ratio close to 1 disregarding the type of cells studied.

II.2.c.3. Specificity vs. other glycopeptides

The specificity of the effects of oritavancin on lipid accumulation in fibroblasts was tested by repeating these experiments with vancomycin and teicoplanin. To make the comparison relevant to the clinical use of these antibiotics, we used concentrations of 50 and 100 mg/L, corresponding to the maximal serum concentrations commonly observed in patients without gross abnormal elimination or distribution parameters and receiving conventional treatments with these antibiotics. Table 9 shows typical results obtained after 3 days of incubation, in comparison with oritavancin. Changes observed with vancomycin and teicoplanin were not statistically significant, in contrast to what was seen with oritavancin.

Table 9: **Accumulation of phospholipids and cholesterol in rat fibroblasts incubated for 3 days with glycopeptides**

(Van Bambeke *et al.*, 2005)

glycopeptide	extracellular concentration (mg/L)	% of control values	
		phospholipids	cholesterol
vancomycin	50	93 ± 7	105 ± 26
teicoplanin	100	109 ± 10	127 ± 28
oritavancin	25	136 ± 10	272 ± 47

II.2.c.4. Additional studies: mechanism of the accumulation of phospholipids

Many cationic amphipathic drugs are known to cause phospholipidosis by inhibiting the activity of lysosomal phospholipases. We therefore investigated whether oritavancin was able to inhibit phospholipid catabolism on an *in vitro* model of liposomes, using as enzymatic source a soluble fraction of purified liver lysosomes (see [Mingeot-Leclercq *et al.*, 1990] for a description of the method).

In particular, we tested the influence of oritavancin on the activity of phospholipase A1 towards phosphatidylcholine included in liposomes made of a mixture of lipids representative of biological membranes, but with variable content in negatively-charged phospholipids (represented by phosphatidylinositol in our model). Figure 31 shows that oritavancin was indeed able to inhibit the enzymatic activity on a concentration-dependent manner (in the 50 – 400 µM range, corresponding to concentrations of 90 - 717 mg/L). Moreover, higher drug concentrations were needed for inhibiting the enzymatic activity to a given extent when the vesicles were enriched in acidic phospholipids (viz. IC_{50} values, which were twice higher when vesicles contained 18 % (PI 3) rather than 12 % (PI 2) of phosphatidylinositol).

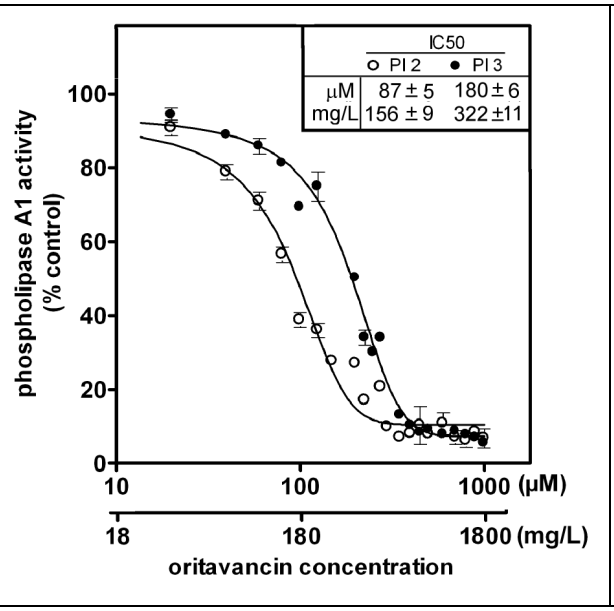


Figure 31: Influence of oritavancin on the activity of phospholipase A1 from purified lysosomes towards ^{14}C-phosphatidylcholine incorporated into negatively-charged liposomes made of cholesterol:sphingomyelin:phosphatidylcholine:phosphatidylinositol 5.5:4.4:3 or 5.5:4:4:2 (in molar ratio).

Results are expressed as % of the activity measured in the absence of the drug and are means ± SD of 3 independent experiments. Error bars that are not visible are included in the symbols. The inset shows the IC_{50} values calculated from the sigmoid regression of the curves (R^2 > 0.97).

II.2.c.5. Conclusion: accumulation of oritavancin causes cellular alterations

This study first of all shows that oritavancin accumulates to exceptional levels not only in macrophages, but also in non-phagocytic cells, like embryo-derived fibroblasts. Interestingly enough, the accumulation levels seen are of the same order of magnitude in these two cell types, suggesting that internalization occurs through a non cell-specific process. We suggested indeed that oritavancin uptake results from adsorptive endocytosis, which drives the drug in large amounts in the lysosomes.
Accordingly, one is struck by the fact that abnormal elements appearing in oritavancin-treated cells, beyond their highly variable aspects and shapes, seem always to be located in membrane-bounded structures, strongly suggesting that we are dealing here with a metabolic disorder of lysosomes and related vacuoles.

Lysosomes are endowed with digestive capabilities towards most naturally occurring polymers (de Duve and Wattiaux, 1966). The highly heterogeneous aspect of the material seen in lysosomes and related vacuoles upon oritavancin treatment suggests, therefore, that several lysosomal catabolic pathways are affected by this drug. The images seen, actually, are reminiscent of two situations in which the activities of several lysosomal enzymes are simultaneously decreased, namely (i) the mucolipidosis type II (I cell disease [Leroy and Spranger, 1970; Hanai *et al.*, 1971]), a pathology in which newly synthesized lysosomal enzymes are diverted to the extracellular milieu (Neufeld *et al.*, 1977) so as to result in a multiple lysosomal enzyme defect (Lightbody *et al.*, 1971), and (ii) the immunological inhibition of several lysosomal hydrolases in fibroblasts (Tulkens *et al.*, 1970). Our present data should, therefore, trigger further studies aiming at comprehensive measurements of the digestive capabilities of lysosomes in oritavancin-treated cells.

Our biochemical studies, nevertheless, already give hints towards perturbations of the metabolism of cholesterol and of phospholipids caused by oritavancin. Since oritavancin is a cationic amphiphile (Cooper *et al.*, 1996), we may suspect direct interaction of this drug with membrane lipids. This may explain why the cell content in phospholipids and cholesterol increases largely in parallel, and in an almost constant molar ratio with oritavancin accumulation. Cationic amphiphilic drugs, which include a variety of pharmacologically-unrelated compounds but which all display one or several aminofunctions together with a large apolar moiety (Kacew *et*

al., 1997), have been known since long to form complexes with phospholipids layers (Lullmann *et al.*, 1980; Kursch *et al.*, 1983). These drugs, however, tend to induce a specific accumulation of phospholipids in cells, which structurally translates in the deposition of lamellar structures (also called myeloid bodies) that, in many cases, fill up the lysosomes (Drenckhahn *et al.*, 1976; Lullmann *et al.*, 1978; Kodavanti and Mehendale, 1990). A similar situation has also been observed in fibroblasts exposed to macrolides (Van Bambeke *et al.*, 1996b; Montenez *et al.*, 1999), or aminoglycosides (Aubert-Tulkens *et al.*, 1979). Accumulation of cholesterol has also been for some of these agents several cationic amphiphilic drugs (Chatelain *et al.*, 1989; Yoshikawa, 1991; Palmeri *et al.*, 1995), including azithromycin (Montenez, unpublished observations, and Van Bambeke *et al.*, 1998).

A first clue in the elucidation of the mechanism responsible for phospholipid accumulation is provided by the observation that oritavancin is able to inhibit lysosomal phospholipases activity. By analogy with the previous studies of our laboratory dealing with aminoglycosides and macrolides (Mingeot-Leclercq *et al.*, 1988; Mingeot-Leclercq *et al.*, 1990; Van Bambeke *et al.*, 1996b; Montenez *et al.*, 1999), this effect could be a consequence of the binding of the drug to the acidic phospholipids of membranes brought to the lysosomes for being degraded. Our *in vitro* studies show indeed that oritavancin inhibits the phospholipase A1 activity on a way that depends on the amount of acidic phospholipids included in the membrane. Interestingly, the concentrations of oritavancin that are needed to inhibit the enzyme *in vitro* are far below those reached in the lysosomes of the cells exposed to the drug and developing phospholipidosis. We can indeed evaluate that the lysosomal content in oritavancin should be ~ 10-200 mg/ml, based on a cell volume of 5 µl/mg protein and of a lysosomal volume corresponding to 10 % of the total cell volume. This may explain why phospholipidosis is irreversible, the cell-associated amount of the drug remaining sufficient to inhibit the enzyme even after 3 days of chase.

Beyond the molecular mechanisms of oritavancin-induced cellular alterations, which need to be further studied, the present work raises critical questions regarding the potential toxicity of this antibiotic to patients. Our observations were made with concentrations of oritavancin close to the expected C_{max} in humans but maintained continuously for several days, creating AUC considerably larger that what could be seen during conventional, discontinuous administration. Oritavancin accumulated by cells, however, leaks out only very slowly and partially, so that discontinuous administration may not much protect against sustained accumulation in the long term.

Oritavancin is also reported to be highly protein-bound in human serum (see table 3), so that cell uptake *in vivo* could be much lower if related to free concentration only. We, unfortunately, lack of data concerning the tissular accumulation of oritavancin in animals or humans. We explained previously, however, that J774 macrophages incubated with 20 mg/L oritavancin in the presence of 10 % calf serum (as in the present conditions) accumulate roughly the same amount of drug than those maintained with 3-5 mg/L oritavancin in the absence of serum. Even though extrapolation to human cells remains largely uncertain, cellular accumulation seems to be a key event, since no significant cell alteration has been reported for vancomycin and teicoplanin, which accumulate to much lower amounts in cultured cells (Maderazo *et al.*, 1988; Van der Auwera *et al.*, 1988). Interestingly, however, cell toxicity has been observed for vancomycin in fibroblasts from I-cell disease patients (Dumontel *et al.*, 1998), which show an impaired lysosomal catabolism.

The link between accumulation of phospholipids, cholesterol or other undigested substrates in lysosomes and cell toxicity remains largely unsettled. Nevertheless, phospholipid accumulation is commonly recognized as associated with the potential toxicity of several drugs accumulating in kidney (aminoglycosides, e.g. [Mingeot-Leclercq and Tulkens, 1999]), lung (amiodarone, e.g. [Reasor and Kacew, 1996]) or liver (diethylaminoethoxyhexestrol, e.g. [Yamamoto *et al.*, 1971]). We also know that excesses of both free and esterified cellular cholesterol are toxic, as illustrated by their involvement in the Niemann-Pick disease type C (Palmeri *et al.*, 1995; Morris and Carstea, 1998), or in atherosclerosis (Kruth, 2001; Tabas, 2002). In this respect, the screening of the lipidogenic capacity of drugs on cell culture models is nowadays considered as an early step in the assessment of their safety profile for pharmaceutical research and drug development (Casartelli *et al.*, 2003). The present study should, therefore, trigger more comprehensive studies aimed at better defining the true toxic potential of oritavancin.

II.3. General discussion : can we dissociate intracellular activity and cellular toxicity ?

II.3.a. Current view of oritavancin fate in eukaryotic cells

Figure 32 depicts our current view of the fate of oritavancin in eukaryotic cells. The drug enters cells by endocytosis (the efficiency of this process is probably improved by adsorption at the cell surface). It is then stored in lysosomes, where it can exert its antibiotic activity towards bacteria thriving in this compartment (like *S. aureus*), but also alter cellular metabolism and cause the deposition of lipids and the accumulation of material of still undetermined nature.

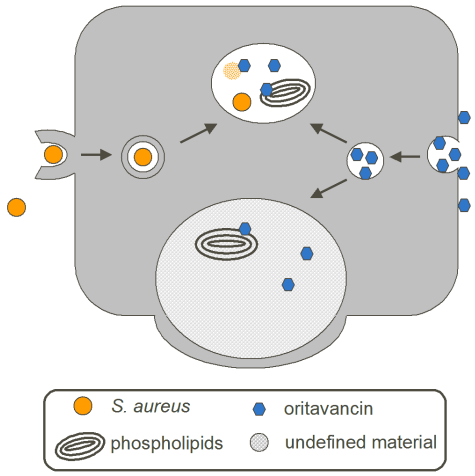


Figure 32: schematic view of the fate of oritavancin in eukaryotic cells. The drug enters cells by (adsorptive ?) endocytosis, is driven to the lysosomes where (a) it exerts its antibiotic activity against intracellular bacteria, and (b) causes the deposition of lipids and other undefined material, leading to the appearance of gigantic vesicles. The trapping of the drug in this accumulated material may be partly responsible for its unexpectedly low intracellular activity.

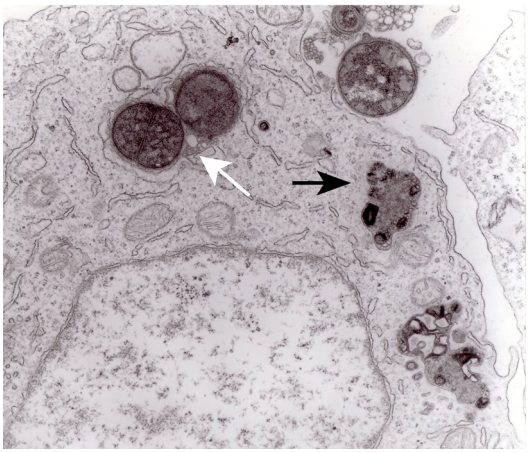


Figure 33: electron microscopic appearance of an infected macrophage exposed for 24 h to 25 mg/L oritavancin. The concomitant presence of bacteria and of morphological alterations suggests that a reduced intracellular bioavailability may partly impair the antibiotic activity (the white arrow points to intracellular bacteria; the black arrow, to the material accumulated).

Figure 33 illustrates that toxicological alterations develop in infected macrophages exposed to oritavancin in conditions where it exerts a marked intracellular activity (24 h, extracellular concentration, 25 mg/L [see figure 22]). This suggests that part of the intracellular drug may be trapped in this accumulated material, and become therefore unavailable for exerting its antibiotic activity. More importantly, it also raises question regarding the safety of this drug in its conditions of clinical use.

We will therefore compare oritavancin with other lysosomotropic antibiotics in terms of cellular activity and toxicity.

II.3.b. Comparison with other lysosomotropic antibiotics

Accumulation of antibiotics in lysosomes has the potential of allowing fighting intracellular bacteria (Tulkens, 1991), but also of eliciting toxic effects and metabolic alterations (de Duve *et al.*, 1974). The present study exemplifies this situation, which was already observed in previous studies dealing with aminoglycosides (Aubert-Tulkens *et al.*, 1979) or macrolides (Van Bambeke *et al.*, 1996b), two other antibiotics preferentially accumulating in lysosomes. In the next pages, we have therefore used available data for oritavancin, gentamicin (as a typical aminoglycoside) and azithromycin (as the best studied macrolide) to analyze correlation between accumulation and activity or toxicity. For this purpose, we concentrate on data on intracellular *S. aureus* on the one hand, and on phospholipidosis, on the other hand.

II.3.b.1. Comparing activity

Table 10 presents on a synoptic fashion the two parameters that are usually considered as critical for intracellular activity, namely (i) the intrinsic antibacterial potency (MIC) at pH mimicking both the extracellular and intracellular pH (within the lysosomes where both the antibiotics and the bacteria are thought to be localized), and (ii) the cellular accumulation (as obtained at an extracellular concentration corresponding to the drug Cmax in human serum). We also calculate the ratio of the cellular concentration to the MIC, in relation with the observed activity (expressed as variation from the original inoculum over a 24-h incubation).

Table 10: relation between intracellular activity against *S. aureus* in THP-1 macrophages and cellular concentration for three lysosomotropic antibiotics

data from (Barcia-Macay *et al.*, 2005)

Antibiotic	MIC (µg/ml)		extracellular concentration (µg/ml)	cellular accumulation	cellular concentration		Δ log CFU from time 0
	pH 7.4	pH 5.4			µg/ml	X MIC (pH 5.4)	
gentamicin	0.50	16	18	4.4	79	5	-1.0
azithromycin	0.50	512	0.5	38	19	0.04	-0.2
oritavancin	0.25	0.25	25	148	3700	14800	-2.9

We can see that, in these conditions, oritavancin is the most active drug, reducing about 1,000-fold the intracellular inoculum, whereas azithromycin is only static. Gentamicin shows an intermediate behavior. We also observe that there is no direct

correlation between drug accumulation and activity. Indeed, azithromycin is less active than gentamicin, in spite of its higher accumulation. This poor activity could be however easily explained by (i) the marked defeating effect of acidity on azithromycin activity and (ii) the fact that macrolides are bacteriostatic (see chapter I.2.a.). Oritavancin is undoubtedly the most active drug (but its activity appears limited in view of its very large intracellular concentration [as deduced from its cell content expressed in multiple of its MIC at acidic pH]).

This apparently lack of correlation between accumulation and activity actually stems simply from the fact that experiments illustrated in table 10 were made at only one extracellular concentration, which is often the case in many reports. The picture changes entirely if we examine a large range of concentrations (figure 34).

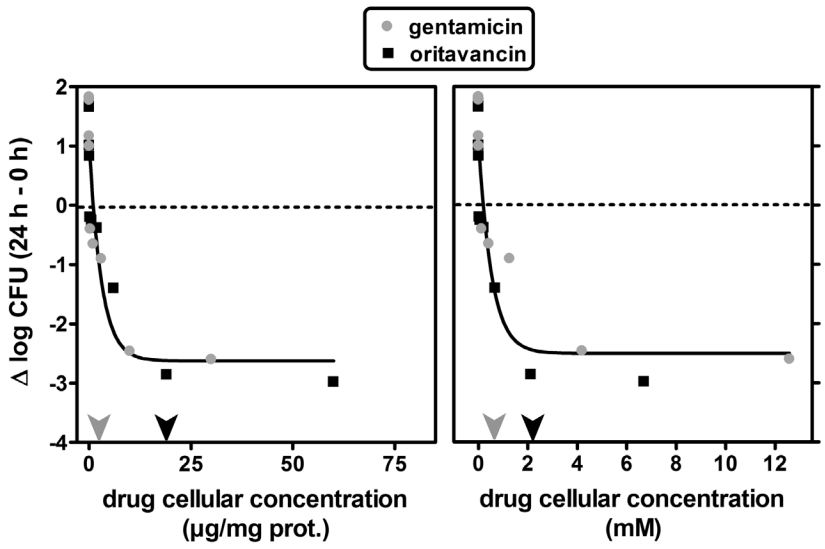


Figure 34: relation between the intracellular concentration of two concentration-dependent antibiotics accumulating in lysosomes (gentamicin [grey] and oritavancin [black]) and their intracellular activity against *S. aureus* in human macrophages. The activity is measured by the change in CFU after 24 h incubation in the presence of increasing concentrations of each antibiotic. The antibiotic cellular concentration is expressed either in µg/mg prot (*left panel*) or in mM (*right panel*). The arrows point to the cellular concentration reached upon incubation with an extracellular concentration corresponding to the human Cmax (18 µg/ml for gentamicin; 25 µg/ml for oritavancin).

A single half-sigmoid dose response is fitted on the whole data in each case (R^2 = 0.88)

data from (Barcia-Macay *et al.*, 2005)

A single half-sigmoid dose-response can indeed nicely be fitted on data for gentamicin and oritavancin, demonstrating clear concentration-dependency for these antibiotics in the intracellular milieu (azithromycin was not included in the graph since it is always

bacteriostatic). Of interest is also the fact that a plateau (at about 3 log decrease in CFU) is reached for both drugs (for gentamicin, however, this plateau is observed for cellular concentrations obtained for cells exposed to extracellular drug concentrations far above the Cmax; it has therefore no clinical significance). This similarity in behavior is in contrast with large differences in intrinsic activity, as assessed by the determination of the MIC at pH 5.4 (i.e. the pH of phagolysosomes). This may indicate that the bioavailability of gentamicin in phagolysosomes is considerably larger than that of oritavancin, or that intracellular *S. aureus* is considerably less sensitive to oritavancin in phagolysosomes than in broth. Interestingly, the relationship is not markedly modified if considering molar rather than mass concentration, indicating that the difference in molecular weight between gentamicin and oritavancin is not the reason for their difference in intracellular activity.

II.3.b.2. Comparing phospholipidosis

Table 11 compares the capacity of the three antibiotics to cause phospholipidosis in fibroblasts. The conditions were selected so as to reach a grossly similar molar content of each drug in cells.

Table 11: relation between capacity to cause phospholipidosis in cultured fibroblasts and cellular concentration for three lysosomotropic antibiotics

data from (Van Bambeke *et al.*, 1996b; Van Bambeke *et al.*, 1998; Van Bambeke *et al.*, 2005)

Antibiotic	culture conditions	Drug cellular concentration			total phospholipid content (% control)
		µg/mg prot	nmol/mg prot	µM	
gentamicin	500 µg/ml, 4 days	6.2	13.0	2600	142
azithromycin	5 µg/ml; 7 days	10.5	14.1	2815	125
oritavancin	25 µg/ml; 3 days	32.0	17.8	3570	135

We know that phospholipidosis induced by all three drugs is due to their capacity to impair the activity of lysosomal phospholipases (see [Mingeot-Leclercq *et al.*, 1990; Van Bambeke *et al.*, 1996b] for gentamicin and azithromycin).

We have therefore compared the ability of these three drugs to inhibit the main phospholipase (phospholipase A1) when assayed towards phosphatidylcholine included in liposomes. Table 12 compares their IC_{50} (concentrations causing a 50 % reduction in enzymatic activity), using data from previous publications for comparators (Van Bambeke *et al.*, 1996b).

For all three drugs, table 12 shows that higher drug concentrations are needed for enzymatic inhibition when liposomes are enriched in acidic phospholipids, suggesting that neutralization of the negative charge of the bilayer is a key determinant in this process (as discussed previously for gentamicin and azithromycin [Mingeot-Leclercq *et al.*, 1990; Van Bambeke *et al.*, 1996b; see also Piret *et al.*, 2005]). The data presented in table 11 also indicate that the concentrations reached in the lysosomes of the cells (considering that these organelles represent less than 10 % of the cell volume and that most of these drugs is localized in the lysosomes) largely exceed what is needed to obtain a full inhibition of phospholipase activity *in vitro.*

Table 12 : comparison of the capacity of three lysosomotropic antibiotics to inhibit lysosomal phospholipase A1 activity *in vitro* *

antibiotic	IC 50 (µM)	
	PI 2 [a]	PI 3 [a]
gentamicin	28 ± 3	102 ± 10
azithromycin	34 ± 3	101 ± 1
oritavancin	87 ± 5	180 ± 6

* towards phosphatidylcholine incorporated in vesicles made of cholesterol : phosphatidylcholine : sphingomyelin : phosphatidylinositol in molar ratio of 5.5:4:5:2 (PI 2) or 5.5:4:4:3 (PI 3)

As for activity, we then examined the change in phospholipid content over a wide range of drug cellular concentrations (figure 35), using the data generated in this study for oritavancin together with those published previously in the same model for gentamicin (Aubert-Tulkens *et al.*, 1979) and azithromycin (Montenez, 1996; Van Bambeke *et al.*, 1998). This analysis shows that while the three drugs behave differentially on a mass basis, they all have a similar propensity to induce phospholipidosis when compared on a molar basis. This observation is at variance with the differences in IC_{50} shown in table 12, which could indicate that other factors than the simple inhibition of phospholipase A1 activity may be critical. We can however safely conclude that there is a clear correlation between drug cellular concentration and phospholipid overloading.

The question which should now be answered is whether intracellular activity and propensity to induce cellular alterations can be dissociated, or whether, *a minima*, we could obtain significant antibacterial activity with no or minimal lipid overloading. Examining the range of cellular concentrations that can be reached *in vivo*, figure 35 also shows that these conditions will definitely cause a marked phospholipid accumulation with oritavancin, whereas gentamicin and azithromycin will be less deleterious.

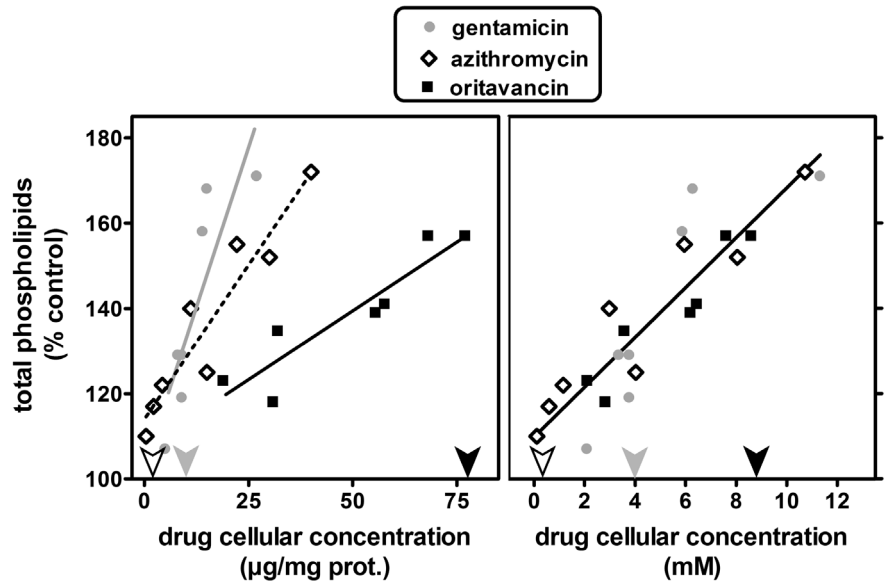


Figure 35: relation between the intracellular concentration of three lysosomotropic antibiotics (gentamicin [grey], azithromycin [white], and oritavancin [black]) and their capacity to cause phospholipids accumulation in rat embryo fibroblasts. Phospholipidosis is expressed in % of control values. The antibiotic cellular concentration is expressed either in µg/mg prot (*left panel*) or in mM (*right panel*). The arrows on each graph point to the highest cellular concentration that can be probably reached *in vivo* in target organs for toxicity, under conditions relevant of the clinical use of these drugs; see [El Mouedden *et al.*, 2000] for gentamicin, [Van Bambeke *et al.*, 1998] for azithromycin, and [Van Bambeke *et al.*, 2005 or figure 28] for oritavancin).

A linear regression is fitted on data for each individual antibiotic when cellular contents are expressed in µg/mg prot (slope: 2.99 ± 0.79, R^2 = 0.75 for gentamicin; slope: 1.45 ± 0.20, R^2 = 0.89 for azithromycin; slope: 0.65 ± 0.12, R^2 = 0.85 for oritavancin), but a single linear regression can be fitted on the whole data when cellular contents are expressed in molar concentrations (slope: 5.84 ± 0.65, R^2 = 0.81).

constructed from data of (Aubert-Tulkens *et al.*, 1979; Montenez, 1996; Van Bambeke *et al.*, 1998; Van Bambeke *et al.*, 2005)

Thus, the conclusion is inescapable that the intracellular activity that oritavancin may offer will be associated with clear-cut cellular changes. We do not know however how these *in vitro* data will translate *in vivo* in terms of toxicity.

II.3.c. Perspectives for future research

The next paragraphs give driving forces of experimental work that could be done for refining the model proposed to describe the interaction between oritavancin and eukaryotic cells.

II.3.c.1. Learning more on oritavancin cellular pharmacokinetics

We suspect that oritavancin enters cells by adsorptive endocytosis, essentially on the basis of the comparison of its clearance with that of tracers of fluid phase and adsorptive endocytosis. The adsorption site is probably of low affinity since a simple washing with physiological serum is sufficient to bring the cell-associated concentration back to a value close to zero when cells are incubated with the drug at 4°C, indicating that the bulk of the drug located at the cell surface has been removed. Some key experiments to better define the nature of this interaction could consist in:

a) measuring the binding of oritavancin to lipids

Determining drug binding to liposomes with a composition mimicking that of the plasma membrane (Esko and Raetz, 1983) would be a useful approach, which will allow for comparisons with our previous studies dealing with aminoglycosides (Mingeot-Leclercq *et al.*, 1990), macrolides (Van Bambeke *et al.*, 1996b; Montenez *et al.*, 1999), or prodrugs of β-lactams (Chanteux *et al.*, 2003b).

b) evaluating the binding of oritavancin to proteins

Using cell membranes as starting material would allow to detect in parallel an interaction with surface proteins, based on the rationale that the endocytosis of non-antibiotic glycopeptides or glycoproteins is facilitated by an interaction of their saccharidic moiety with lectins (Baenziger and Fiete, 1982).

II.3.c.2. Learning more on oritavancin cellular pharmacodynamics

Three main axes of research could be developed in this area.

a) exploring the mechanism of the bactericidal activity in broth

Preliminary data with telavancin and other alkyl derivatives suggest they could induce unspecific perturbations of bacterial membranes (Allen and Nicas, 2003) and

disturb their integrity (Debabov *et al.*, 2004; Higgins *et al.*, 2005). This hypothesis could be further substantiated by evaluating the capacity of glycopeptides to alter membrane permeability (Van Bambeke *et al.*, 1993; Mingeot-Leclercq *et al.*, 2001), using liposomes with a composition mimicking that of bacterial membranes (Hayami *et al.*, 1979; Rozgonyi *et al.*, 1981; Rozgonyi *et al.*, 1990; Sakayori *et al.*, 2003) or vesicles reconstituted from bacterial membranes (Huijbregts *et al.*, 1996), and including lipids coupled to peptides terminating in D-Ala-D-Ala (as in glycopeptide susceptible strains) or in D-Ala-D-Lac or D-Ala-D-Ser (as in vancomycin-resistant enterococci) (Cooper and Williams, 1999) to facilitate glycopeptide anchoring in the membrane.

b) deciphering the reasons for the reduction of activity intracellularly

Despite the fact oritavancin is one of the most active antibiotics against intracellular *S. aureus,* there is a clear discrepancy between its cellular concentration and the level of bactericidal effect observed. This reduced activity (as compared to the extracellular one) could be due to inappropriate characteristics of each of the partners involved in intracellular activity, namely:

b.1) <u>the antibiotic</u>

Figure 33 suggests that part of the intracellular oritavancin could be trapped in the material it causes the deposition of, leading to reduced intra-organelle bioavailability. This possibility could be examined by evaluating the bactericidal activity of oritavancin in extracellular medium supplemented with cellular extracts, by measuring the intracellular free fraction of the drug, or by determining its cellular disposition in infected cells having developed marked phospholipid overloading.

b.2) <u>the bacteria</u>

The delayed intracellular growth of *S. aureus* suggests that bacteria need to adapt to their new environment after phagocytosis. Among the metabolic modifications that could be explored, current literature data point to the induction of a Small Colony Variant (SCV) phenotype in the intracellular milieu (Vesga *et al.*, 1996). These variants are known to be less susceptible to antibiotics, in relation with major impairment of their oxidative metabolism (Chuard *et al.*, 1997; Mitsuyama *et al.*, 1997; Baumert *et al.*, 2002). Formation of intracellular biofilms could also be envisaged as a key mechanism. Bacteria growing on biofilms are indeed characterized by a lower susceptibility to antibiotics, leading even to the appearance of an antibiotic tolerant

phenotype (Keren *et al.*, 2004) and of persistent populations (Fux *et al.*, 2005). Quite interestingly, intracellular biofilms of *E. coli* have been recently discovered and associated to the persistent character of some bladder infections (Anderson *et al.*, 2003).

b.3) the host cell

Infected macrophages display major modifications in their genetic expression (McCaffrey *et al.*, 2004), in particular in genes encoding for defense mechanisms. Modulating these defense mechanisms by appropriate inhibitors (Carryn, 2003) would therefore help to detect how they can cooperate with or inhibit antibiotic activity.

c) enlarging our field of investigations to clinical strains and animal models

We have demonstrated that new glycopeptides may be useful for the eradication of both the extracellular and intracellular forms of *S. aureus.* Our experience is however limited so far to a fully-susceptible laboratory strain, and needs to be extended to strains presenting resistance characters, in particular, MRSA, VISA, and VRSA (see II.1.a.2. for further details on these organisms).

Likewise, we do not know how the activity determined in cellular models can translate *in vivo*, where pharmacokinetic issues make the game more complex. In this context, the most appropriate models would consist in recurrent infections for which intracellular foci are suspected to play an important role (mastitis, osteomyelitis, skin and soft tissue infections, or endocarditis), based on the demonstration of the capacity of the bacteria to invade the corresponding cell types *in vitro* (Menzies and Kourteva, 1998; Mempel *et al.*, 2002; Brouillette *et al.*, 2003; Ellington *et al.*, 2003).

II.3.c.3. Learning more on oritavancin cellular toxicity

a) Unraveling the mechanisms responsible for cholesterol accumulation

While phospholipid accumulation seems related to the capacity of oritavancin to inhibit lysosomal phospholipase activity, the mechanism responsible for the accumulation of cholesterol is not yet elucidated. Accumulation of free cholesterol has been reported upon exposure to several cationic amphiphilic drugs (Chatelain *et al.*, 1989; Yoshikawa, 1991; Palmeri *et al.*, 1995). Accumulation of cholesterol esters is a less common observation with cationic amphiphiles, but has been seen in cells exposed to chloroquine (Matsuzawa and Hostetler, 1980). Following the different

steps of cholesterol traffic and regulation of metabolism in cells (Brown and Goldstein, 1986; Wierzbicki, 2003; Soccio and Breslow, 2004), we may envisage several mechanisms, among which the followings appear worthwhile to investigate: stimulation of the hydroxy methyl glutaryl -CoA reductase activity (Lange and Steck, 1994), specific increased storage of cholesterol esters by stimulation of acyl-coenzyme A:cholesterol acyl transferase activity (Cheng *et al.*, 1995), alteration of the fate of LDL, as a consequence of a modification in the number of cell surface receptors, an alteration of endocytosis capacity, or a perturbation of lysosomal degradation (Brown and Goldstein, 1986).

b) Identifying the nature of the accumulated material and its origin

The highly pleiotropic aspect of the material detected in cells exposed to oritavancin strongly suggests that many more substances than phospholipids and cholesterol are accumulated. We underlined previously the fact that this appearance of cells is reminiscent of situations like I-cell disease, in which the activity of several lysosomal enzymes is grossly decreased. A systematic search looking for levels of activity of these enzymes could therefore be rewarding to track down the origin of this quite exceptional type of cellular alteration. In parallel, identification of the chemical nature of the material stored could also be undertaken (Meikle *et al.*, 2003).

c) Searching for target organs of toxicity in animal models

Our *in vitro* data suggest a potential for toxicity of oritavancin, but which needs to be carefully documented in animal models. These will however need to be envisaged with a full deployment of appropriate morphological and biochemical methods.

II.3.c.4. Characterizing the interaction between oritavancin and lipids

We have insisted on the importance of the interaction between glycopeptides and membranes, considering it as determinant in the capacity of the drug to accumulate in eukaryotic cells and to cause toxicity, but also to exert bactericidal activity. A fine-tuned analysis of this interaction will therefore be interesting to improve our knowledge of the pharmacological properties of this new class of drugs, and also to try to delineate structure-activity and structure-toxicity relationships, as done in our laboratory in the past to characterize the interactions between lipids and

aminoglycosides, macrolides, or prodrugs of β-lactams (Mingeot-Leclercq *et al.*, 1989; Schanck *et al.*, 1992; Van Bambeke *et al.*, 1993; Mingeot-Leclercq *et al.*, 1995; Van Bambeke *et al.*, 1995; Montenez *et al.*, 1996; Van Bambeke *et al.*, 1996a; Montenez *et al.*, 1999; Chanteux *et al.*, 2003b; Berquand *et al.*, 2004).

II.3.c.5. Extending studies to other glycopeptides

To refine our knowledge of the structure-activity or –toxicity relationships in this new family of antibiotics, the studies done or foreseen with oritavancin will need to be extended to other derivatives. Telavancin is our first candidate, because of its current availability. Preliminary data suggest that it accumulates as well in macrophages (although at a slower rate than oritavancin [figure 36]), where it also shows a cidal activity towards intracellular *S. aureus* (figure 37).

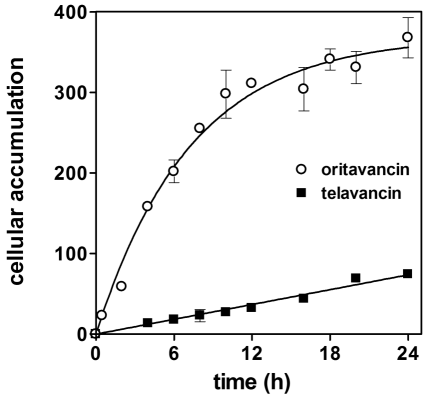


Figure 36: kinetics of accumulation of telavancin and oritavancin in macrophages exposed to extracellular concentrations mimicking their respective Cmax in humans (25 mg/l for oritavancin; 90 mg/l for telavancin).

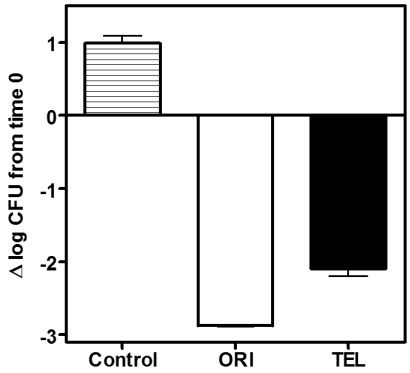


Figure 37: activity against intracellular *S. aureus* of telavancin and oritavancin in macrophages exposed during 24 h to extracellular concentrations mimicking their respective Cmax in humans (25 mg/l for oritavancin; 90 mg/l for telavancin).

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III. Antibiotic active efflux from eukaryotic cells : consequences for pharmacokinetics and pharmacodynamics.

Experimental studies with macrolides and quinolones in macrophages

Based on the following publications

Reviews:

- F. Van Bambeke, E. Balzi & P.M. Tulkens. 2000. Antibiotic efflux pumps (commentary). *Biochem. Pharmacol.* 60, 457-470.
- F. Van Bambeke, Y. Glupczinsky, P. Plésiat, J.C. Pechère & P.M. Tulkens. 2003. Antibiotic efflux pumps in procaryotic cells: impact for resistance to antibiotic treatments. *J. Antimicrob. Chemother*. 51:1055-1065.
- F. Van Bambeke, J.M. Michot & P.M. Tulkens. 2003. Antibiotic efflux pumps in eucaryotic cells: Impact on antibiotic cellular pharmacokinetics, pharmacodynamics and toxicodynamics. *J. Antimicrob. Chemother.* 51:1067-1077.

Primary papers:

- C. Seral, J.M. Michot, H. Chanteux, M.P. Mingeot-Leclercq, P.M. Tulkens & F. Van Bambeke. 2003. Influence of P-glycoprotein inhibitors on the accumulation of macrolides in J774 murine macrophages. *Antimicrob. Agents Chemother.* 47: 1047-1051.
- C. Seral, S. Carryn, P.M. Tulkens & F. Van Bambeke. 2003. Influence of P-glycoprotein and MRP efflux pump inhibitors on the intracellular activity of azithromycin and ciprofloxacin in macrophages infected by *Listeria monocytogenes* or *Staphylococcus aureus. J. Antimicrob. Chemother.* 51:1167-1173.
- J.M. Michot, F. Van Bambeke, M.P. Mingeot-Leclercq, & P.M. Tulkens. 2004. Efflux of the fluoroquinolone antibiotic ciprofloxacin from J774 macrophages through MRP-like transporter. *Antimicrob. Agents Chemother.* 48:2673-2682.
- J.M. Michot, C. Seral, F. Van Bambeke, M.P. Mingeot-Leclercq, & P.M. Tulkens. 2005. Influence of efflux transporters on the accumulation and efflux of 4 quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. *Antimicrob. Agents Chemother.* In press.

III.1. Review on efflux pumps and antibiotics

Biological membranes have most likely appeared very early on during evolution to isolate hydrophilic microdomains from the surrounding medium, allowing catalyzed reactions to occur in an effective manner. Biomembranes, accordingly, constitute efficient barriers towards hydrophilic molecules, most of which can only penetrate cells by specific inward transport systems or see their entry restricted to the endocytic pathway. *A contrario*, biomembranes are easily crossed by amphiphilic compounds since these are able to diffuse through both the hydrophilic and the hydrophobic domains of the bilayer. It is therefore not surprising that mechanisms were devised, also very early, to protect cells from the disordered invasion by amphiphilic molecules, many of which are endowed with biological activities leading to potentially harmful effects. A major mechanism in this respect is constituted by active outward transport.

Although efflux systems have been known since many years, their importance, both in terms of number and variety of substrates, has become clearly recognized only very recently. Based on sequence similarities with known transporters and with proteins possessing at least 2 transmembrane segments, it has indeed been calculated that 15-20 % of the genome of *Escherichia coli* or of *Saccharomyces cerevisiae* may actually code for transporters (Paulsen *et al.*, 1998a; Paulsen *et al.*, 1998b). At least 300 gene products are proposed to effectively transport known substrates, out of which approximately 20 to 30 transport antibiotics and other drugs (see <http://www.biology.ucsd.edu/~msaier/transport/>).

Drugs are often amphiphilic, whether by selection or by design, to ensure their wide tissue distribution and/or their penetration in membrane-protected compartments. It appears therefore natural that many drugs should fall in this category of exogenous compounds for which efflux mechanisms, globally referred to as 'drug efflux pumps', are numerous and fairly active (Kolaczkowski and Goffeau, 1997). Thus, more and more membrane-spanning proteins involved in the outward transport of a surprisingly large variety of drugs have been recognized and characterized over the last years in almost all cell types, from prokaryotes and archebacteria through fungi and higher eukaryotes. This now raises even the question to know which drug is not transported. For eukaryotic cells, drug efflux pumps have been viewed by many authors as complementing the cytochrome P_{450}- or other enzyme-based detoxification systems (Ishikawa *et al.*, 1997, e.g.) to achieve efficient protection against 'chemical invaders'. Both systems, indeed, show broad specificity and may even work in synergy (see the

concept of concerted barrier in enterocytes, e.g [Wacher *et al.*, 1998; Hall *et al.*, 1999]). Drug efflux, indeed, decreases the load on enzyme-mediated detoxification systems, thereby avoiding their saturation, while chemical modifications brought by the enzyme-based systems, which usually increase the amphiphilicity of drugs, provide drug pumps with better substrates (Ishikawa *et al.*, 1997; Suzuki and Sugiyama, 1998). Moreover, most drug efflux pumps have a broad substrate specificity and may therefore deal a wide range of drugs of completely unrelated pharmacological classes.

We have focused our interest on antibiotics since the role of drug-efflux mechanisms, as a major cause of bacterial resistance, has only recently been recognized. It therefore needs to be stressed and examined in details if one wishes to cope with this major challenge in anti-infective therapy. Quite interestingly also, it now appears that antibiotic transport mechanisms play important roles in eukaryotic cells by modulating the pharmacological and toxicological profile of antibiotics. Thus, the identification and characterization of the corresponding genes and gene products, in bacteria as well as in eukaryotic cells, has not only an immediate, fundamental interest, but also opens interesting perspectives for new and more rational developments in chemotherapy.

III.1.a. Organization and phylogeny

Transporters can be classified on the basis of three main criteria, namely the energy source, the phylogenic relationship, and the substrate specificity (figure 1). A 4-digits nomenclature has recently been proposed, constructed in analogy with the enzyme nomenclature, and in which the first group of digits refers to the mode of transport and energy source, the second and the third to the phylogeny [superfamilies and families], and the fourth to the substrate (Paulsen *et al.*, 1998a; Paulsen *et al.*, 1998b; Saier, Jr., 2000; see also the corresponding web site at: <http://www.biology.ucsd.edu/~msaier/transport/>). The so-called '**primary active transporters**' use various forms of energy and make the bulk of drug-efflux pumps in eukaryotic cells (Paulsen *et al.*, 1998a; drug efflux transporters are classically energized by ATP). The '**secondary active transporters**', acting as synports and antiports (i.e. coupling the drug efflux to the downhill transport of an ion along a concentration gradient) are predominant in bacteria (Paulsen *et al.*, 1998b). Within each of these two main classes, phylogenic studies have led to the recognition of

superfamilies, families and clusters, in correlation with their substrate specificity (Paulsen *et al.*, 1998b). Yet, most drug efflux pumps confer a multidrug resistance phenotype, corresponding to the large variety of substrates the transporters may recognize, including several classes of antibiotics as well as non-antibiotic drugs. Antibiotic-specific efflux pumps appear restricted to those organisms producing antibiotics and are often an integral part of the corresponding biosynthetic pathway (Mendez and Salas, 1998; Mendez and Salas, 2001).

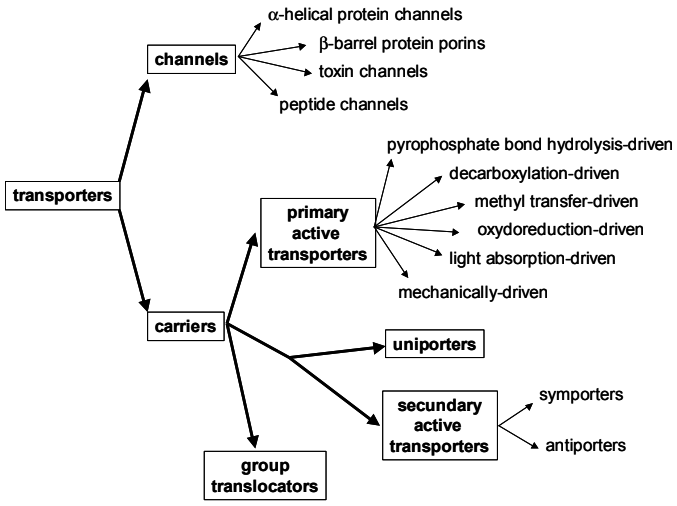


Figure 1: Scheme illustrating the currently recognized types of transporters found in nature.

These proteins are initially divided into channels and carriers.
Carriers are subdivided into primary active carriers, secondary active carriers (including uniporters), and group translocators that modify their substrates during transport.

Primary sources of chemical energy that can be coupled to transport include pyrophosphate bond (i.e. ATP) hydrolysis, decarboxylation, and methyl transfer. Oxidation-reduction reactions, light absorption, and mechanical devices can also be coupled to transport.

Secondary active transport is driven by ion and other solute (electro)chemical gradients created by primary active transport systems. The only well-established group-translocating system found in nature is the bacterial phosphoenolpyruvate:sugar PTS, which phosphorylates its sugar substrates during transport.

(Saier, Jr., 2000)

Table 1 lists the main drug efflux pumps acting on antibiotics described so far, within the corresponding families of drug transporters. We also mention in which organisms they are mainly found (common bacteria, the special category of antibiotic-producing organisms, common fungi, and mammalian cells).

The secondary active transporters being of a simpler structure will be presented first. In this group, pumps acting on antibiotics are mainly found in the so-called RND (**R**esistance/**N**odulation/**D**ivison) superfamily, and the MFS (**M**ajor **F**acilitator **S**uperfamily), but a few of them belong to other families, among which the DMT (**D**rug/**M**etabolite **T**ransporters) [and its SMR (**S**mall **M**ultidrug **R**esistance) subfamily in particular], the POT (**P**roton-dependent **O**ligopeptide **T**ransporters), the OAT (**O**rganic **A**nion **T**ransporters), the MATE (**M**ulti **A**n**t**imicrobial **E**xtrusion) subfamily of the MOP (**M**ultidrug/**O**ligosaccaridyllipid/**P**olysaccharide flippases), and the MET (**M**ultidrug **E**ndosomal **T**ransporter) family. SMR have so far been found only in

bacteria (Paulsen *et al.*, 1996b), whereas MET (Hogue *et al.*, 1996; Hogue *et al.*, 1999) seem restricted to superior eukaryotes. Other families are widely distributed, but antibiotic efflux pumps are not described in all organisms (viz. the RND [Tseng *et al.*, 1999]). SMR and MET are considered as the smallest transporters, possessing only 4 transmembrane segments.

- SMR can be divided in two groups of gene products, one of them is immediately related to drug efflux whereas the other is not, which suggests that evolution from the ancestor transporter towards a gene product with a drug efflux phenotype occurred only once (Paulsen *et al.*, 1996b; Saier, Jr. *et al.*, 1998). Highly conserved motifs are involved in transport activities as well as in binding of the substrates.
- MET present a general organization similar to that of SMR but are characterized by signal motifs rich in tyrosines at their C-termini, which direct them to intracellular compartments of eukaryotic cells (Hogue *et al.*, 1999).
- Members of the RND superfamily share common topological features, namely 2 large extracytoplasmic loops and 12 transmembrane segments resulting from an internal duplication of a gene encoding a 6-transmembrane segment (Tseng *et al.*, 1999 for review). All the known members of this superfamily have a function of efflux transporter, but proteins conferring multidrug resistance are grouped in 2 of the 7 families recognized in this superfamily. Thus, the HAE1 (**H**ydrophobic **A**mphiphilic **E**fflux 1) family is largely predominant and includes the well-known drug efflux pumps of Gram-negative with very broad substrate specificity. They probably all derive from a single ancestor (Paulsen *et al.*, 1996a; Saier, Jr. *et al.*, 1998). In contrast, there is only one member characterized so far in the HAE2 family. Yet, M*ycobacterium tuberculosis* possesses 10 genes encoding proteins of this family, which could explain the poor sensitivity of this organism to many common antibiotics, if all of them were to correspond to antibiotic efflux pumps (Tseng *et al.*, 1999).
- The situation is more complex for drug efflux pumps belonging to the MFS (Marger and Saier, Jr., 1993; Pao *et al.*, 1998). Drug-specific and multidrug efflux pumps appear indeed randomly interspersed in families (see e.g. the DHA2 [also called DHA14] family), indicating that narrowing and broadening of specificity occurred repeatedly during evolution (Paulsen *et al.*, 1996a; Saier, Jr. *et al.*, 1998). Yet, they are also thought to derive from a common ancestor. Moreover, sequence analysis suggests that a simple duplication of a gene encoding a 6-transmembrane segments protein led to the appearance of the 12-transmembrane segments family (DHA1 [also called DHA12] family). The 14-transmembrane segments family (DHA2 [also called DHA14]) then evolved from the insertion of an increasingly hydrophobic central loop of the DHA12 precursor into the membrane (the DHA12 actually displays a long intracytosolic peptide loop running between the 6th and the 7th transmembrane segments which may have provided the necessary scaffold for these two additional membrane spanning segments seen in DHA14). Some of the conserved motifs throughout the MFS have been shown to play a role in activity.

Within the group of **primary active transporters**, the ABC (**A**TP **B**inding **C**assette) drug efflux pumps have been extensively classified in families according to structure homology (Higgins, 1992; Decottignies and Goffeau, 1997; Croop, 1998; Taglicht and Michaelis, 1998; van Veen and Konings, 1998; Dean *et al.*, 2001; Bouige *et al.*, 2002; Klokouzas *et al.*, 2003; see also <http://nutrigene.4t.com/humanabc.htm>). They have also been phylogenetically distinguished on the basis of their import or export activity (Saurin *et al.*, 1999). If import pumps are present only in prokaryotes, efflux pumps are maintained in both prokaryotes and eukaryotes, suggesting that selection of the transport directionality has occurred before divergence between prokaryotes and eukaryotes (Saurin *et al.*, 1999). ABC domains generally present a high degree of homology whereas transmembrane domains differ between transporters and might contribute to define their substrate specificity (Higgins, 1992).

Considering efflux pumps only, two families, the MDR (**M**ulti **D**rug **R**esistance exporters) and the CT2 (**C**onjugate **T**ransporters 2) deserve special attention since they correspond to the most studied and pharmacologically important transporters in eukaryotic cells (an exemplary phylogenic tree of the human ABC transporter proteins is given at figure 2). They also show functional interchangeability between different types of organisms (van Veen *et al.*, 1998).

- MDR, which comprise the well-known P-glycoprotein in eukaryotes (PgP, a product of the *MDR*1 gene; table 1), is responsible for the efflux of a wide range of drugs besides antibiotics, including anticancer agents.
- The CT2 family, comprising MRP (**M**ultidrug **R**esistance **P**roteins) in eukaryotes and Yor1 in *Saccharomyces cerevisiae,* is also involved in the efflux of many drugs including again antibiotics and anticancer agents (Ishikawa *et al.*, 1997; Taglicht and Michaelis, 1998; Cole and Deeley, 1998; Kolaczkowski *et al.*, 1998). This family is phylogenetically close to the CFTR **(C**ystic **F**ibrosis **T**ransmembrane conductance **R**egulator) family, a chloride channel which, however, does not transport drugs (its mutation causes cystic fibrosis).
- The **P**leiotropic **D**rug **R**esistance (PDR) transporters share several biochemical features with the human PgP (Decottignies and Goffeau, 1997; Croop, 1998; Taglicht and Michaelis, 1998) and constitute the major class of ABC drug efflux pumps in yeasts and fungi. Recently, an eukaryotic member of this family called BCRP (**B**reast **C**ancer **R**esistance **P**rotein) has been recognized has a multidrug resistance protein with a substrate specificity partially overlapping that of P-glycoprotein and conferring resistance to many anticancer agents (Litman *et al.*, 2000; Sarkadi *et al.*, 2004).
- Finally, proteins from the DrugE1 and DrugRA1 families are involved in drug-specific efflux in antibiotic-producing organisms (Saier, Jr. *et al.*, 1998; Saurin *et al.*, 1999) while those of the DrugE2 or DrugRA2 families are rather found in non-antibiotic producing bacteria.

Table 1: Classification[a] and illustrative examples of drug efflux pumps extruding the antibiotics used in clinical practice [b]

Transporter class	Mode of transport	(Super)family [c]	(Sub)family [c]	Bacteria	Antibiotic producers	Fungi	Mammals
2 Electrochemical potential-driven transporters	2.A Porters (symports, antiports, uniports)	2.A.1 **MFS** Major Facilitator Superfamily	2.A.1.2. **DHA1** Drug:H+ Antiporters-1 12 spanners (DHA12)	• TetA, B, E of *E. coli* (chl, nal, tet) • TetC of *P. aeruginosa* (tet) • TetH of *P. multocida* (tet) • CmlA of *P. aeruginosa* (chl) • Bcr of *E. coli* (sulf) • NorA of *S. aureus* (chl,fq, tet) • Blt of *B. subtilis* (chl, fq)		• CaMDR1 of *C. albicans* (azo, chl) • Flr1 of *S. cerevisiae* (azo)	• VMAT1 of *Rattus norvegicus* (monoamines)
			2.A.1.3. **DHA2** Drug:H+ Antiporters-2 14 spanners (DHA14)	• EmrB of *E. coli* (nal) • MdfA of E. *coli* (ag, chl, ery, fq, rif, tet) • LfrA of *M. smegmatis* (fq) • TetK of *S. aureus* (tet)	• Ptr of *S. pristinaespiralis* (pris, rif) • LmrA of *S. lincolnensis* (lin) • Pur8 of *S. lipmanii* (pur) • RifP of *A. mediterranei* (rif)	• Atr1 of *S. cerevisiae* (azo)	
			2.A.1.13 **MCP** Monocarboxylate Porter				• MCT1 (βlac)
			2.A.1.14. **ACS** Anion:Cation Symporter				• Npt1 of *Mus musculus* (βlac)
			2.A.1.19. **OCT** Organic Cation Transporters				• Oct1 of *Rattus norvegicus* (organic cations, fq) [human homologs: Oct1-2] • Oat1of *Rattus norvegicus* (organic anions, βlac) [human homologs: Oat1-3]
			2.A.1.20. **SET** Sugar Efflux Transporters	SetA of *E. coli* (sugars; ag)			
			2.A.1.21. **DHA3** Drug:H+ Antiporters-3 12 spanners	• MefA of *S. pyogenes* (14ml, 15ml, ole) • MefE of *S. pneumoniae* (14ml, 15ml) • Cmr of *C. glutamicum* (chl, ery, pur, tet) • TetV of *M. segmatis* (tet) • Tap of *Mycobacterium* (tet)			

Transporter class	Mode of transport	(Super)family [c]		(Sub)family [c]		Bacteria	Antibiotic producers	Fungi	Mammals
2 Electrochemical potential-driven transporters	2.A Porters (symports, antiports, uniports)	**2.A.6.**	**RND** Resistance Nodulation Division	**2.A.6.2.**	**HAE1** Hydrophobe Amphiphile Efflux	• Acr of *E. coli* (βlac, chl, ery, fus, fq, rif, tet, ml) • Mex of *P. aeruginosa* (ag, βlac, inhib βlac'ase, chl, fq, fus, ml, rif, tet) • MtrD of *N. gonorrheae* (βlac, chl, ery, fus, rif, tet) • AmrB of *B. pseudomalei* (ag, ery)			
				2.A.6.5.	**HAE2** Hydrophobe Amphiphile Efflux		• ActII3 of *S. coelicolor* (actinorhodin)		
		2.A.7.	**DMT** Drug/Metabolite transporters	**2.A.7.1.**	**SMR** Small Multidrug Resistance	• EmrE of *E. coli* (ery, sulf, tet) • Mmr of *M. tuberculosis* (ery)			
		2.A.17	**POT** Proton-dependent Oligopeptide Transporters	**2.A.17.4.**	**PepT**				• PepT1 of *R. norvegicus* (βlac)
		2.A.60	**OAT** Organo Anion Transporter	**2.A.60.1.**	**OATP**				
		2.A.66	**MOP** Multidrug/Oligosaccharidyl-lipid/Polysaccharide flippases	**2.A.66.1.**	**MATE** Multi Antimicrobial Extrusion	• NorM of *V. parahaemolyticus* (ag, fq)			
		2.A.74	**MET** Multidrug Endosomal Transporter	**2.74.1.**					• MTP of *Mus musculus* (ery)

Transporter class	Mode of transport	(Super)family [c]	(Sub)family [c]	Bacteria	Antibiotic producers	Fungi	Mammals
3 Primary active transporters	3.A P-P-bound-hydrolysis driven transporters	3.A.1. **ABC** ATP Binding Cassette	3.A.1.105. **DrugE1** Drug exporter-1		• OleC of *S. antibioticus* (ole)		
			3.A.1.117. **DrugE2** Drug Exporter-2	• LmrA of *L. lactis* (drugs)			
			3.A.1.120. **DrugRA1** Drug Resistance ATPase-1		• OleB of *S. antibioticus* (ole) • SrmB of *S. ambofaciens* (ml) • TIrc of *S. fradiae* (tyl)		
			3.A.1.121. **DrugRA2** Drug Resistance ATPase-2	• MsrA of *S. epidermidis* (ery)			
			3.A.1.201. **MDR (ABCB)** Multidrug Resistance Exporter				• MDR1 of *H. sapiens* (fq, lm, ml, rif, tet)
			3.A.1.205. **PDR** Pleiotropic Drug Resistance			• Pdr5 of *S. cerevisiae* (azo, chl, ery, lm, tet) • Snq2 of *S. cerevisiae* (azo) • CDR1 of *C. albicans* (azo, chl) • AtrA, B of *A. nidulans* (ag, azo)	• BCRP of *H. sapiens* (anticancer agents)
			3.A.208 **CT2 (ABCC)** Conjugate Transporter			• Yor1 of *S. cerevisiae* (ery, tet) • Ycf1 of *S. cerevisiae* (conjugates)	• MRP of *H. sapiens* (conjugates, βlac, fq, rif,)

[a] This classification, based on that proposed by Saier, considers first the mode of transport and energy source, and within each mechanism ranks the transporters phylogenetically. The first digit in each number refers to the transporter class, the second one, to the mode of transport, the third and fourth ones, to the (super)family and the (sub)family. Note that mechanisms # 1 and # 4 and the corresponding transporters are not shown here since they do not correspond to drug efflux pumps. In each family, we present only those subfamilies where antibiotic transporters have been evidenced. This classification is regularly updated (see http://www-biology.ucsd.edu/~msaier/transport/).

[b] illustrative examples of known antibiotic transporters ; substrate lists are not exhaustive but correspond to the present state of knowledge (only the main clinically relevant antibiotics and antifungals transported are shown). Abbreviations: ag, aminoglycosides; azo; azoles; βlac; β-lactams; inhib βlac'ase , inhibitor of βlactamase; chl; chloramphenicol; ery; erythromycin; fq; fluoroquinolones; fus, fusidic acid; lm; lincosamides; ml; macrolides (14ml, macrolides with a 14 atoms macrocycle; 15ml, macrolides with a 15 atomes macrocycle); nal, nalidixic acid; ole, oleandomycin; pris, pristinamycin; pur, puromycin; rif; rifampicin; sulf; sulfamides; tet; tetracyclines, tyl, tylosine.

[c] some superfamilies and families are sometimes referred to as families and subfamilies when the number of members in the group is small.

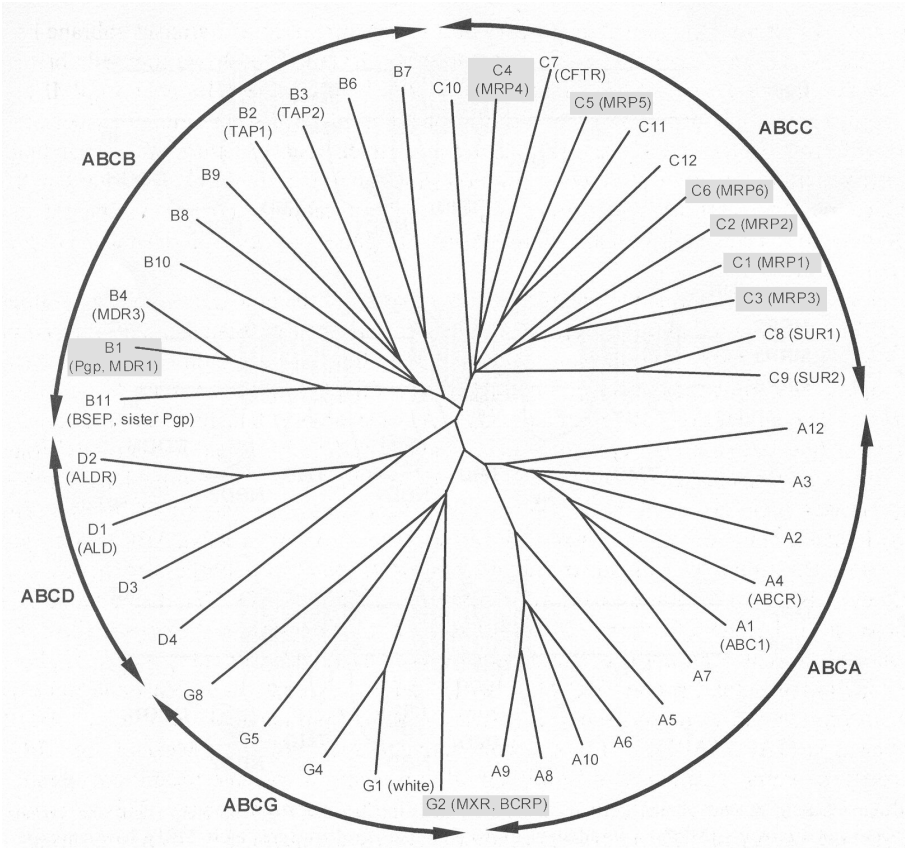


Figure 2: Phylogenetic tree of the human ATP-binding cassette (ABC) transporter proteins.
On the basis of sequence homology, ABC proteins are classified into 7 families, among which ABCA, ABCB, ABCC, ABCD, and ABCG contain the transporter proteins. Those involved in multidrug resistance are highlighted on a grey background.

(Homolya *et al.*, 2003)

III.1.b. Structure and function

Figure 3 shows in a combined fashion the topological organization of the main antibiotic-extruding pumps presented in table 1 together with a schematic view of their mode of operation and the type of antibiotics transported. The mechanisms of transport and of substrate recognition remain, however, largely unknown in most instances, and many of the current views are based on extrapolations from data obtained with transporters of physiological substrates (the assumption is that proteins deriving from a common ancestor have maintained sufficient similarity not only of structure but also of function throughout evolution). Purification and obtainment of crystals is indeed a very difficult task for membrane-embedded proteins like efflux pumps. This explains why structural and functional studies are only beginning to be published for a limited number of transporters, giving a first insight on their mode of organization and mechanism of extrusion.

SMR
RND
MFS
ABC
Topology
Mechanism
Antibiotics
12 TMS
14 TMS
MDR(1)
MRP(1)
COOH
NH2
ATP
ADP + Pi
GSH
lipophilic, multicationic substrates
amphiphilic, charged substrates
amphiphilic, mono- or dicationic substrates [anionic substrates for OAT]
amphiphilic, neutral or cationic substrates
organic, anionic substrates [sometimes, hydrophobic, neutral or mildly cationic substrates]
tetracyclines
erythromycin
sulfadiazine
fluoroquinolones
erythromycin
rifampicin
β-lactams
fusidic acid
chloramphenicol
aminoglycosides
fluoroquinolones
lincosamides
pristinamycin
macrolides

Figure 3:
Topology, mechanism of action and typical substrates for 4 classes of antibiotic efflux pumps
Topology: the membrane is represented in grey, with the extracellular milieu at the top of each scheme, and proteins as a chain of circles, the closed ones corresponding to conserved motifs (identified by letters). In ABC, the locations of the ATP binding cassettes are indicated by two black circles. The 5 transmembrane segments at the N-terminal part of MRP (in the discontinuous line square) are present in MRP1, MRP2 (also called cMOAT), MRP3, MRP6 and MRP7. Numerous other organizations of ABC and transmembrane domains have been proposed (for instance, a mirror image of that shown here for the Pgp, or ABC transporters in which transmembrane segments and ATP binding domains are not fused).
Mechanism of action: SMR, RND and MFS are drug-H^+ antiporters. H^+ probably exchanged from a conserved glutamate (E) of the 'a' conserved domain in SMR and from a conserved arginine (R) of the 'b' conserved domain in MFS. Recognition of cationic drugs may imply the same conserved glutamate for SMR and a conserved acid residue (glutamate or aspartate; E in the figure) in the 'd' domain for MFS. In RND, 3 conserved residues seem to be involved in H^+ echange. ABC transporters involved in drug efflux use ATP as energy source. In all types of transporters, the drug seems to be extracted from the membrane rather than from the cytosol. The transporter then could act as a flippase (catalyzing the flip-flop of the drug from the inner to the outer face of the membrane) or as a 'vacuum cleaner' (moving the drug from the membrane to the central domain of a channel closed to the cytosolic face of the membrane but open to its extracellular face, as already shown for MDR [also known as PgP]). MRP require also the presence of glutathion, which could be conjugated to the drug, prior or during extrusion.
Antibiotics: classes of antibacterial agents for which transport has been described for at least one pump in each family are grouped according to their general physico-chemical character.

(Van Bambeke *et al.*, 2000)

Secondary transporters are mostly but not exclusively involved in drug efflux from prokaryotes. They are all energized by electrochemical forces. SMR, MET, RND, and most MFS transporters are driven by proton gradient (H^+-antiports), while POT act as H^+-symports, MOP, as Na^+-symport, and OAT as uniport or anion exchangers.

- Because of its small size (~ 100 residues), the SMR pump EmrE from *Escherichia coli* is an excellent example of optimized structure, in which a minimal number of essential residues insures substrate recognition and transport function. Accordingly, it has been extensively studied as a paradigm of efflux transporter.

  In crystals, EmrE is organized in asymmetric homodimers, with a substrate binding chamber formed from 6 α helices, accessible both from the aqueous phase and laterally from the lipid bilayer (Ubarretxena-Belandia *et al.*, 2003). These dimers are functional, but higher olimeric structures may exist *in vivo*, like trimers (as suggested by functional studies [Rotem *et al.*, 2001]) or dimers of dimers (i.e. tetramers, as deduced from structural studies [Ubarretxena-Belandia and Tate, 2004]). Based on structural data as well as on site-directed mutagenesis studies, a putative mechanism of drug transport has been established for the EmrE transporter (figure 4). It answers to a model of translocation cycle, which could involve the following steps : (i) exchange between the drug and a proton fixed on a charged residue; (ii) translocation of the drug by a series of conformational changes driving it through an hydrophobic pathway; (iii) replacement of the drug by a proton in the external medium and return to the initial conformational state (Paulsen *et al.*, 1996a; Mordoch *et al.*, 1999). The overall result of the transport is therefore an exchange between the drug and a proton (antiport). The residue responsible for proton-exchange in SMR could be a

conserved glutamate present in the first transmembrane segments (Grinius and Goldberg, 1994; Ninio *et al.*, 2001); apparently, all the glutamate residues of the oligomer are functionally equivalent and do participate to the transport (Soskine *et al.*, 2004). Yet, a cluster of amino-acids in the vicinity of this glutamate also participate to the proton- and substrate-binding sites (Gutman *et al.*, 2003).

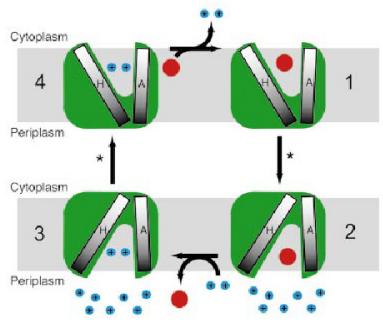


Figure 4. Transport mechanism in ErmE.

Cartoon of EmrE structural changes during efflux, based upon the 3D structural analysis, with substrate as a large red sphere and protons as small blue balls. Binding of substrate from the inner leaflet (1) and conformational change (*) involving a change of tilt of helice H relative to helix A, opens the binding chamber to the periplasm (2). Proton binding displaces the substrate into the periplasm (3), and a second proton-driven conformational change (*) reorients the binding site to face the cytoplasm.

(Ubarretxena-Belandia *et al.*, 2003)

- The same mechanism probably applies to MFS, but the proton exchanger may be a conserved arginine (Paulsen and Skurray, 1993). The internal cavity of the transporter would be mostly negative, based on modeling studies (Vardy *et al.*, 2004), and a conserved acidic residue may be involved in the recognition of positively-charged substrates (Edgar and Bibi, 1999). This explains why both SMR and MFS preferentially extrude cationic molecules (see below).

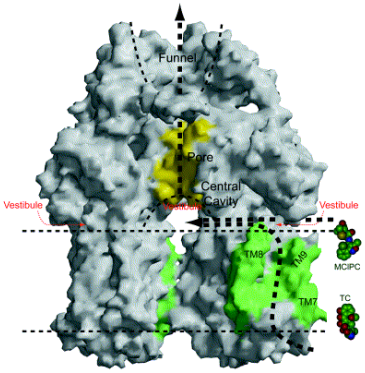
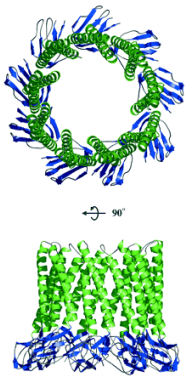
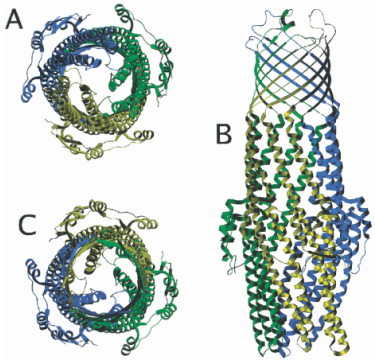
- RND transporters have also been extensively studied over the last years. Actually, these pumps, widely spread in Gram-negative microorganisms, are organized in tripartite proteic complex, with the pump it-self being localized in the inner membrane, a 'membrane fusion protein' connecting the inner membrane to the outer membrane, and a 'outer membrane protein' crossing this outer membrane. Such an organization allows the extrusion of the substrate directly in the external medium, bypassing the periplasm and thereby amplifying the efficacy of the transporter. It is now proposed to be a common feature for RND, MFS, and ABC efflux pumps in Gram-negative bacteria, the 'membrane fusion proteins' differing between transport systems, whereas the 'outer membrane adaptor proteins' are probably common to all three types of transporters (Paulsen *et al.*, 1997; Nishino *et al.*, 2003).

  Like SMR and MFS, RND possess highly conserved charged residues in their transmembrane segments, which may play an important role in substrate binding or proton transport (Guan *et al.*, 1999). The inner membrane protein is also characterized in Gram-negative bacteria by two large extracellular loops. These were first thought to interact with two other proteins (Guan *et al.*, 1999), and are

now recognized as also playing an important role in drug recognition (Mao *et al.*, 2002; Elkins and Nikaido, 2002; Tikhonova *et al.*, 2002).

The crystal structure of the three types of proteins constituting the tripartite complex has been recently published, respectively for the AcrB (Murakami *et al.*, 2002), and TolC (Koronakis *et al.*, 2000) constituents of the AcrAB-TolC efflux pump from *E. coli* and for the MexA (Higgins *et al.*, 2004) constituent of the MexAB-OprM pump from *P. aeruginosa* (figure 5). This constitutes the first example of fully elucidated structure of a multidrug efflux transporter. These pioneer data have a potentially wide interest, in particular for the understanding of the mode action of this complex building.

Figure 5: structure of RND efflux pumps

cut way view of the surface of the inner membrane protein	**structure of the periplasmic protein**	**structure of the outer membrane protein**
		
AcrB (trimeric protein) appears like a jellyfish. Each protomer comprises ▪ a transmembrane region, arranged in a ring-like manner with a central hole and a funnel-shaped groove ▪ a periplasmic headpiece, divided in o a pore domain forming a pore prolonging that formed in the transmembrane region and presenting vestibules large enough to accommodate substrates and communicating with the periplasm o a TolC docking domain, forming a funnel ending in hairpins that can connect with the bottom of the TolC protein. Broken lines indicate the putative substrate recognition pathway. Helices comprising a groove are highlighted in yellow and green. Some substrates are depicted as space-filling models placed in the inner and outer leaflets of the cytosolic membrane. MPIC, cloxacillin; TC, tetracycline. (Murakami and Yamaguchi, 2003)	Each protomer of MexA consists in ▪ a β-barrel ▪ a lipoid domain ▪ a α-helical hairpin. Monomers pack to form a cylinder, with helices able to associate with the periplasmic protein, β-sheets constituting the larger part of the funnel, and the lipid domain involved in the interaction with the inner membrane protein. Top: view of the MexA monomer, with the α-helical hairpin to the right, the lipoid domain central, and the β-barrel to the left. Bottom: model for the assembly of the α-helical hairpins into a barrel. α-helices are in green, β-barrels, in blue. (Higgins *et al.*, 2004)	TolC trimerizes to form a cylinder (protomers are colored individually) that is open at the outer membrane end but comes to a narrow constriction at the periplasmic end. View normal to the plane of the membrane (B); looking down the channel from the outer membrane (A) and periplasmic (C) ends of the protein. (Borges-Walmsley *et al.*, 2003)

Based on these structural studies and on first experimental evidences, a still largely putative sequence of events has been proposed to explain the mechanism of extrusion of drugs by RND transporters (Murakami and Yamaguchi, 2003; figure 6):

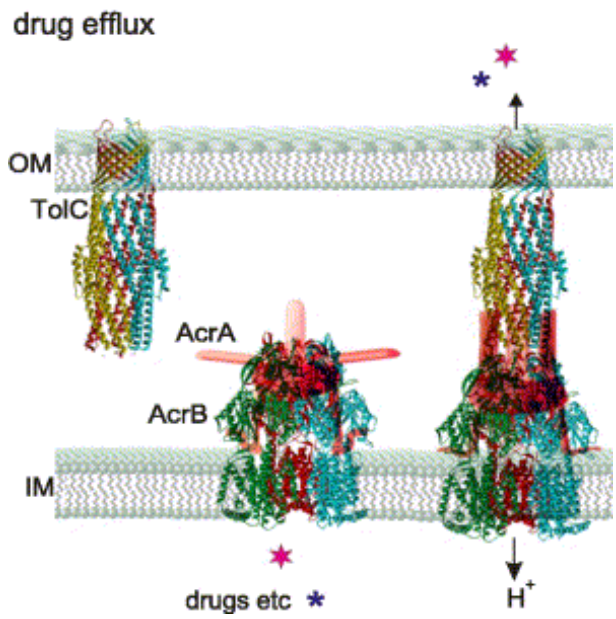


Figure 6a: assembly of TolC dependent machineries

Model of drug efflux achieved by reversible interaction of TolC or a homologue with substrate-specific inner membrane (IM) complexes (translocases). These contain a common adaptor protein and an energy-providing protein (in drug efflux, most commonly a proton antiporter). In this way a contiguous exit route is assembled from the cytosol to the outside environment. It is proposed that a key event is opening the TolC entrance. The assembly process may require the cell proton-motive force, but substrate translocation is driven by the specific activities of the translocase.

(Murakami *et al.*, 2002)

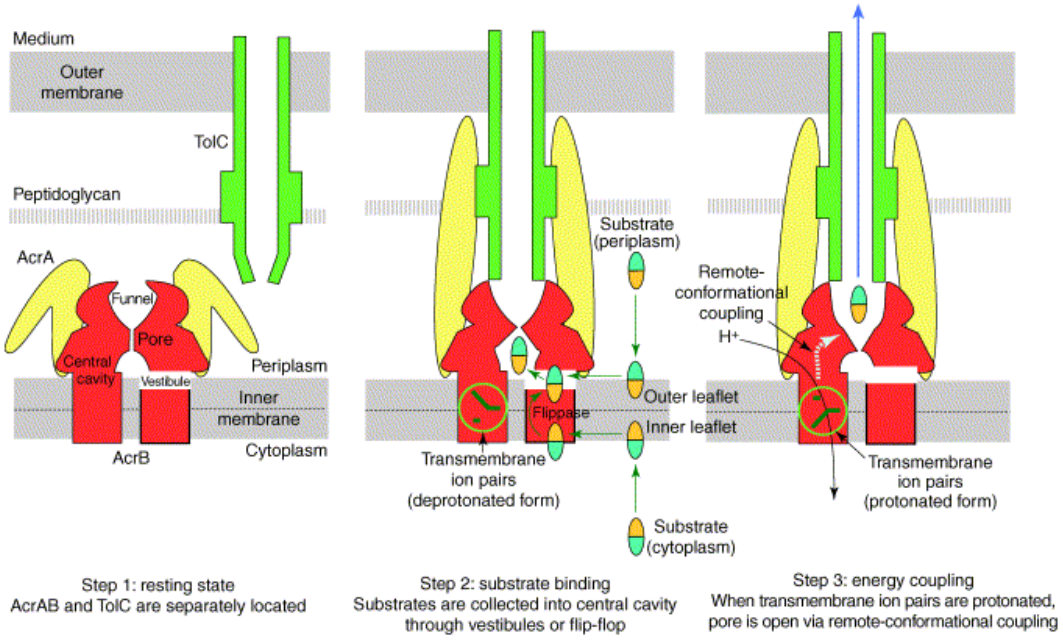


Figure 6b: Proposed mechanism for AcrAB–TolC-mediated drug export.

AcrAB and TolC at first exist independently. AcrA probably binds to the side of AcrB in the folded-down form. TolC might be fixed on the peptidoglycan, whereas the AcrAB complex can move horizontally. When AcrB binds to the substrate, the conformation of the top of AcrB is changed to enable docking of AcrB with TolC. When the AcrAB complex docks with TolC, the AcrA molecule extends and covers the side of TolC to reinforce docking. Finally, protons are captured at the transmembrane ion pairs and remote conformational coupling makes the extramembrane pore open. This is followed by active transport of substrates from the cavity to the funnel and then to the TolC tunnel.

(Murakami and Yamaguchi, 2003)

- uptake of the substrate (figure 7):
  this may occur from the inner leaflet of the cytosolic membrane, for antibiotics that do enter bacterial cells, or from the outer leaflet for antibiotics which are poorly diffusible (like aminoglycosides) or which do not need to enter bacteria to exert their activity because their target is extracellular (like β-lactams). Drugs expelled from the inner leaflet would undergo a flip-flop movement through the groove present in the transmembrane domain of the pump, and rejoin the vestibule through which substrates taken at the outer leaflet travel to the central pore. Accordingly, RND seem to act not only as "membrane vacuum cleaners", but also as "periplasmic vacuum cleaners" (Li *et al.*, 1994; Nikaido, 1996). RND have a broader substrate specificity than any other type of multidrug resistance protein, in particular the mammalian ones (P-glycoprotein and MRP). This may be due to the presence of a large binding site insuring substrate recognition in the periplasmic part of AcrB, located at the level of the central cavity (Tikhonova *et al.*, 2002; Yu *et al.*, 2003a; Yu *et al.*, 2003b).

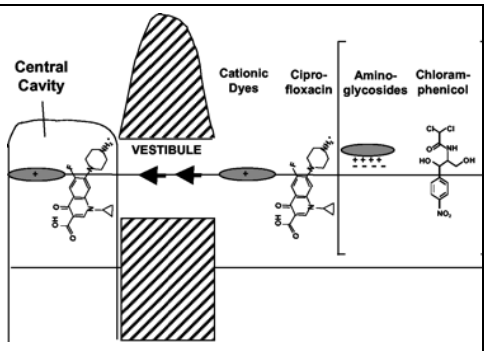


Figure 7: Hypothetical path of substrate capture by AcrB and related pumps.

The crystal structure of AcrB trimer bound to cationic dyes strongly suggests that such dyes, bound to the acidic surface of the bilayer, travel through the vestibule and bind to the wall of the central cavity without much change in its location or orientation in relation to the bilayer surface. A similar mechanism is likely to apply for ciprofloxacin, which may partially partition into the outer leaflet of the bilayer before diffusing through the vestibule and binding to the cavity. For the other substrates shown in the brackets, the crystal structure of the liganded AcrB is not yet available. However, it seems likely that the polycationic aminoglycosides become adsorbed to the polyanionic surface of the bilayer and are captured by some RND pumps. Similarly, amphiphilic molecules with dipolar lipophilic domains, such as chloramphenicol, may partition partially into the outer leaflet of the bilayer and be captured by lateral diffusion through vestibules. PL, phospholipids.

(Yu *et al.*, 2003a)

- assembling of the tripartite complex: substrate binding may induce a conformational change of AcrB, enabling the lateral movement of the AcrAB complex, its contact with TolC, and then the unfolding of AcrA. The later extends in the periplasm, reinforcing the interaction between TolC and AcrB.

- transport: substrate would be transported from the central cavity through the pore formed by TolC. The driving force is given by protons. Thus, the protonation of the Asp-Lys ion pair may drastically reduce the affinity of the pump for its substrate, generate a twisting motion of the transmembrane helices and induce a subsequent opening of the pore and of the TolC tube (Andersen *et al.*, 2002; Murakami *et al.*, 2004).

ABC transporters are rather spread in eukaryotic cells and were originally described for their capacity to confer resistance to anticancer agents. They usually contain two ATP binding cassettes (ABC domains), and derive their energy from the hydrolysis of ATP. Sequence identity is low among ABC proteins, especially in their transmembrane domains, which is consistent with the notion that this divergence is responsible for their varied transport functions. In contrast, nucleotide binding domains are relatively conserved, so as to insure their ATP hydrolysis function.

- In this family, most structural and functional studies have been performed with P-glycoprotein, which was the first multidrug efflux pump evidenced in a resistant cancer cell line (Juliano and Ling, 1976). P-glycoprotein is made of 2 homologous halves, each containing 6 transmembrane domains and 1 nucleotide binding domain. It is glycosylated at 3 sites, which seems necessary for its proper trafficking to the cell surface (Schinkel *et al.*, 1993). Mutational studies, labeling with photoaffinity substrate analogues, and cross-linking with thiol reactive substrates indicate that the 2 halves interact to form a single transporter (van Veen *et al.*, 2001; Loo and Clarke, 2001b; Safa, 2004).

  The exact mechanism of drug transport is still hypothetical, but presents striking similarities with that described here above for antiporters. Among the different models that have been proposed, the two most likely ones indeed present ABC efflux pumps as acting either like flippases, or like hydrophobic 'vacuum cleaners'. These models contrast with the old, more conventional models of efflux pumps acting a simple pore-like channels oriented towards export, which pick up their substrate from the cytosol and orient it towards the outside of the cell thanks to a regulator valve. They indeed both assume that the drug is primarily extracted from the inner phase of the membrane. Strong membrane anchoring is probably the only common requirement for substrates of drug efflux pumps. Several lines of experimental evidences, based most often, however, on studies with non-antibiotic substrates, support this assumption. First, the only common feature of pump substrates is a liposolubility that must be sufficient to insure a proper penetration in the bilayer (Higgins and Gottesman, 1992). Second, substrate or inhibitor molecules can compete with lipophilic fluorophores for insertion in the membrane (Higgins and Gottesman, 1992; Wadkins and Houghton, 1993). Third, lipophilic fluorophores are themselves substrates of the pumps (Bolhuis *et al.*, 1996). Fourth, the rates and kinetics of efflux are not directly related to the cytosolic drug content (Ashida *et al.*, 1998). In the flippase model, the drug is thought to reach the protein from within the membrane, but would then be flipped to the outer layer (as proposed for phosphatidylcholine flip-flop under the action of flippases). In the ‘vacuum cleaner’ model, the drug is thought to freely move into the lipid phase of the membrane, reaching then the protein and its central channel from where it would be actively expelled outwardly. Some functional studies tend to privilege the flip-flop hypothesis, since the physiological transporters of phospholipids and of glutathion conjugates, which

belong to the MDR and CT2 families, respectively, are known as flippases (Smith *et al.*, 1994; Sokal *et al.*, 1998; Zhou *et al.*, 1999). In contrast, the structural characteristics of P-glycoprotein (in particular its three-dimensional structure solved at a low resolution) rather favor the hypothesis of vacuum cleaner. Indeed, the twelve transmembrane domains seem organized in such a way that they form a large aqueous pore, open to the extracellular medium but closed towards the cytosol (somewhat like an empty, open bottle or beaker floating on the surface of water; see figure 9 [Rosenberg *et al.*, 1997; Rosenberg *et al.*, 2003]). In addition, transmembrane segments are rich in aromatic amino acids which could help the hydrophobic substrates to travel into this channel (Pawagi *et al.*, 1994).

Putting thus together experimental, computational, and structural data leads to the following view of the mechanism of action and drug recognition for P-glycoprotein (figure 8).

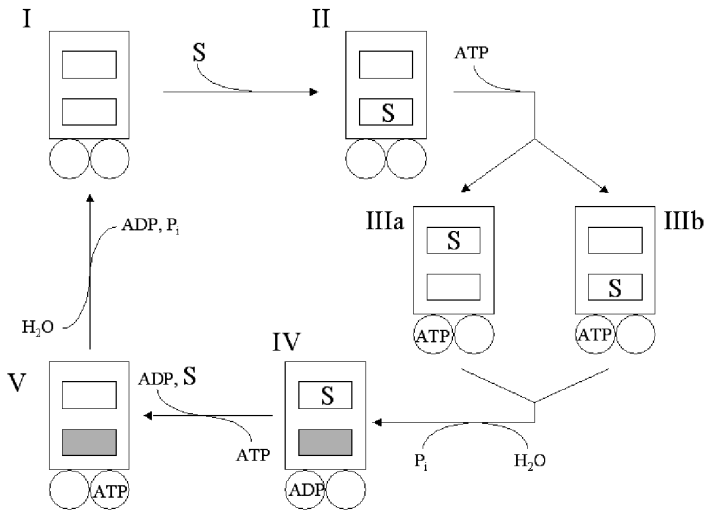


Figure 8: mechanism of P-glycoprotein functioning.

In its resting state (I) the protein is free of any nucleotide or drug. It has been shown that affinity of ATP for P-gp is increased by substrate binding (Rosenberg *et al.*, 2001b) and a contrario ATP binding to P-gp reduces the availability of the drug binding site (Sauna and Ambudkar, 2000). This implies that drug binding precedes ATP binding. The transport cycle is initiated by drug binding to a high-affinity site in the TM domains (state II) from the inner leaflet of the bilayer (Shapiro *et al.*, 1997). In a second step ATP binding occurs in one of the NBDs, presumably in the NBD located in the NH_2-terminal half (Vigano *et al.*, 2003). From this state two paths are conceivable, each of them supported by experimental data. In one path (IIIa) ATP binding would mediate a reorganization in the whole protein lowering the affinity of the drug binding site for its substrate and causing the translocation of the substrate to a low affinity binding site. This is supported by a study on LmrA (van Veen *et al.*, 1998) and by the electron microscopy (EM) structure of P-gp (Rosenberg *et al.*, 2001b). In the other path (IIIb) it is only after ATP hydrolysis that conformational changes happen, including migration of the substrate to a low affinity binding site. This is supported by dissociation constant measurements (Sauna and Ambudkar, 2000) and cysteine disulfide cross-linking (Loo and Clarke, 2002). After ATP hydrolysis, a further conformational change occurs (structure (IV); Rosenberg *et al.*, 2001b) from which the substrate could be released to the extracellular medium. On release of ADP a second ATP molecule binds to the other NBD (V). After hydrolysis of this molecule P-gp returns to its original configuration. The protein is then reset for another cycle.

(Sauna and Ambudkar, 2000).

Figure 9 further compares the conformation of the protein in its resting and activated state.

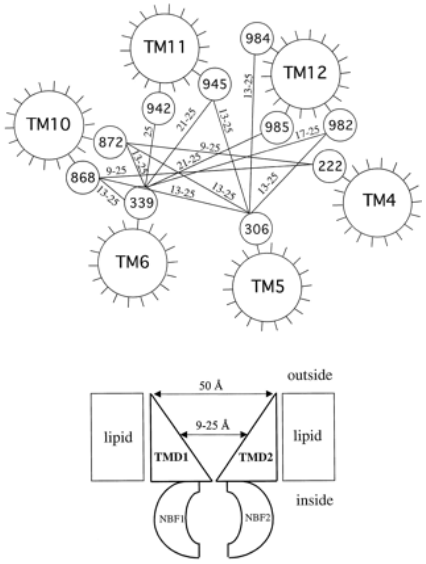


Figure 9a: Model of the binding site of P-gp constructed from cross-linking experiments .

Upper panel, top view of P-gp where the residues of the TM segments are arranged as α-helical wheels. The numbered circles refer to the positions of residues that were shown to contribute to the drug-binding site of P-gp The numbered lines represent the sizes (Å) of the cross-linker arms used to cross-link the two residues.
Lower panel: cross-sectional view of P-gp. The four domains are shown as follows: NBF1, NH_2-half nucleotide-binding domain; NBF2, COOH-half nucleotide-binding domain; TMD1, NH_2-half transmembrane domain containing TMs 1-6; TMD2, COOH-half transmembrane domain containing TMs 7-12.

(Loo and Clarke, 2001b)

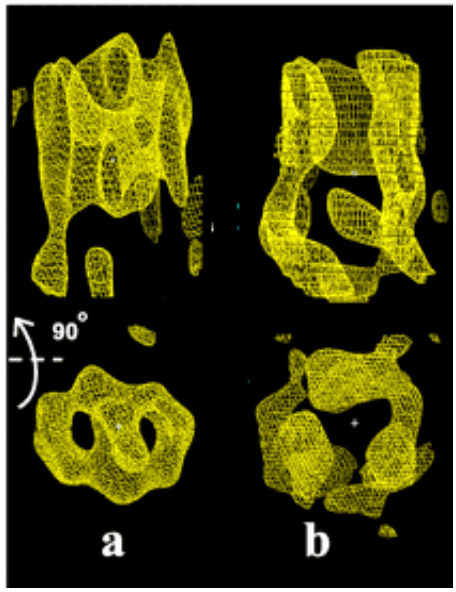


Figure 9b: Three-dimensional structure of P-gp in the absence (a) or in the presence (b) of a non hydrolysable analog of ATP

In each case, a side view (top panels) and a top view, looking down onto the equivalent of the extracellular surface (bottom panels) are shown. In the absence of ATP, the protein looks like a barrel closed at its lower part (bottom panel).
In the presence of the ATP analog, three domains are visible in the protein, with a lateral channel appearing between 2 of them.

(Rosenberg *et al.*, 2003)

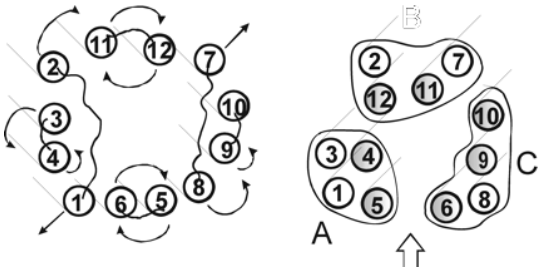


Figure 9c: Simplified schematic presentation of the arrangement of transmembrane domains (TMs) upon P-gp functioning.

left: inactive state; right: active state. The extracellular linkers are shown about proportional to their length. The small arrows direct to possible rotation/lateral movement of TMs during the transition from the inactive to the active state. Clusters A, B, C correspond to those observed in the X-ray structure of the nucleotide-bound P-gp, and the big arrow points to the gap.

(Pajeva *et al.*, 2004)

Thus, in sharp contrast with the image, observed in resting conditions, of a barrel surrounding a central pore closed at its cytosolic face, the transmembrane domains of the active protein appear as segregated in three domains, also enclosing a central pore (figure 9b). However, the pore is less obviously closed at its bottom and a gap open to the lipid phase appears between 2 domains, highly suggestive of a possible route for drugs to travel from the lipid phase to the central pore of the pump. In this new conformation, helices 4-6 and 10-12 would face the central pore (figure 9c; Pajeva *et al.*, 2004), which is consistent with biochemical

crosslinking data showing that these helices form the drug binding domain (Loo and Clarke, 2000; Loo and Clarke, 2001a). The latter is organized as central chamber with a funnel shape (Loo and Clarke, 2001b), narrow (9-25 Å) at its cytosolic side and widest (50 Å) at the extracellular side (figure 9a). This binding pocket would be large enough to accommodate at the same time different substrates, with different anchoring points (Loo *et al.*, 2003b). It is therefore proposed that substrate-itself would induce an auto-fit mechanism, where its size and shape would change the packing of the transmembrane helices (Ambudkar *et al.*, 2003). Drug binding itself induces further conformational modifications, both in the transmembrane domains and in the nucleotide binding domains, with a continuous cross-talk between the two types of domains (Loo *et al.*, 2003a; Loo *et al.*, 2003c). In particular, modifications in the transmembrane domain conformation will contribute to the "fitting" of the binding site to the substrate, while changes in the conformation of the nucleotide binding domains would stimulate ATP hydrolysis (as proposed in step IIIb hereabove), which would in turn modify the conformation of the transmembrane segments, reducing the affinity of the binding site for the substrate and favoring its extrusion.

In the direct line of these studies, a putative pharmacophore, common for most P-glycoprotein substrates or inhibitors, is progressively being built. Thus, it is known for long time that P-glycoprotein substrates are all hydrophobic, with a molecular mass of 300-2000 Da (Ford and Hait, 1990), anionic substrates/inhibitors being in principle not transported. More recently, the comparison of the biophysical properties of P-glycoprotein substrates/inhibitors and their theoretical models have led to the identification of common determinants for substrate/inhibitor candidates (Seelig, 1998; Ekins *et al.*, 2002; Pajeva and Wiese, 2002; Garrigues *et al.*, 2002b). Thus, these studies converge to the idea that recognition of substrates/inhibitors does not depend on precise chemical motifs, but rather on the shape, size, and distribution of the polar and hydrophobic elements on the molecule, making possible the recognition of several and variable chemical structures. The various binding sites may be distributed between 2 pharmacophores.

Consensus motifs include the following determinants (figure 10), located at appropriate distance from one another:

- at least 2 hydrophobic (alkyl or aromatic) groups [aromatic being often found in inhibitors)
- 1 to 3 H-bound acceptors
- 1 H-bound donor, which does probably not necessarily need to be protonated.

Binding affinity is related to the number of interacting points.

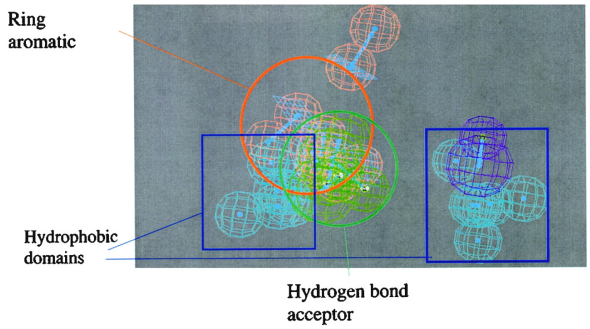


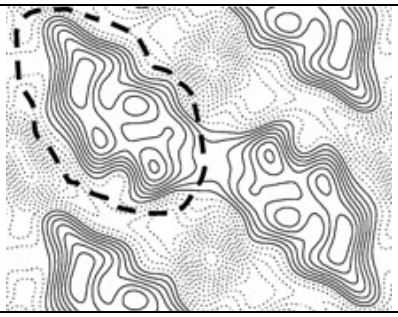
Figure 10: Five merged P-gp pharmacophores (from inhibitors) defining common areas of hydrophobicity, hydrogen bonding, and ring aromatic features (orange, ring aromatic feature; green, hydrogen bond acceptor; purple, hydrogen bond donor; and cyan, hydrophobic features).

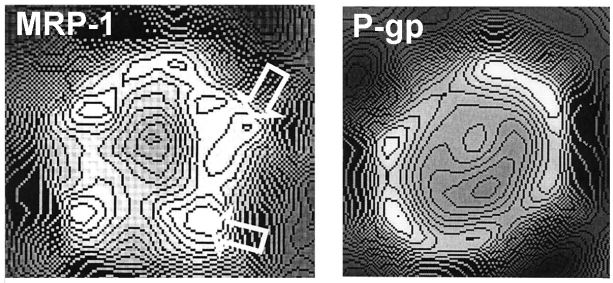
(Ekins *et al.*, 2002)

- MRP were more recently discovered, as other multidrug resistance proteins with a substrate specificity partly overlapping and partly complementary to that of P-glycoprotein. About 15 years after the isolation of the first representative in this family (Cole *et al.*, 1991; Cole *et al.*, 1992), 10 members have been identified, among which 7 have already been shown to confer resistance to anticancer agents (Ambudkar *et al.*, 2003 for review). Focusing on MRP1, it is composed of a large core segment similar to that of P-glycoprotein, but possesses a third membrane spanning domain with 5 transmembrane helices, the N-terminus having a unusual extracellular location (see figure 3). Such an extension is also found in MRP2, 3, 6, and 7 but not in the other ones (Kruh and Belinsky, 2003).

  Structural data on MRP are scarce. Only a low resolution electron microscopy study of MRP1 has been published so far (figure 11 [Rosenberg *et al.*, 2001a]). It suggests that the protein may organize in dimers (at least in two-dimensional crystals of the protein reconstituted in lipids), but this does not necessarily mean that it keeps this organization in the membrane.

Figure 11





Projection maps of MRP1 in negative stain at 25-Å resolution, calculated from images of two-dimensional crystals of MRP1. One unit cell contains two molecules of MRP1. The solid lines indicate density above the mean; dotted lines indicate density below the mean value. A putative monomer is shown by the bold dotted lines

Comparison of solubilized MRP1 and P-gp projection structures predicted to correspond to the extracellular face of the proteins. Both structures were filtered to the same resolution and similarly contoured to facilitate a comparison between the two. The arrows point to the main differences between the two proteins.

(Rosenberg *et al.*, 2001a)

Each monomer seems to display a central depression (putative pore) largely open at the cytoplasmic face, presumably involved in drug transport. When compared to P-glycoprotein, the two halves do not appear perfectly symmetric, probably because of the presence of 5 additional transmembrane segments in MRP1.

The precise mechanism of extrusion by MRP has neither been explored in details, but two major differences with P-glycoprotein have been evidenced. First, the two ATP-binding cassettes are in this case not equivalent, the substrate-induced ATP hydrolysis taking obligatory place in NBD2, NBD1 playing mostly a regulatory role

(Hou *et al.*, 2002; see also Altenberg, 2004 for review). Second, the activity of MRP1 depends also on the presence of glutathion, but its exact role is still unsettled. Moreover, it may differ according to the type of substrate transported, but its weak ionic character seems critical in any cases (Cole and Deeley, 1998).

Current working hypotheses (summarized by Kruh and Belinsky, 2003) suggest:

- that glutathione is simply co-transported (this mechanism may occur for uncharged substrates; Rappa *et al.*, 1997)
- that glutathione causes a allosteric modification of the protein, favoring the ATP binding and/or hydrolysis as well as the binding and transport of anionic substrates (Manciu *et al.*, 2003)
- that drugs which are not recognized as such are conjugated to glutathione before to be transported (Ishikawa, 1992).

Interestingly also, the oxidation product of glutathione (GSSG) is a good MRP1 substrate (Leier *et al.*, 1996). The pump may therefore appear as a homeostatic mechanism preserving the cellular redox balance.

- BCRP belongs to the category of ABC 'half-transporters', because the active transporter is actually a homodimer made of two polypeptides, each consisting in a single nucleotide binding domain and one transmembrane domain comprising 6 transmembrane helices. Like the other ABC multidrug efflux pumps, BCRP can transport large, hydrophobic, positively- or negatively-charged molecules (see Sarkadi *et al.*, 2004 for review). Its implication in antibiotic efflux is therefore most probable, even though it has not yet been documented.

III.1.c. Antibiotic substrate specificity

Figure 12 identifies the main groups of transporters (also referred to as superfamilies) that have been shown so far to effectively act upon antibiotics. Two of these superfamilies (MFS and ABC) span the prokaryote-eukaryote boundary, but with specific members in each kingdom. It must be remembered, however, that most transporters have been identified only very recently, so that the discovery of many more families with both prokaryotic and eukaryotic members would not be surprising in the near future.

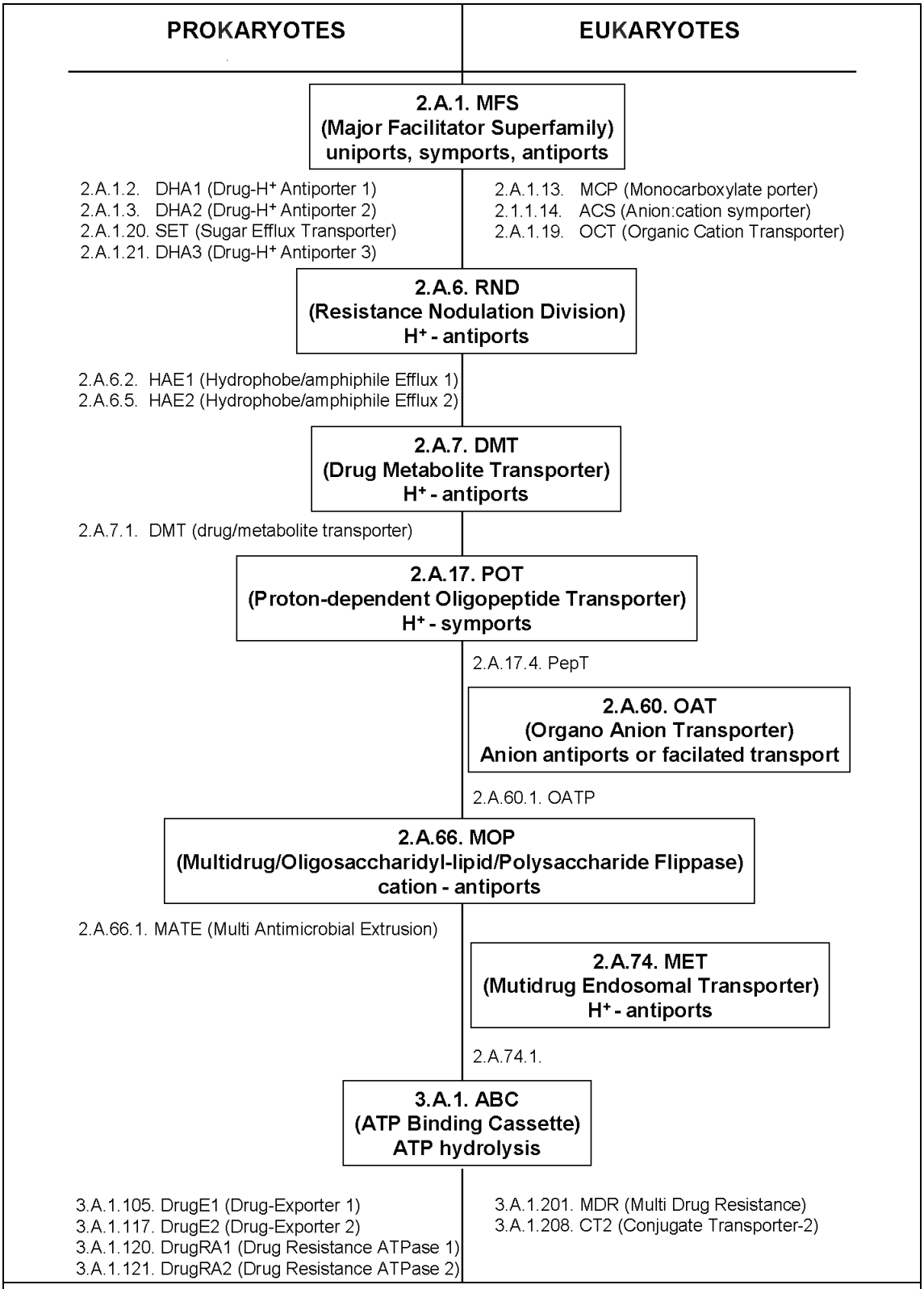


Figure 12: Occurrence and distribution of antibiotic transporters in prokaryotic and eukaryotic cells.

The nomenclature is based on the classification of Saier (described in the following website : <http://www.biology.ucsd.edu/~msaier/transport/>). The transporters are grouped in so-called superfamilies (3 digits nomenclature; bold characters), 6 of which are found in prokaryotes and eukaryotes. Within each superfamily, only those families (four digits nomenclature; normal characters) transporting antibiotics are shown. OAT and MET are not found in prokaryotes.

(Van Bambeke *et al.*, 2003a)

Substrate recognition is ensured by specific domains within the transmembrane spanning segments of the protein. In Gram-negative bacteria, in which efflux transporters are organized as multicomponent systems (Poole, 2000), the substrate specificity is probably dependent on the protein located in the inner membrane, which is actually the efflux pump, but works in conjunction with a periplasmic fusion protein and an outer membrane protein so as to directly expel its substrates through the two membrane of the bacteria (figure 13).

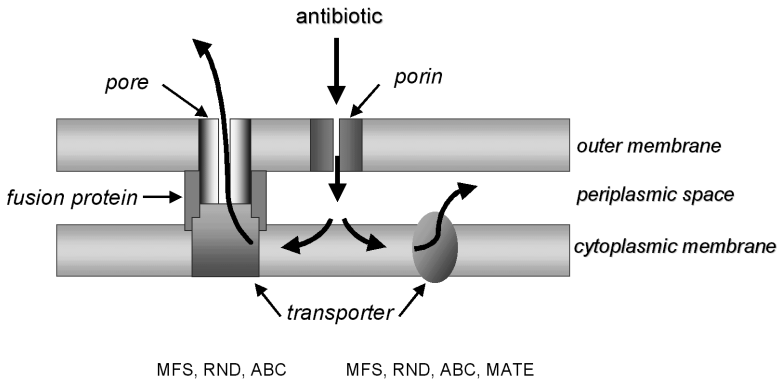


Figure 13: Topology of multicomponent (left) and monocomponent (right) efflux pumps as they can be found in MFS, RND, and ABC superfamily and in MFS, RND, ABC, and MATE superfamilies, respectively.

Multicomponent efflux pumps are specific to Gram-negative bacteria, since their particular organization allows extruding antibiotics directly in the extracellular medium. Arrows show antibiotic directions of transport. MFS, RND, SMR and MATE are energized by ions gradients and ABC, by ATP hydrolysis.

(Van Bambeke *et al.*, 2003a)

As mentioned previously, a key characteristic of the efflux pumps is the variety of molecules they may transport. Focusing now on antibiotics, two main categories of transporters can be distinguished:

- those which present a relatively narrow substrate specificity (typically, they transport a given class of antibiotics). Examples are found within pumps belonging to the MFS superfamily for both prokaryotic and eukaryotic cells, and to the ABC superfamily for prokaryotic cells (table 1)
- those which present a very wide spectrum, recognizing antibiotics from chemically unrelated families, together with other drugs or substrates. Illustrative examples thereof are the multidrug resistance proteins from the ABC superfamily in eukaryotes, and RND in prokaryotic cells.

Especially in the latter case, it is clear that only very minimal common structural determinants are necessary to obtain detectable transport. As explained before, a unifying hypothesis is that most if not all transporters recognize molecules with a polar, often slightly charged head associated with a hydrophobic domain (see how the main antibiotic classes fit with these features in figure 14).

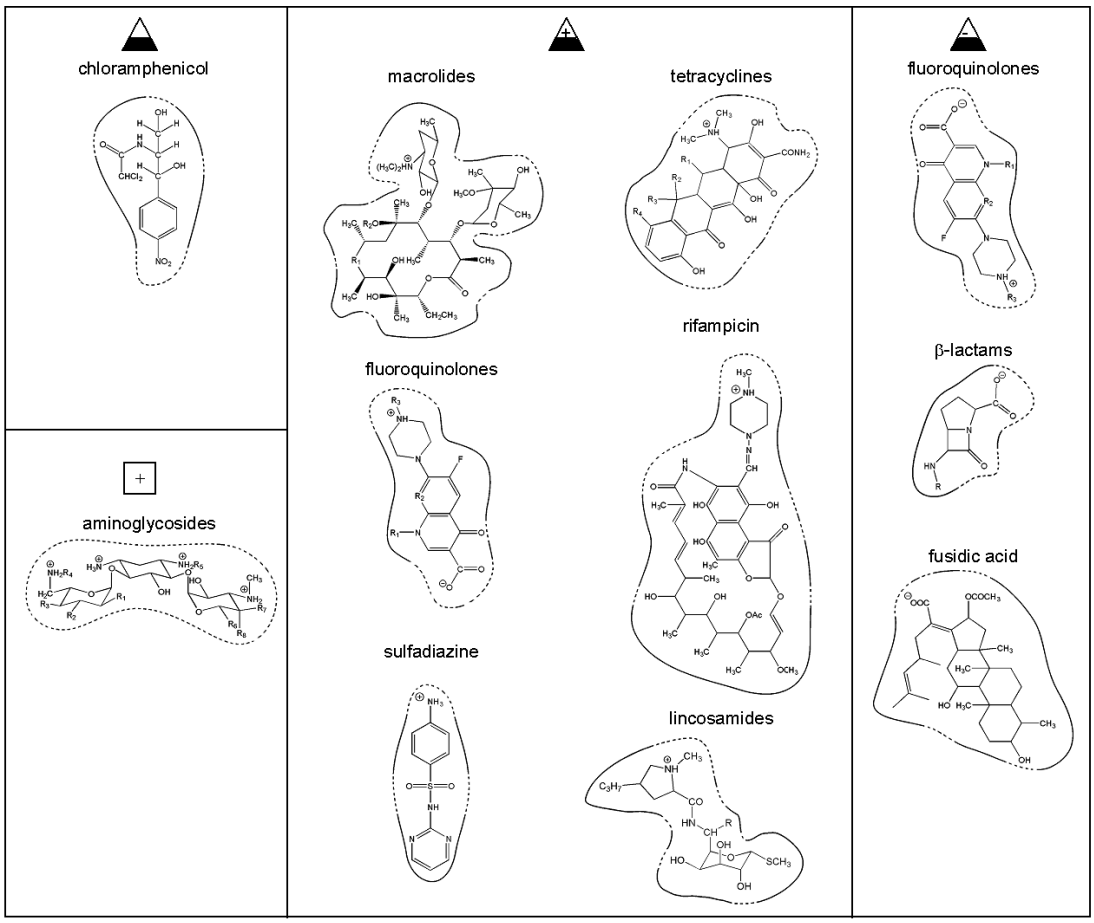


Figure 14: Structural formulae of the main antibiotics for which efflux has been demonstrated through at least one type of the transporters described in table 1 and figure 3. The molecules are presented so as to evidence their amphipathic character when appropriate (the zones considered more lipophilic are surrounded by a solid line, and those considered more hydrophilic, by a dotted line. The charged groups are systematically oriented upwards (fluoroquinolones are represented twice since these can act as cations as well as anions, and are accordingly transported by RND, MFS, MDR and MRP transporters). The local pH, which may vary largely from one type of organism to another and from the precise location of the pumps, strongly influences the ionization of these groups and their role in recognition by the transporters. The structures shown emphasize the common characters of each class of antibiotics, since structural variations within each class (denoted by the existence of variable substituents [R]) do not appear to markedly, systematically and specifically alter their recognition properties.

(Van Bambeke *et al.*, 2000)

Unfortunately, the importance of lipophilia is often difficult to ascertain, with only very few available systematic studies comparing homogenous series of drug derivatives. It, nevertheless, appears striking for the RND multidrug efflux pumps when considering the data available on the β-lactams, for which a transport ranking of cloxacillin > nafcillin > penicillin G > carbenicillin > penicillin N (the latter being almost not transported) has been clearly demonstrated (figure 15). This ranking is in close relationship with the lipophilicity of their side chain, which itself guarantees their partition in the membrane (Nikaido *et al.*, 1998).

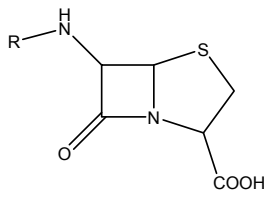
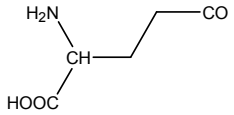
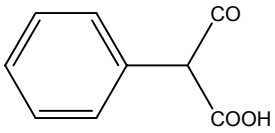
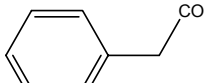
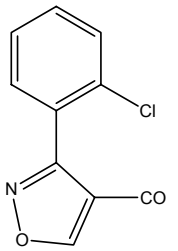
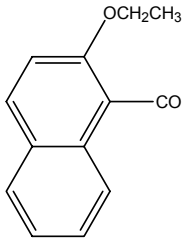


Figure 15:

Relationship between structure, lipophilicity of the side chain of selected penicillins and MIC of bacteria resistant by efflux

(Nikaido *et al.*, 1998)

R (Side chain)	molecule	side-chain partition coefficient	MIC ratio[a]
	penicillin N	0	1
	carbenicillin	80	4
	penicillin G	270	32
	cloxacillin	890	256
	nafcillin	4200	128

[a] ratio between the MIC of a *S. typhimurium* resistant mutant (AcrAB-TolC overproducer) and of its susceptible parental strain

But the situation may be different for highly diffusible antibiotics in interaction with narrower spectrum transporters. Thus, a study comparing the efflux of quinolones by the NorA pump from *S. aureus* (MFS superfamily) comes to the conclusion that the extrusion of these antibiotics is not correlated with their hydrophobicity, but is rather inversely proportional to the bulkiness of substituent in position 7 and the bulkiness and hydrophobicity of substituent in position 8, as illustrated for selected examples in figure 16 (Takenouchi *et al.*, 1996).

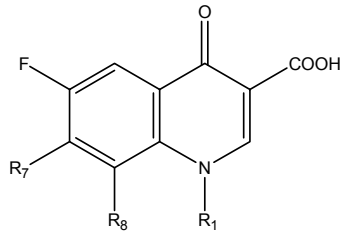
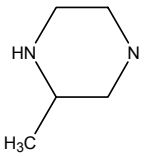
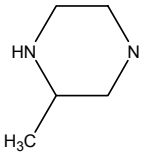
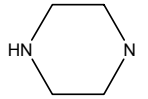
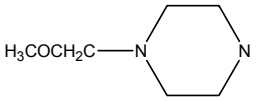
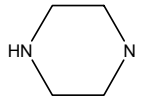


Figure 16:

Relationship between structure, bulkiness, and hydrophobicity of selected quinolone substituents and MIC of bacteria resistant by efflux.

(Takenouchi *et al.*, 1996).

molecule	R_1	R_7	R_8	MIC ratio[a]
(a)			OCF_3	2
(b)			OCH_3	4
ciprofloxacin			H	16
(c)	C_2H_5		H	16
norfloxacin	C_2H_5		H	64

[a] ratio between the MIC of a *S. aureus* resistant mutant (NorA overproducer) and of its susceptible parental strain.

While some antibiotic classes like quinolones, tetracyclines, macrolides, or chloramphenicol, are substrates of a large array of transporters, others are very rarely actively expelled from the cells, and from bacteria in particular. This is the case for aminoglycosides (Aires *et al.*, 1999; Moore *et al.*, 1999), but this can probably be explained by the high hydrophilicity of these antibiotics which prevents their entry into cells by non-specific diffusion. Aminoglycoside antibiotics indeed largely mimic polyamines -an essential substrate for many types of cells- and use their inward transport system for entering both bacteria [for activity] and specific eukaryotic cells [causing toxicity] (see Mingeot-Leclercq and Tulkens, 1999; Mingeot-Leclercq *et al.*, 1999 for reviews); aminoglycoside-producing organisms generally tend to protect themselves not by efflux pumps, but by the production of aminoglycoside-inactivating enzymes (Cundliffe, 1992). Similarly, the absence of efflux pumps acting on

glycopeptide antibiotics has been explained by the fact that these bulky, largely hydrophilic drugs act in the outerspace of Gram-positive bacteria, and never cross the bacterial membrane (glycopeptides are inactive against Gram-negative bacteria precisely because they cannot cross the outer membrane of these organisms [Hancock and Farmer, 1993]). Again, resistance in producing microorganisms is mediated by the production of enzymes allowing for the synthesis of modified peptidoglycan precursors with a lowered affinity for glycopeptides (Marshall *et al.*, 1998; see chapter II for further details on the mechanisms of resistance to glycopeptides). Antibiotic producing organisms make however exception, since efflux pumps have been evidenced in those synthesizing aminoglycosides or glycopeptides, and are required for the exportation of these antibiotics from the cytosol were they are synthesized (see e.g. Barrasa *et al.*, 1995; Pootoolal *et al.*, 2002).

Beyond these specific considerations, it must also be emphasized that a given antibiotic may be a substrate for different types of pumps, so that (i) it may be expelled by different organisms for which no common transporter has been identified so far (giving the false impression that the transporter is ubiquitous), and (ii) that modulation of the activity of a given transporter may be compensated by a modulation in the opposite direction of another transporter, with therefore no or little change in the cellular accumulation of the drug (giving the false impression that the drug is not transported). Moreover, a given pump may extrude not only different antibiotics within a same class but also different classes of antibiotics. Finally, a single cell may possess a vast and complex arsenal of efflux pumps allowing for the extrusion of a very broad spectrum of drugs (viz. *E. coli* and *S. cerevisiae,* the complete genome sequencing of which has revealed the existence of more than 250 putative transporters monopolizing 10 % of the whole genetic material [Paulsen *et al.*, 1998a; Paulsen *et al.*, 1998b]).

III.1.d. Antibiotic efflux pumps in prokaryotic cells: impact for resistance to antibiotic treatments

III.1.d.1. Why antibiotic transporters in bacteria ?

The first description of antibiotic transporters in bacteria has resulted from the study of resistance to tetracyclines in the early eighties (McMurry *et al.*, 1980). Soon after, several transporters were identified based on the same approach, i.e. through the unraveling of resistance mechanisms towards various classes of antibacterials, including successively the macrolides (Ross *et al.*, 1990), the fluoroquinolones (Yoshida *et al.*, 1990), the β-lactams (Li *et al.*, 1994) and, more recently, the aminoglycosides (Aires *et al.*, 1999). It eventually led to the concept that efflux must be considered as a common and basic mechanism of resistance, in the same line, and perhaps even more ubiquitous than target modification or production of antibiotic-inactivating enzymes. The reason why antibiotics are subject to efflux may actually be simply that most of them share the necessary basic structural features for effective recognition. These determinants are most often the simple combination of an amphipathic character and the presence of an ionisable function. In this context, antibiotics appear as occasional substrates of transporters aimed at protecting cells from exogenous, diffusible molecules. Other examples of potentially harmful substrates include biocides (Poole, 2002) or bile salts for *Enterobacteriaceae* (Thanassi *et al.*, 1997).

But efflux pumps can also be viewed as machineries developed by cells to extrude poorly diffusible or toxic endogenous molecules from the cytosol, and the latter may actually constitute some of the natural substrates of these transporters. Using *P. aeruginosa* PAO1 wild-type (WT) and its efflux mutants, it was indeed shown with models of epithelial cells and of murine endogenous septicemia, that most mutants demonstrated significantly reduced invasiveness and decreased capacity to kill mice. Invasiveness was restored by complementation of the mutants with the wild genes or by adding culture supernatants from epithelial cells infected with WT (Hirakata *et al.*, 2002). In the same line, the quorum-sensing auto-inducer 3-oxo-C12-homoserine lactone, which plays a key role in *P. aeruginosa* pathogenesis, is regulated by the MexAB-OprM efflux system (Rahmati *et al.*, 2002). These effects can however be counterbalanced by the reduced fitness which may occur as a consequence of the extrusion of bacterial metabolites (Sanchez *et al.*, 2002) or by a

decreased intracellular concentration of auto-inducing virulence factors (Kohler *et al.*, 2001) as shown in MDR-overproducing mutants of *P. aeruginosa.* Finally, combining both self-protecting and physiological roles, transporters also export bacterial toxins (Sharff *et al.*, 2001) as well as antibiotics from antibiotic producers (Cundliffe, 1992).

III.1.d.2. Main antibiotic transporters in pathogenic bacteria

Table 2 shows, on a systematic fashion, the antibiotic transporters positively identified in some important human pathogens. For each of them, we present the necessary elements of identification and a non-exhaustive list of antibiotics transported. Many other efflux pumps are suspected to exist, based either on phylogenetic comparison with established transporters or on phenotypic characterization (see for example Piddock *et al.*, 2002). Examination of table 2 shows three major features. First, several transporters recognize very different classes of antibiotics. This holds especially true for RND transporters and is the basis for the cross-resistance of several bacteria to apparently structurally unrelated drugs (substituting one class of antibiotics by another will not be a satisfactory answer to resistance detected in these isolates). Second, a given species (typically, *P. aeruginosa* and *E. coli*) may express quite different drug transporters, leading again to multiresistant phenotypes. Third, a given antibiotic may be recognized by different pumps, and some classes such as tetracyclines, macrolides, fluoroquinolones, and chloramphenicol appear as quasi-universal substrates. Yet, individual drugs, within a given class, may show specific behaviors with respect to recognition by transporters (see table 3). This has raised passionate debates concerning apparently closely related molecules, but may simply illustrate the critical impact of apparently minor chemical modifications in the recognition process of substrates by transporters.

Table 2a: classification and substrate specificity of antibiotic transporters identified in selected human pathogens (Gram-positive)

updated from (Van Bambeke et al, 2003a)

pathogen	transporter	super family	TC number[b]	antibiotics												
				β-lactams					FA	AG	Tet	OX	ML	SG	LM	CP	Rif	Q		SM	TM
				peni	ceph	carb	n-bac	inhib β-ase										NA	FQ		
S. aureus	NorA	MFS	2.A.1.2.10																		
	TetK-L	MFS	2.A.1.3.6																		
	MdeA	MFS																			
	MsrA[5]	ABC	3.A.1.121.1																		
S. pneumoniae	MefA	MFS	2.A.1.21.1																		
	MefE	MFS																			
	PmrA	MFS																			
	TetK-L	MFS																			
S. pyogenes	MefA	MFS	2.A.1.21.2																		
L. monocytogenes	MdrL	MFS																			
	Lde	MFS																			
	TetK-L	MFS																			
M. tuberculosis.	Mmr	SMR	2.A.7.1.2																		
	Tap	MFS																			
	LfrA	MFS	2.A.1.3.3																		
	TetK-L	MFS																			
	DrrB	ABC	3.A.1.105.1																		
Enterococcus spp	Mef?[6]	MFS																			
	TetK-L	MFS																			
	EmeA	MFS																			
	LfrAB	ABC																			
	Lsa	ABC																			

Green : transport demonstrated for at least 1 antibiotic of the class; red: absence of transport for the antibiotic class

Table 2b: classification and substrate specificity identified in selected human pathogens (Gram-negative)

updated from (Van Bambeke et al, 2003a)

pathogen	transporter	super family	TC number[b]	antibiotics														
				β-lactams					FA	AG	Tet	OX	ML	SG	LM	CP	Rif	Q		SM	TM
				peni	ceph	carb	n-bac	inhib β-ase										NA	FQ		
H. influenzae	hmrM	MATE																			
	TetB, K	MFS																			
	AcrB-like	RND																			
Neisseria spp	NorM	MATE	2.A.6.1.10																		
	MtrD	RND	2.A.6.2.5																		
Salmonella spp	AcrB	RND																			
	TetA-D	MFS																			
	FloR	MFS																			
E. coli	EmrE	SMR	2.A.7.1.3																		
	YdhE	MATE	2.A.66.1.3																		
	TetA-E	MFS	2.A.1.2.4																		
	Bcr	MFS	2.A.1.2.7																		
	MdfA	MFS	2.A.1.2.19																		
	YceL	MFS	2.A.1.2.21																		
	YidY	MFS	2.A.1.2.22																		
	EmrB	MFS	2.A.1.3.2																		
	YebQ	MFS	2.A.1.3.17																		
	SetA[8]	MFS	2.A.1.20.1																		
	Fsr	MFS	2.A.1.35.1																		
	AcrB	RND	2.A.6.2.2																		
	AcrD	RND	2.A.6.2.7																		
	AcrF	RND	2.A.6.2.1																		
	YegN	RND	2.A.6.2.12																		
	YhiV	RND	2.A.6.2.13																		
	MacB	ABC	3.A.1.122.1																		
P. aeruginosa	CmlA	MFS	2.A.1.2.3																		
	TetA,C,E	MFS																			
	MexB	RND	2.A.6.2.6																		
	MexD	RND	2.A.6.2.15																		
	MexF	RND	2.A.6.2.16																		
	MexK	RND	2.A.6.2.17																		
	MexY	RND																			

Green : transport demonstrated for at least 1 antibiotic of the class; red: absence of transport for the antibiotic class

Table 3: usual difference in affinity towards efflux pumps for antibiotics representative of the main classes

(Van Bambeke *et al.*, 2003a)

antibiotic class	affinity for efflux pumps			references
	high	variable [a]	low	
penicillins [b]	nafcillin, cloxacillin, penicillin G		carbenicillin	(Nikaido *et al.*, 1998)
cephalosporins [b]	cefalotin, cefotaxime, ceftriaxone		cefazolin, cephaloridin	(Nikaido *et al.*, 1998)
carbapenems	meropenem	imipenem		(Kohler *et al.*, 1999)
macrolides	14 - and 15 - membered		16 –membered, ketolides	(Clancy *et al.*, 1996; Capobianco *et al.*, 2000; Giovanetti *et al.*, 2000)
tetracyclines	tetracycline	minocycline	glycylcyclines [c]	(Mendez *et al.*, 1980; Someya *et al.*, 1995; Roberts, 1996)
quinolones	ciprofloxacin, norfloxacin	ofloxacin, levofloxacin	cinafloxacin, gatifloxacin, gemifloxacin, moxifloxacin [d], garenoxacin	(Takenouchi *et al.*, 1996; Piddock *et al.*, 1998; Schmitz *et al.*, 1998; Heaton *et al.*, 1999; Lomovskaya *et al.*, 1999; Schmitz *et al.*, 2002)

[a] depending on the efflux pump

[b] ranking corresponding to the degree of lipophilicity of the side chain

[c] low affinity substrate of MexD in *P. aeruginosa* and AcrB and AcrF in *E. coli*

[d] low affinity substrate of a still unidentified efflux transporter in *S. aureus*

III.1.d.3. Impact on resistance to antibiotic treatments

Generally speaking, efflux mechanisms confer a low to moderate level of resistance only (1-to 64-fold increase in MIC; Lomovskaya *et al.*, 1999; Mazzariol *et al.*, 2000) so that their clinical relevance has been questioned (Lynch III and Martinez, 2002). The following points need, therefore, to be carefully taken into consideration.

- the intrinsic (or natural) resistance of many bacteria to antibiotics depends on the constitutive or the inducible expression of active efflux systems. A typical example is given by *P. aeruginosa*, which was thought for a long time to be poorly susceptible to a large range of antibiotics of different classes because of the relative impermeability of its outer membrane to drugs.

Yet, the simple disruption of the gene coding for the MexB pump dramatically increases the susceptibility of *P. aeruginosa* to β-lactams, tetracyclines, fluoroquinolones, and chloram-phenicol (table 4; Li *et al.*, 1995).

Table 4: **Change in MIC of various antibiotics in *P. aeruginosa* upon disruption of the MexAB-OprM efflux pump**

(Li *et al.*, 1995)

antibiotic	MIC (µg/ml)	
	Wild type	MexAB-OprM disrupted
carbenicillin	32	< 0.25
ceftriaxone	64	8
tetracycline	8	0.5
norfloxacin	> 8	0.1
ciprofloxacin	2	0.2
chloramphenicol	16	8

Likewise, disruption of the gene coding for mdrL in *Listeria monocytogenes* causes a 10-fold decrease of the MIC of cefotaxime (Mata *et al.*, 2000), which suggests that the intrinsic resistance of *L. monocytogenes* to cephalosporins may be due to other mechanisms beside the existence of low affinity of *L. monocytogenes* PBP's towards this subclass of β-lactams. Finally, the poor susceptibility of *Haemophilus influenzae* to macrolides may partly result from the presence of an efflux mechanism ([Peric *et al.*, 2003]; beside the well known effect of acidity on the activity of macrolides, a CO_2-containing atmosphere being necessary to grow this bacteria). This suggests new avenues to design new macrolides with enhanced activity against *H. influenzae*.

- concomitant expression of several efflux pumps in a given bacterial species may lead to apparently "high level" resistance phenotypes when considering the shared substrates. This has been observed in Gram-negative bacteria, in which the multicomponent efflux pumps of the RND superfamily transport antibiotics from the cytosolic leaflet of the inner membrane to the periplasmic space, and the single component efflux pumps of the MFS superfamily promote efflux from this space to the external medium (Lomovskaya, 2002).

- efflux may also co-operate with other resistance mechanisms to confer not only high level but also broad-spectrum resistance.

  For example, in *E. coli,* expression of class C β-lactamase confers resistance to first and second generation cephalosporins, and expression of pump AcrB causes resistance to most penicillins. The global result is that the organism becomes only susceptible to third or fourth generation cephalosporins (Mazzariol *et al.*, 2000).

Table 5: **Susceptibility of *P. aeruginosa* and isogenic mutants to a selected carbapenem antibiotic**

(Okamoto *et al.*, 2001)

phenotype			Faropenem MIC (µg/ml)
AmpC	MexAB-OprM	OmpF	
-	-	+	0.5
-	-	-	1
+	-	-	32
-	+	-	256
+	+	-	512
+	++	-	4096

Likewise, the high intrinsic penem resistance of *P. aeruginosa* results from the interplay between the outer membrane barrier (production of OmpF porin), the active efflux system MexAB-OprM, and AmpC β-lactamase (table 5; Okamoto *et al.*, 2001).

A more indirect but probably very effective mode of co-operation is exemplified by the combination of DNA gyrase and/or topoisomerase IV mutation and efflux in the development of resistance to fluoroquinolones. Whereas single target mutations confer only a low level of resistance, the reduction in the intrabacterial concentration of fluoroquinolones through expression of one or several efflux pumps may result in MICs exceeding breakpoints (see [Le Thomas *et al.*, 2001] for example). Thus, resistant strains from clinical sources (Yoshida *et al.*, 1994) often display a combination of multiple mutations in the target genes and overexpression of efflux transporters (Jalal and Wretlind, 1998). More critically, the exposure of the targets to insufficient drug concentrations will favor the selection of mutants.

Table 6: **Frequency of levofloxacin-resistant mutants in *P. aeruginosa* with deletion of the efflux pump operons**

(Lomovskaya *et al.*, 1999)

Pump status			Levofloxacin MIC (µg/ml)	Frequency of resistant mutants
MexAB	MexCD	MexEF		
+	+	+	0.25	$2\ 10^{-7} – 4\ 10^{-7}$
-	+	+	0.015	$2\ 10^{-7} – 4\ 10^{-7}$
+	-	+	0.25	$2\ 10^{-7} – 4\ 10^{-7}$
+	+	-	0.25	$2\ 10^{-7} – 4\ 10^{-7}$
-	+	-	0.015	$1\ 10^{-7} – 2\ 10^{-7}$
-	+	+	0.25	$2\ 10^{-6}$
-	-	+	0.015	$1\ 10^{-9}$
-	-	-	0.015	$< 1\ 10^{-11}$

Thus, constitutive expression of efflux pumps acting on fluoroquinolones probably explains the high frequency of mutations leading to resistance in Gram-negative bacteria. This has been particularly well demonstrated for levofloxacin and *P. aeruginosa*. Disruption of the genes of 3 RND pumps not only brings the MIC from 0.25 to < 0.02 µg/ml, but, most strikingly, reduces the frequency of appearance of first-step mutants from $2x10^{-7}$ to $< 10^{-11}$ (table 6; Lomovskaya *et al.*, 1999). It must, however, be emphasized that all pumps must be simultaneously inactivated to obtain such an effect since the lack of activity of one can be easily compensated by overexpression of other ones with overlapping spectra (Li *et al.*, 2000).

- antibiotics can serve as inducers and regulate the expression of some efflux pumps at the level of the gene transcription or of the mRNA translation, by interacting with regulator systems (Roberts, 1996). Transporters may also become overexpressed as a result from mutations occurring in these regulators (Poole, 2000; this mechanism may be the predominant one for resistance of *P. aeruginosa* to fluoroquinolones in cystic fibrosis patients [Jalal *et al.*, 2000]). More importantly, global regulations may be involved (Pumbwe and Piddock, 2000), causing the overexpression of several independent genes (regulons; George, 1996). For instance, the *mar* regulon in *E. coli* may not only control the expression of AcrB, but probably also that of other drug-specific transporters and porins, together with numerous other proteins involved in stress responses (see [Barbosa and Levy, 2000] and the references cited therein). Worryingly also, mutations in regulator genes might even result in constitutive expression of several efflux pumps, causing multiple resistance.

- resistance by efflux can be easily disseminated. In several cases, indeed, the genetic elements encoding efflux pumps and their regulators are located on plasmids (such as Tet transporters in Gram-positive bacteria), on conjugative or transformable transposons located either on plasmids (Tet transporters also, but in Gram-negative bacteria), or in the chromosome (as *mef* genes in *S. pneumoniae*; Del Grosso *et al.*, 2002). Importantly, efflux-mediated resistance mechanisms can spread between phylogenically very distant species. This is exemplified by the macrolide-mediated efflux which not only has moved among *Streptococci* but also to Gram-negative bacteria (Luna *et al.*, 2000). Co-transfer with genes for other resistance mechanisms may also take place if these are present together on large mobile genetic elements.

III.1.d.4. Strategies for the future of antibiotherapy

Efflux mechanisms should now be taken fully into account in the evaluation of new antibiotics as well as for the future of chemotherapy in the long term. At the level of the clinical microbiology laboratory, suspicion of efflux as cause of resistance should ideally be confirmed either phenotypically or genotypically for anticipating cross-resistances during therapy. However, the phenotypic characterization of efflux may prove difficult or impossible in multiresistant isolates producing several mechanisms. The clear-cut situation of macrolides and streptococci, in which efflux-

based resistance is limited to 14- and 15-membered macrolides (Farrell *et al.*, 2002) may be unique. Conversely, demonstrating resistance to a large variety of unrelated agents can suggest the presence of wide-spectrum transporters (see [Ziha-Zarifi *et al.*, 1999] for an example with clinical isolates). Unfortunately, several of the antibiotic substrates recognized by many efflux pump systems (such as chloramphenicol, tetracyclines, or trimethoprim) are no longer tested in clinical laboratories because of lack of immediate therapeutic interest. More genotypic characterization could yield important information for surveillance studies. For instance, PCR has been successfully used to unambiguously identify efflux-based resistance mechanisms to macrolides (Sutcliffe *et al.*, 1996), tetracyclines (Aminov *et al.*, 2002), and fluoroquinolones (Jalal and Wretlind, 1998) and finger printing has allowed to follow the spread of the corresponding resistant clones (Jalal and Wretlind, 1998). Further development of other new genotypic tests allowing to improve the characterization of efflux mechanisms in clinical isolates would be welcome in this context.

Moving now to the evaluation of new antibiotics, exploring their potential recognition by typical efflux transporters must now be in the forefront of their preclinical and clinical assessment. All other pharmacological and toxicological properties being equal, the aim, here, will be to favor the selection of derivatives that are poor substrates of the efflux pumps and do not induce their overexpression. Table 3, which illustrates the relative affinity of current and newly introduced antibiotics for efflux transporters, reveals indeed that most recent antibiotics in each class are less recognized than older ones.

The development of pump inhibitors as adjuvant therapy also represents an interesting area for drug discovery, somewhat like β-lactamase inhibitors brought new life to β-lactams (Ryan *et al.*, 2001). This approach, however, appears very challenging because of potential effects on efflux transporters also present in eukaryotic cells. It must be emphasized that most presently available inhibitors display strong pharmacological activities in eukaryotic cells systems and are therefore unusable in clinical practice. Typical examples include reserpine (Brenwald *et al.*, 1997), omeprazole (Aeschlimann *et al.*, 1999b), phenothiazines (Molnar *et al.*, 1997), sertraline (Munoz-Bellido *et al.*, 2000), verapamil (Cohn *et al.*, 1995), and siderophores (Rothstein *et al.*, 1993). It is therefore essential to develop molecules not only specifically designed to inhibit prokaryotic transporters, but also to stay away from molecular structures close from those of drugs. Another difficulty comes from the

fact that the pharmacokinetic/dynamic properties of the pump inhibitors will need to closely match those of the companion antibiotic. Actually, only three main categories of compounds have been uncovered so far (Lomovskaya and Watkins, 2001). The first group comprises compounds specifically raised against the Tet transporters for tetracyclines (namely tetracycline derivatives substituted in position 13 and probably acting as competitive inhibitors [Nelson and Levy, 1999] and a non-competitive inhibitor built on a indan nucleus [Hirata *et al.*, 1998]. The second group is represented by a family of flavonolignans derived from a natural alkaloid from *Onopordon corymbosum* (Guz *et al.*, 2001). These molecules inhibit the NorA transporter of *S. aureus.* As flavones are also known to inhibit the eukaryotic P-glycoprotein, considerable lead optimization studies will be needed to obtain safe compounds. The third category is represented by peptides acting upon the RND transporters of *P. aeruginosa* (Renau *et al.*, 1999). These inhibitors selectively improve the activity of antibiotics that are substrates of the MexB efflux pump (quinolones, macrolides, chloramphenicol, and to a lesser extent, tetracycline or carbenicillin [Lomovskaya *et al.*, 2001]), which suggests a high level of specificity (Barrett, 2001). They do not interact with the eukaryotic P-glycoprotein. A desirable step forward would be to consider the design of wider spectrum inhibitors acting on pumps present in both Gram-positive and Gram-negative bacteria and belonging to different phylogenetic families. A prudent use of these inhibitors will however be essential, to avoid fast emergence of resistance to them, which will most probably also emerge. Laboratory mutants of *Bacillus subtilis* have indeed already been isolated with resistance to the inhibitory activity of reserpine (Ahmed *et al.*, 1993).

III.1.e. Antibiotic efflux pumps in eukaryotic cells: Impact on antibiotic cellular pharmacokinetics, pharmacodynamics and toxicodynamics

III.1.e.1. Why are antibiotics transported in eukaryotic cells ?

Even though eukaryotic cells are not the primary pharmacological targets of antibiotics, the concepts we have developed teach us that the existence of efflux pumps transporting these drugs in eukaryotic cells is not surprising. Active transporters probably originally serve to facilitate the transmembrane transport of endogenous molecules, such as phospholipids (by acting as a flippase), cytokines, metabolic intermediates or nutrients, act as influx mechanism for polar compounds, or insure the true transport functions across epithelial barriers in pluri-cellular organisms. However, they can also transport drugs, which are recognized based on physico-chemical properties, such as hydrophobicity, aromaticity, hydrogen binding capacity, and an ionisable character (within a given spatial environment) rather than on defined chemical or pharmacological properties, as in classical enzyme-substrate or ligand-receptor recognition (Breier *et al.*, 2000; Zheleznova *et al.*, 2000; Ekins *et al.*, 2002; Salerno *et al.*, 2002; Stouch and Gudmundsson, 2002). In all these situations, antibiotics, as other drugs, really appear as opportunistic substrates (Johnstone *et al.*, 2000).

III.1.e.2. Occurrence and general properties of antibiotic transporters in eukaryotic cells

Table 7 gives a summary of the main characteristics of the transporters that have been described as interacting with antibiotics in eukaryotic cells. It also gives, for each type of transporter, the best-characterized non-antibiotic drug substrates and, when known, the non-drug substrates, often tentatively identified as the physiological substrates. Figure 17 shows the distribution of these transporters among the main cell types. Whereas some transporters are considered ubiquitous (for example, MDR1 and MRP1), many others show quite specific distribution. Moreover, the function of these transporters depends on their orientation. Accordingly, drug movement must not only be analyzed in terms of influx or efflux at the level of a single cell, but also at that of the whole organism.

Efflux-oriented transport is mainly facilitated by the so-called multidrug transporters. If localized at the brush border membrane of polarized cells (for example, MDR1, MRP2), they will cause an accelerated clearance, although MDR1 in choroid cells (Rao *et al.*, 1999) is responsible for an increased concentration of its substrates in the CNS. Conversely, they will cause a retention of the drug in the organism if they are located at the basolateral surface of polarized cells (for example, MRP1 and MRP3 [Borst *et al.*, 1999]). Moreover, some transporters, such as NPT1, found at the apical membrane of some cells but at the basolateral membrane of others, will have opposite effects on drug clearance, depending on the organ in which they are found. This explains, for instance, why β-lactams, which are substrates for this transporter, may be secreted at the level of the kidney but be reabsorbed at that of the liver (Yabuuchi *et al.*, 1998; Uchino *et al.*, 2000).

Table 7 : Transporters involved in the transmembrane passage of antibiotics in eukaryotic cells

(Updated from Van Bambeke et al, 2003b)

superfamily	family	source of energy	transporter	Physiological substrates	Typical examples of non antibiotic substrates	Typical examples of inhibitors	Examples of recognized antibiotics	organ where transport has been demonstrated
3.A.1. ABC.	**3.A.1.201.** Multidrug Resistance Exporter	ATP hydrolysis	MDR1	phospholipids cytokines	anthracyclines vincristine methotrexate	verapamil cyclosporin A GF120918 LY335979	**fluoroquinolones** levofloxacin sparfloxacin grepafloxacin ofloxacin, ciprofloxacin hsr-903 **macrolides** erythromycin clarithromycin roxithromycin, josamycin, azithromycin, spiramycin **beta-lactams: lipophilic cephalosporins** cefoperazone , ceftriaxone , cefazoline (ceftadzidime , cefradine , cefotetan) dicloxacillin **tetracyclines** tetracycline **streprogramins** pristinamycin **trimethoprim**	 kidney intestine liver transfected cells brain intestine, transfected cells, liver, brain kidney, transfected cells, transfected cells cancer cells transfected renal cells transfected cells intestine transfected renal cells
	3.A.1.208. Conjugate-Transporter-2	ATP hydrolysis; (glutathion as co-factor for some of them)	MRP1	phospholipids leukotrienes glucurono- and glutathion-conjugates	anthracyclines vincristine methotrexate	probenecid gemfibrozil MK-571	**fluoroquinolones** difloxacin ofloxacin **rifampicin** **macrolides** erythromycin	 cancer cells transfected cells cancer cells cancer cells
			MRP2	bilirubine-conjugates leukotrienes glucurono- and glutathion-conjugates	anthrocyclines vincristine methotrexate	probenecid	**fluoroquinolones** hsr-903, grepafloxacin **beta-lactams** cefodizime **rifampicin** **macrolides** azithromycin	 liver liver hepatic cells liver, intestine
			MRP3	glycocholate, bile salts glucurono-conjugates	methotrexate etoposide purine and nucleotides analogues	probenecid	**rifampicin**	hepatic cells
			MRP5	cyclic nucleotides		probenecid sildenafil	**rifampicin**	hepatic cells

superfamily	family	source of energy	transporter	Physiological substrates	Typical examples of non antibiotic substrates	Typical examples of inhibitors	Examples of recognized antibiotics	organ where transport has been demonstrated
2.A.1. MFS	2.A.1.13. Monocarboxylate Porter Family	Monocarboxylate uptake/efflux port	MCT1	lactate, pyruvate, mevalonate	pravastatine	mersalyl acid	**beta-lactams** cefdinir, carindacillin (prodrug of carbenicillin)	intestine
	2.A.1.14. Anion:Cation Symporter family	anion:cation symport	NPT1	phosphate	foscarnet	phosphono-formic acid	**beta-lactams** **anionic > zwitterionic** cloxacillin, cefoperazone , cefpiramide ,nafcillin , dicloxacillin , apacillin , penicillin G , cefixime ,(ceftizoxime, cephalexin , ampicillin, cephradine, cyclacillin , cephalotine , cephaloridine) faropenem	liver kidney
	2.A.1.19. Organic Cation Transporter family	ion uniport or ion:H^+ symport	OAT1	bile salt prostaglandins cyclic nucleotides	anionic drugs steroids NSAID diuretics	probenecid	**(fluoro)quinolones** nalidixic acid, ofloxacin, cinoxacin **beta-lactams** cephaloridine, cephalotine, cephazoline, cephalexine, carbenicillin, cefoperazone, ceftriaxone, cefadroxil, cefamandole	kidney kidney
			OAT2	prostaglandins	zidovudine NSAID methotrexate	probenecid	**tetracycline** **cephalosporins** cephaloridine, cephalotine, cefoperazone, cefotaxime cefamandole	kidney kidney
			OAT3	anionic neuro-transmitters	anionic drugs cimetidine	indocyanine green	**beta-lactams** penicillin G cefazoline, cefoperazone, cephalothin, cephaloridine, cefadroxil, cefamandol	brain kidney
			OAT4	prostaglandins		probenecid	cefazoline, cefoperazone, cephalothin, cephaloridine, cefadroxil, cefamandol	kidney
			OCT1	neuro-transmitters vitamins	cationic drugs	tetraethyl-ammonium	**Fluorquinolones** Ofloxacin	kidney (rat)
		cation:Na^+ symport	OctN2	carnitine	quinidine verapamil	quinidine verapamil	**beta-lactams** **(quaternary ammonium)** cephaloridin , cefepime , cefluprenam (cefocelis , cyclacillin)	kidney
2.A.60. Organo Anion Transporter family	2.A.60.1.	anion uniport or anion:anion antiport	Oatp1	bile salts steroid hormones	digoxin	indocyanine green	**rifampicin**	liver
2.A.17. Proton Dependent Oligopeptide Transporter family	2.A.17.4.	peptide:H^+ symport	PEPT1	peptides	protease inhibitors quinapril	sulfonylureas	**beta-lactams** ceftibuten, cyclacillin , cefadroxil, cefamandol, cephradin, cefaclor, cefuroxime axetil, cefixime, cephalotin, cefalexin, ampicillin	intestine
			PEPT2	peptides	valacyclovir	sulfonylureas	**beta-lactams** **(aminogroup on the phenyl ring)** cefalexine; cefadroxyl, cephradine cefadroxyl , cyclacillin	kidney

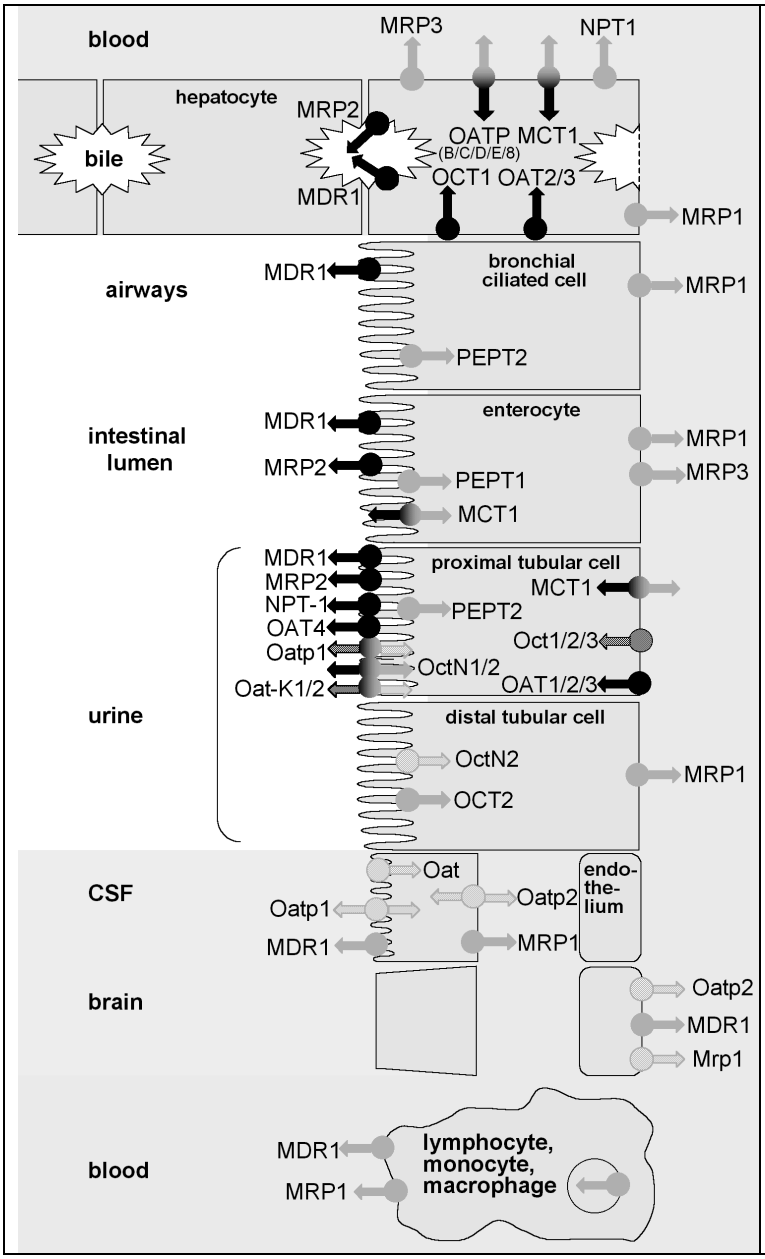


Figure 17: Schematic representation of the main transporters potentially involved in antibiotic movement at the level of epithelial cells in the main organs (liver, bronchial tree, intestine, kidney), the blood-brain barriers, and in leucocytes (PMN are not considered here since the role of drug transporters in these cells is unclear). Black arrows denote transport towards extracorporeal compartments such as urine, bile, intestine and airways (i.e. transporters involved in drug elimination from the body). Grey arrows describe uptake processes from extracorporeal fluids into cells (i.e. allowing drugs to accumulate in tissues), or from cells to body fluids (i.e. causing the drug to be transported from one body fluid to another [from blood to cerebrospinal fluid, e.g.]). The level of expression of each transporter may differ between species (arrows with a checkerboard background indicate transporters that have been, so far, evidenced in animals only). The direction of transport of bidirectional transporters may differ according to the cell type.

(Van Bambeke *et al.*, 2003b)

Influx transporters located at the basolateral membrane will increase the drug concentration within the epithelial cells. If these are bordering the external medium (Sekine *et al.*, 1997; Sekine *et al.*, 1998; Urakami *et al.*, 1998), an increased clearance can be obtained provided the drug can diffuse out of these cells. An excellent example is OAT1 which is responsible for the tubular secretion of β-lactams (Jariyawat *et al.*, 1999; Takeda *et al.*, 1999). Conversely, an inwards transporter localized at the brush border membrane of epithelial cells can indirectly increase the systemic concentration of its substrates by driving them into these cells, from where they can diffuse into the blood (Liang *et al.*, 1995; Liu *et al.*, 1995).

Bidirectional transporters have also been found and these can take various roles depending on their localization (Tsuji *et al.*, 1993; Li *et al.*, 1999; Masuda *et al.*, 1999; van Montfoort *et al.*, 1999).

III.1.e.3. Modulation of the absorption and elimination of antibiotics

The role of drug transporters in the modulation of antibiotic pharmacokinetics has been mainly studied for β-lactams, fluoroquinolones and, to a lesser extent, macrolides. β-lactams are known as generally being poorly reabsorbed with, however, a few noticeable exceptions. These concern derivatives that have been shown to be substrates for either PEPT1 (oral cephalosporins or ampicillin [Bretschneider *et al.*, 1999]; see also details in table 1) or MCT (as is the case for carindacillin, the oral prodrug of carbenicillin [Li *et al.*, 1999]). In the same way, OctN2 and PEPT2 have been shown to facilitate the reabsorption of β-lactams from the renal tubular filtrate (Ganapathy *et al.*, 1995; Aeschlimann *et al.*, 1999a; Ganapathy *et al.*, 2000), thereby prolonging their plasma half-life. OctN2 recognizes derivatives with a quaternary ammonium substituent (such as cephaloridine), whereas PEPT2 transports cephalosporins with an amino group in the substituent of the cephem nucleus (such as cefadroxil; Ganapathy *et al.*, 2000). A critical role of drug transporters in the elimination of β–lactams through the renal and hepatobiliary tracts has also been suggested with transporters located at the basolateral and apical levels (Sathirakul *et al.*, 1994; Jariyawat *et al.*, 1999). A concerted action, implying pairs of transporters localized at both the basolateral and apical poles of the hepatocytes, has also been proposed for the fluoroquinolone grepafloxacin (although fluoroquinolones are most probably able to freely diffuse across membranes) and its glucurono-conjugates (Sasabe *et al.*, 1997; Sasabe *et al.*, 1998). Similarly, clearance of both macrolides and fluoroquinolones can be accelerated by the action of MDR1 or MRP2, in the intestine, kidney, liver or central nervous system (Ito *et al.*, 1997; Cormet-Boyaka *et al.*, 1998; Sasabe *et al.*, 1998; Takano *et al.*, 1998; Wakasugi *et al.*, 1998; Murata *et al.*, 1999).

III.1.e.4. Barrier effects

Transporters identified at the blood-brain and the blood-CSF barriers probably play a key role in clearing the central nervous system from drugs and other toxins (Schinkel, 1999; Sugiyama *et al.*, 1999; Terasaki and Hosoya, 1999; Aeschlimann *et*

al., 1999a; Lin, 2004 and figure 17) This is probably most important for β-lactams and fluoroquinolones. Indeed an inverse correlation has been observed between the propensity of fluoroquinolones to induce seizures (De Sarro *et al.*, 1999) and their rate of efflux from the CNS (Ooie *et al.*, 1997). Active efflux may be detrimental for treatment of meningitis and other infections of the CNS. For example, failures with cephalothin have been attributed to the active efflux of this molecule (Suzuki *et al.*, 1997).

Drug inactivating mechanisms and drug transporters may also combine to cause more efficient barrier effects. This concept, which is well known in the case of resistant bacteria (see above; III.1.d.3), is now increasingly recognized in mammals, where intestinal and liver transporters co-operate with cytochrome P450-based metabolism to decrease quickly and effectively the amount of active molecule present in the body (Dietrich *et al.*, 2003). Thus, Phase I metabolism adds polar functions on drug molecules, which are further transformed into bioconjugates by Phase II enzymes. The increased polarity of metabolites favors their recognition by efflux pumps (Benet *et al.*, 1999), as demonstrated with MRP2 for grepafloxacin (Sasabe *et al.*, 1998). This has led to the concept of "Phase III" elimination of drugs (figure 18; Ishikawa, 1992).

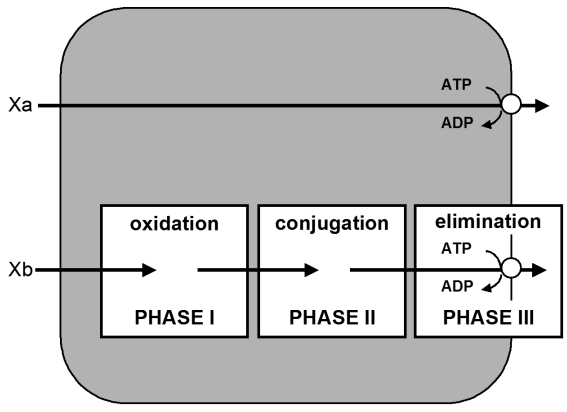


Figure 18: metabolism and elimination of xenobiotics by two distinct pathways.

Xa: xenobiotics that are exported by P-glycoprotein;

Xb: xenobiotics that are
- bioactivitated in Phase I,
- conjugated with glutathione in Phase II,
- and thereafter eliminated from the cell by MRP2.

(Ishikawa, 1992)

Interestingly, the orphan nuclear receptor SXR, which is activated upon exposure to substrates common to cytochromes P450 and MDR, can co-regulate the expression of these two clearance systems (Synold *et al.*, 2001). The subsequent change in their activity may shed a new light on the specific mechanisms of some drug-antibiotic interactions (Leveque and Jehl, 1995). For instance, rifampicin reduces the blood level of several drugs by inducing both cytochromes P450 and MDR expression (Hebert *et al.*, 1992), whereas erythromycin increases that of digoxin, by inhibiting the activity of both proteins (Kim *et al.*, 1999).

III.1.e.5. Modulation of cellular accumulation of antibiotics

The intracellular concentration of antibiotics is considered to be an important determinant in their activity against intracellular organisms (Tulkens, 1990; Nix *et al.*, 1991). Monocytes, macrophages and lymphocytes have been shown to express MRP and MDR transporters (Ichibangase *et al.*, 1998; Puddu *et al.*, 1999; Jones *et al.*, 2001) (table 7), which have the potential to decrease the cellular antibiotic concentrations and to impair their activity. This has been seen for fluoroquinolones, macrolides, streptogramins, lincosamides, and rifampicin, in cells overexpressing MDR1 and infected by *Listeria monocytogenes* (Nichterlein *et al.*, 1998). On the contrary, gemfibrozil, an inhibitor of organic anions transporters, significantly improves the activity of fluoroquinolones against the same bacteria (Rudin *et al.*, 1992). The impact of efflux pumps on antibiotic activity is, however, more difficult to predict when considering bacteria localized in the phagosomal (*Legionella pneumophila, Mycobacteria* spp), or lysosomal (*Staphylococcus aureus, Salmonella* spp) compartmeņts. Efflux pumps are indeed also found in intracellular structures and could therefore modify the subcellular distribution of their substrates Kim *et al.*, 1997; Oh and Straubinger, 1997). We do not know, however, to what extent the cellular pools correspond to active proteins (Molinari *et al.*, 1994; Molinari *et al.*, 1998; Larsen *et al.*, 2000).

Modification of intracellular accumulation may also be associated with corresponding changes in toxicity. A well-known example is given by the β-lactams that are substrates for the renal OAT (such as cephaloridine). These are more nephrotoxic than other cephalosporins (Takeda *et al.*, 1999) related to their increased accumulation in proximal tubular cells (Jariyawat *et al.*, 1999). On the other hand, a lower hepatic concentration of rifampicin or erythromycin through the activity of MDR1 lowers their ability to modulate the cytochrome P450 activity (Schuetz *et al.*, 1996; Schuetz *et al.*, 1998). In a wider context, multidrug transporters are also thought to play a protective role against apoptosis induced by several drugs, an effect which, however, could be due to other mechanisms than drug efflux itself (see [Johnstone *et al.*, 2000] for review). It is noteworthy in this context that several antibiotic classes may be apoptogenic, for example, aminoglycosides (Nakagawa *et al.*, 1997; El Mouedden *et al.*, 2000), macrolides (Aoshiba *et al.*, 1995), fluoroquinolones (Aranha *et al.*, 2002), or chloramphenicol (Turton *et al.*, 1999).

III.1.e.6. Strategies for the future

The role of transporters in the modulation of antibiotic pharmacokinetics should be taken into account in the future selection of drugs. In relation to the examples discussed here, a prime example is the design of β-lactams with an increased oral absorption and a decreased elimination. It is unfortunate, however, that the substrate specificities of the intestinal PEPT1 and the renal PEPT2 transporters are not exactly similar (as shown in table 7) (Ganapathy *et al.*, 1995), which may make quite difficult to obtain molecules optimized with respect to both transporters. Another area of interest would be the selection of fluoroquinolones with decreased penetration into or retention within the central nervous system. Structure-activity relationships in this context and design of improved compounds appears, however, difficult, due to the multiplicity of transporters interacting with a given drug (Tamai *et al.*, 2000). Despite this, one recent, successful example might be HSR-903 (Murata *et al.*, 1999; Tsuji, 1999).

Inhibition of transporters may also prove useful. An historical example is probenecid, used for long as a sparing drug against the renal elimination of β-lactams and fluoroquinolones. We know today that this effect is mediated at least by the inhibitory effect probenecid exerts towards OAT and MRP2 (Foote and Halstenson, 1998; Bakos *et al.*, 2000). Similar effects on pharmacokinetics or cellular retention have been observed with gemfibrozil, and several other drugs (for example, verapamil, cyclosporin A), which are now known to be substrates of drug transport that can act as competitive inhibitors. These first-generation inhibitors suffer, however, from severe limitations, due primarily to their own pharmacological activity and to their unspecificity of action (inhibition of several transporters as well as of enzymes involved in Phase I metabolism). The following step consisted therefore in derivatizating these molecules to obtain second generation molecules that do not present any pharmacological activity (Ford and Hait, 1990; Krishna and Mayer, 2000). They remain however able to inhibit cytochrome P450 3A4 as well as many ABC transporters, causing unpredictable pharmacokinetic interactions (Thomas and Coley, 2003). The next goal was thus to design new chemical entities able to inhibit selectively a given class of transporters, without affecting the hepatic metabolism (Thomas and Coley, 2003). This has been partially achieved with third-generation molecules, like the preferential inhibitors of MDR or MRP, for instance (Hyafil *et al.*, 1993; Gekeler *et al.*, 1995; Dantzig *et al.*, 1996; Krishna and Mayer, 2000), some of which have been shown to be noncompetitive inhibitors acting on the ATP-ase activity

of the pump (Martin *et al.*, 1999). Some of these inhibitors are currently being evaluated for their potential use in therapy (Dantzig *et al.*, 2001; Kruijtzer *et al.*, 2002). A major unknown in this area is, however, the detrimental effects impairment of transporters may have on the handling of their natural substrates. There is thus still room ahead for further research aimed at a better understanding of the complex relationships between transporters and the pharmacokinetics, pharmacodynamics and toxicodynamics of antibiotics.

III.2. Experimental studies: active efflux of antibiotics from macrophages

III.2.a. Preliminary data: expression on efflux pumps in macrophages

To illustrate the impact of antibiotic efflux from eukaryotic cells, we have chosen macrophages as a study model (J774; continuous cell line of murine origin [Snyderman *et al.*, 1977]). Macrophages appear indeed as a first-line defense of the organism against bacterial infections, so that any modulation of antibiotic concentration in these cells could have deleterious pharmacological implications. Among the numerous efflux pumps cited in the introduction, we have shown (by immunodetection [confocal microscopy (figure 19) and Western-Blot]) that J774 macrophages express at least two transporters of the ATP-binding cassette family, namely P-glycoprotein, and MRP1. MRP-1 was mainly localized at the cell surface, while P-glycoprotein was found partly at the pericellular membrane and partly associated with discrete cytoplasmic granules of variable sizes and with a perinuclear distribution.

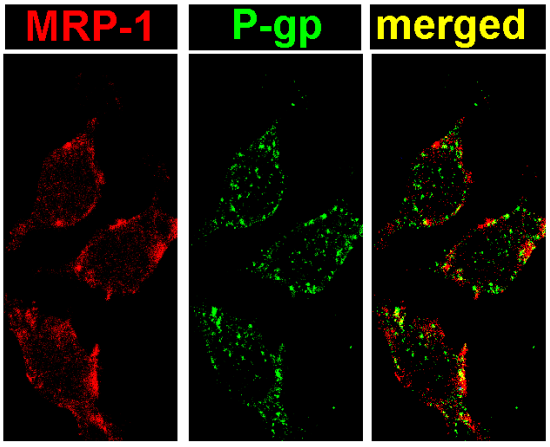


Figure 19: Observation in confocal microscopy of immuno-reactive MRP-1 and P-glycoprotein in J774 macrophages.

Red channel: immunological detection of MRP-1 using rat anti-human MRP1 antibodies and alexa fluor 568 anti-rat secondary antibodies; green channel: immunological detection of P-glycoprotein using rabbit polyclonal anti-P-glycoprotein antibodies and alexa fluor 488 anti-rabbit secondary antibodies

These granules were tentatively identified as making part of the endocytic apparatus (endosomes or lysosomes), since a co-localization of P-glycoprotein and of rhodamine-labeled dextran could be demonstrated (Seral *et al.*, 2003b). When merged images were examined, a large proportion of these granules turned yellow, demonstrating a partial common subcellular localization of both types of transporters. This distribution is consistent with what has been described in other cells. The quantity belonging to intracellular pools could be to maintain a constant amount of the active protein at the cell surface (Larsen *et al.*, 2000).

III.2.b. Active efflux of macrolides by P-glycoprotein

Macrolides are made of a 14-, 15-, or 16-membered lactone ring substituted by two sugars (figure 20). The first sugar (desosamine) bears a protonable aminated function, which confers to all macrolides a cationic character.

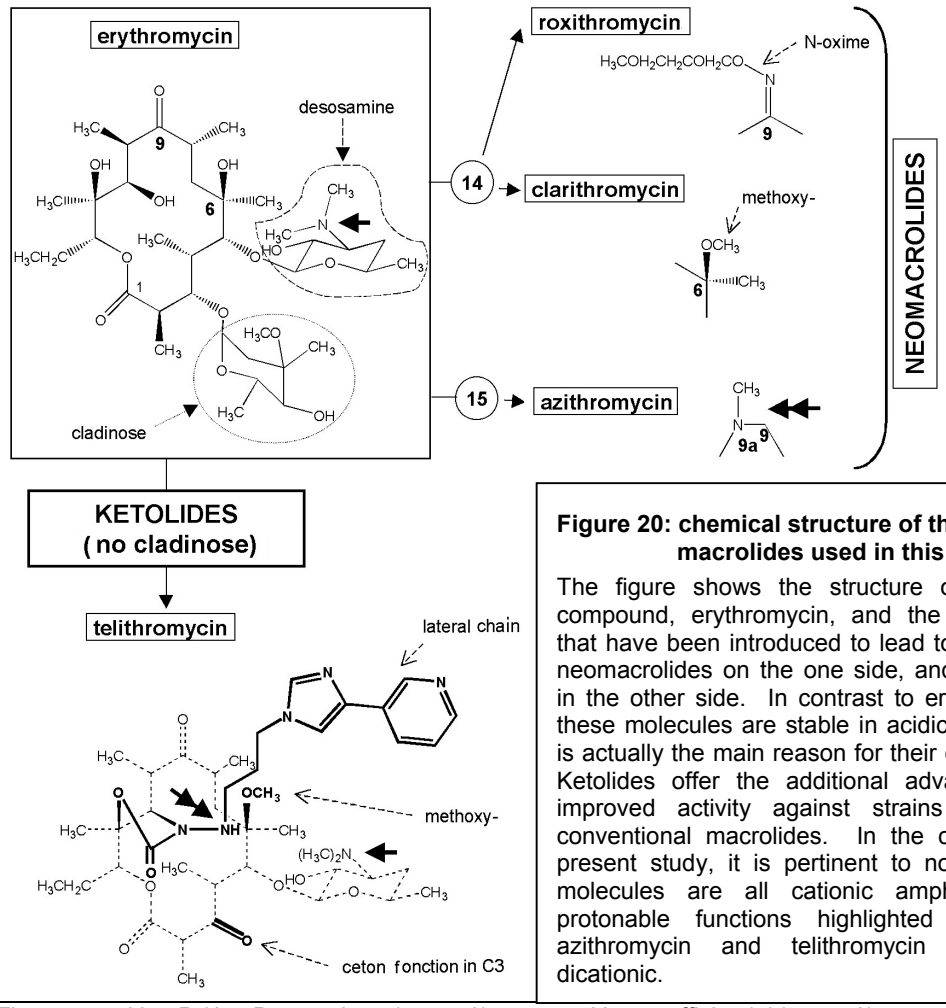


Figure 20: chemical structure of the macrolides used in this study

The figure shows the structure of the parent compound, erythromycin, and the modifications that have been introduced to lead to the family of neomacrolides on the one side, and of ketolides, in the other side. In contrast to erythromycin all these molecules are stable in acidic medium (this is actually the main reason for their development). Ketolides offer the additional advantage of an improved activity against strains resistant to conventional macrolides. In the context of the present study, it is pertinent to note that these molecules are all cationic amphiphiles (with protonable functions highlighted by arrows), azithromycin and telithromycin being even dicationic.

The reported log P / log $D_{pH=7}$ values (octanol/water partition coefficient) / (octanol/water partition coefficient at pH 7; log D = log P - log[1+$10^{(-charge * (pH-pKa))}$]), two accepted measures of the hydrophobic character of organic molecules) are 2.83 ± 0.78 / 1.66, 3.16 ± 0.78 / 2.00, 3.33 ± 0.77 / 0.58 , and 5.09 ± 0.80 / 3.95 for erythromycin, clarithromycin, azithromycin and telithromycin, respectively. All log P, and log D data, calculated with the Advanced Chemistry Development (ACD) Software Solaris V4.67[©1994-2003 ACD], were obtained from Sci Finder Scholar 2004 [American Chemical Society, Washington, DC]).

The second sugar (cladinose) is critical to obtain clinically-useful activity, but its removal can be compensated (that means, activity can be brought back to clinically useful values), if an appropriate side chain is added to the molecule, as done in ketolides. Some derivatives like azithromycin and telithromycin are dicationic, due to the presence of a second protonable aminated function in the main ring on the side chain.

Macrolides are well known to accumulate in large amounts (5 to 100 times) in eukaryotic cells, and in macrophages in particular (Carlier *et al.*, 1987; Carlier *et al.*, 1994; Mor *et al.*, 1994; Pascual *et al.*, 2001). The mechanism proposed to explain this accumulation is that of diffusion-segregation of amphiphilic weak bases in acidic compartments (de Duve *et al.*, 1974).

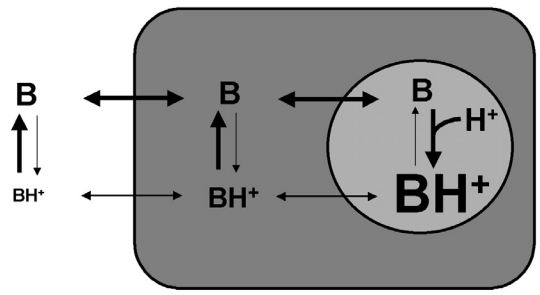


Figure 21: model describing the accumulation of weak bases in the acidic compartments of the cells.

Under its unprotonated form, the basis can freely diffuse through the membranes. The concentration of the basic forms therefore equilibrates in all compartments. However, in acidic compartments, the basis becomes protonated and therefore less diffusible, explaining why these drugs accumulate in the cells as a function of the pH gradient between compartments (extracellular pH [~ 7.4] < cytosolic pH [~ 7.0] >>>> lysosomal pH [~5.4].

Following this model (figure 21), the non-protonated form would easily diffuse through the membranes, gain access to the acidic compartments of the cells (lysosomes) where it would be trapped under its less diffusible protonated form. This mechanism would also explain why a dibasic molecule like azithromycin accumulates to a larger extent than monobasic derivatives.

The diffusion capacity of macrolides through membranes does not preclude recognition by active transporters. On the contrary, and as abundantly explained in the introduction, efflux pumps preferably transport diffusible molecules that have an easy access to the intracellular milieu. Little is known however about the role of efflux transporters in the handling of macrolides by macrophages, but erythromycin has been shown to be transported by P-glycoprotein in Caco-2 intestinal cells (Takano *et al.*, 1998; Saito *et al.*, 2000). In parallel, erythromycin and azithromycin are capable of inhibiting the transport of various substrates of the P-glycoprotein in epithelial cells *in vitro* as well as *in vivo* (Kim *et al.*, 1999; Siedlik *et al.*, 1999; Kiso *et al.*, 2000; Schwarz *et al.*, 2000; Wang *et al.*, 2000; Goldschmidt *et al.*, 2001). On these bases, we have undertaken to evaluate whether macrolides would also be recognized by efflux pumps in J774 macrophages.

III.2.b.1. Pharmacokinetic studies and phenotypic characterization of the macrolide transporter in J774 macrophages

We have thus examined the potential implication of P-glycoprotein and MRP in the accumulation and efflux of five macrolides of clinical interest (see Seral *et al.*, 2003b for experimental details). We used both broad spectrum, non-specific inhibitors of P-glycoprotein and MRP (verapamil and cyclosporin A on the one hand and

probenecid and gemfibrozil on the other hand) as well as the specific P-glycoprotein modulator GF120918 (Hyafil *et al.*, 1993; Krishna and Mayer, 2000).

a) Influence of P-glycoprotein inhibitors on the kinetics of macrolide accumulation and efflux.

In a first series of experiments, we studied whether verapamil was able to alter the kinetics of uptake and efflux of azithromycin. Figure 22 (left panel) shows that azithromycin (5 mg/L) is gradually accumulated by J774 macrophages, reaching a plateau after approximately 3 h (apparent cellular to extracellular concentration ratio of approx. 30 fold).

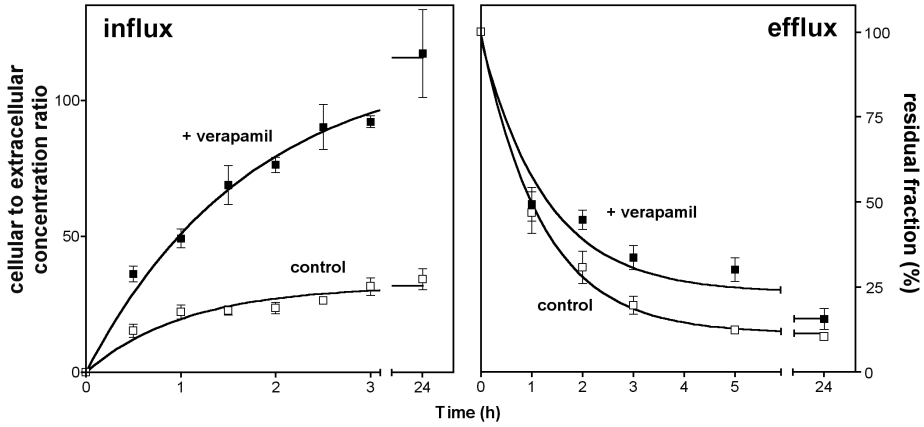


Figure 22: Influence of verapamil on the kinetics of accumulation and efflux of azithromycin in J774 macrophages. *Left panel*: Kinetics of uptake of azithromycin (extracellular concentration = 5 mg/L) in J774 macrophages incubated for up to 24 h in the absence or in the presence of 20 µM of verapamil. *Right panel*: Efflux of azithromycin from J774 macrophages incubated for 3 h with azithromycin in the presence or not of verapamil and then reincubated in an azithromycin-free medium (chase). Open squares, controls; closed squares, cells incubated with 20 µM verapamil during uptake and efflux. Results are expressed as the % of the drug remaining associated to the cells at the end of the loading period. All data are the mean ± SD of three experiments.

(Seral *et al.*, 2003b)

In the presence of verapamil (20 µM, added simultaneously with azithromycin), similar kinetics were observed with, however, systematically larger accumulation values (2-3.5 fold) at all time points (yielding an apparent cellular to extracellular concentration ratio at equilibrium of approx. 100 fold). Similar observations were made in the presence of cyclosporin A and GF120918. Subsequent experiments were therefore performed with cells incubated 3 h with the macrolides under study (5 mg/L) with or without the P-glycoprotein inhibitors. To examine azithromycin efflux, cells loaded with this antibiotic (extracellular concentration, 20 mg/L) in the presence of inhibitors for 3 h were reincubated in antibiotic-free medium in the continuing presence of the same inhibitors. Controls used the same protocol except for the absence of inhibitor.

Figure 22 (right panel) shows that efflux was only modestly impaired by verapamil with a maximal effect at 5 h (approximately 30 % retention *vs* 12 % in controls). Yet, no significant influence of verapamil could be demonstrated at short (1 h) or long (24 h) term periods. Similar results were obtained with the two other inhibitors (cyclosporin A and GF120918). All these experiments were then repeated with telithromycin, with similar results.

b) Dose-effects of P-glycoprotein and of MRP inhibitors on azithromycin accumulation, and comparison between macrolides.

To further characterize this effect, we then ran dose-effect experiments with verapamil, cyclosporin A, or GF120918, and measured their influence on azithromycin accumulation of at equilibrium (figure 23). All 3 inhibitors displayed a similar maximal effect (approximately 4-fold) but with marked differences in potencies. In parallel experiments, we also examined the influence of two known inhibitors of the MRP efflux pump, namely probenecid (2.5 mM) and gemfibrozil (0.25 mM), but did not observe any effect (data not shown).

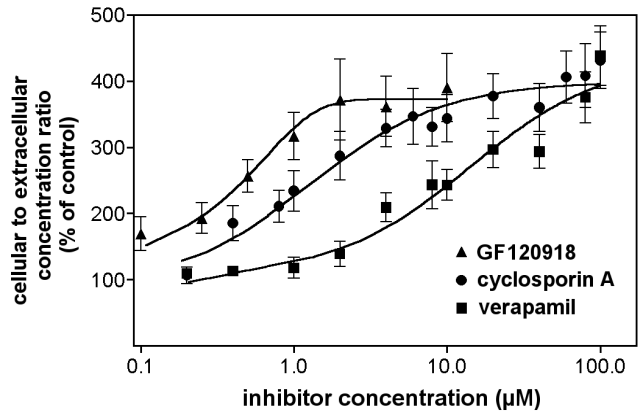


Figure 23: Influence of inhibitors of P-glycoprotein on the accumulation of azithromycin in J774 mouse macrophages.

Cells were incubated in the presence of an extracellular concentration = 5 mg/L for 3 h and with increasing concentrations of P-glycoprotein inhibitors. All data are the mean ± SD of three experiments.

(Seral *et al.*, 2003b)

In the next series of experiments, we compared the effect of the 3 same inhibitors used at a fixed concentration on the accumulation of different macrolides. All antibiotics were used at 5 mg/L, except erythromycin which had to be present at 50 mg/L for detection due to its lower accumulation (Carlier *et al.*, 1987). As observed in figure 24, erythromycin and telithromycin (with respect to cyclosporin A and GF120918), behaved essentially like azithromycin (the effect of verapamil on telithromycin was, however, lower than was seen with the other inhibitors). In sharp contrast, the accumulation of clarithromycin was essentially unaffected. Roxithromycin showed an intermediate behavior.

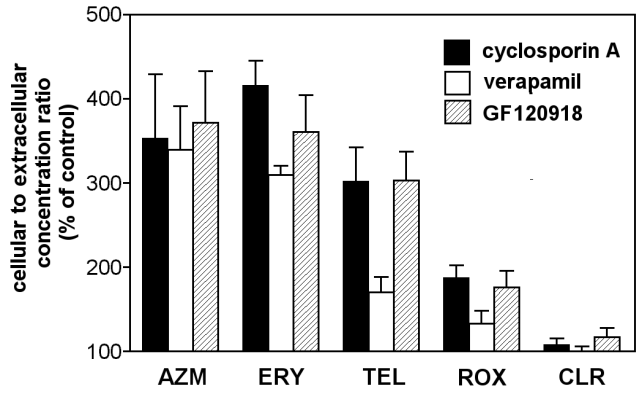


Figure 24: Influence of P-glycoprotein inhibitors on the cellular accumulation of macrolides in J774 macrophages after 3 h of incubation (closed bars, cyclosporin A [20 µM]; open bars, verapamil [20 µM]; hatched bars, GF120918 [2 µM]). The extracellular concentration was 5 mg/L for all macrolides except for erythromycin (50 mg/L). Results are expressed as % of the accumulation observed in the absence of inhibitor. All data are the mean ± SD of three experiments.

(Seral *et al.*, 2003b)

Cellular to extracellular concentration ratios in controls: azithromycin, 20.7 ± 3.7 ; erythromycin, 2.7 ± 0.1, telithromycin, 16.3 ± 1.4; roxithromycin, 19.3 ± 1.7, clarithromycin, 46.6 ± 2.1; values for azithromycin, erythromycin and roxithromycin are consistent with those observed previously with the same cells but using radiolabeled drug (Carlier *et al.*, 1987; Carlier *et al.*, 1994). The effect of all inhibitors on antibiotic accumulation was significantly different for clarithromycin as compared to azithromycin, erythromycin or telithromycin and on roxithromycin as compared to azithromycin or erythromycin ($p < 0.05$). The effect of verapamil on antibiotic accumulation was significantly different from that of the other inhibitors for azithromycin, erythromycin and telithromycin ($p < 0.05$).

c) Influence of ATP depletion and of cell exposure to monensin on azithromycin accumulation

P-glycoprotein activity being ATP-dependent (Rosenberg *et al.*, 1997; Saier, Jr., 2000), we examined the influence of ATP-depletion on azithromycin accumulation in cells (obtained by a preincubation of cells with 2-deoxyglucose and NaN_3).

Table 8: Influence of an ATP-depleting treatment (90 min incubation) and of monensin (20 µM; 3 h incubation) on the cellular accumulation of azithromycin (5 mg/L) in control J774 macrophages and in cells exposed to verapamil (20 µM) [a].

(Seral *et al.*, 2003b)

condition	control cells		cells exposed to verapamil	
control	100.0 ± 7.8	(A)	287.6 ± 20.4	(B)
ATP depletion[b]	254.4 ± 30.7	(C)	310.7 ± 33.9	(D)
monensin	5.9 ±1.0	(E)	3.4 ± 0.6	(F)

[a] Results are expressed as the percentage of the cellular accumulation observed in cells without any treatment (azithromycin alone). Data are the means ± SD of three experiments.

[b] ATP depletion was obtained by 1 h preincubation in the presence of 60 mM deoxyglucose and 5 mM NaN_3, these conditions were maintained during the incubation with azithromycin.

Statistical analysis (one way ANOVA): A vs B, A vs C, A vs E : $p < 0.001$; B vs D, C vs D, E vs F : non-significant

As shown in table 8, ATP depletion caused a marked increase in azithromycin accumulation, which was as important as that seen with 20 µM verapamil. In parallel, cells were exposed to monensin (a carboxylic ionophore known to dissipate transmembrane H^+ gradients in eukaryotic cells [Tartakoff, 1983]). Monensin also almost completely suppressed the accumulation of azithromycin in control cells and in cells incubated with verapamil.

d) Influence of verapamil on the lysosomal and cytoplasmic pHs

As macrolide accumulation in cells is dependent upon transmembrane pH gradients causing them to be preferentially sequestered in lysosomes and related acidic vacuoles, a first hypothesis could therefore be that P-glycoprotein inhibitors increase the pH gradient between lysosomes and the extracellular milieu and/or the cytosol, either directly or by modulating the P-glycoprotein basal activity (pH gradients are indeed reduced between cell compartments upon overexpression of efflux transporters [Larsen *et al.*, 2000]). We therefore examined whether verapamil could increase macrolide accumulation by perturbing the pH gradient between the extracellular milieu and lysosomes. This was investigated using specific fluorescent tracers, the excitation spectra of which varies upon pH changes (namely, the lysosensor yellow / blue [2-(4-pyridyl)-5-((4-(2-dimethylaminoethylamino-carbamoyl) methoxy)phenyl)oxazole]-labeled dextran for lysosomes [Diwu *et al.*, 1999] and BCECF [2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein] for cytosol [Rink *et al.*, 1982]). No significant change from the control values was observed (pH = 7.06 ± 0.93 [n=17] and 4.95 ± 0.13 [n=35] for cytosol and lysosomes in controls, respectively). We can therefore disclose any effect due to pH gradients modification to explain the influence of verapamil on azithromycin accumulation. Moreover, pH variations induced by inhibitors should have affected the accumulation of all macrolides if they were the reason for the modifications observed.

e) Conclusion: role of P-glycoprotein in macrolide cellular pharmacokinetics and possible mechanisms involved

Our data discloses that verapamil, cyclosporin A and GF120918 markedly increase the cellular accumulation of the macrolide antibiotics azithromycin, erythromycin and telithromycin in a model of J774 macrophages, whereas roxithromycin and clarithromycin are respectively less and not affected. While verapamil and cyclosporin A are known not only to inhibit the mammalian P-glycoprotein but also to affect several other cell functions (Krishna and Mayer, 2000), GF120918 is a much more specific inhibitor of P-glycoprotein and is not known, so far, to have other significant pharmacological properties (see Hyafil *et al.*, 1993 and Krishna and Mayer, 2000 for review). Together with the fact that J774 macrophages do express P-glycoprotein, these data strongly suggest that this efflux pump is responsible for the effects observed on the accumulation of azithromycin, erythromycin, and telithromycin.

Since the effects of P-glycoprotein inhibitors could not be attributed to a perturbation of macrolide accumulation by a simple disturbance of pH gradients between subcellular compartments, the more likely hypothesis is therefore that P-glycoprotein defeats the cellular accumulation of certain macrolides thanks to its drug efflux capabilities. This hypothesis is consistent with our observation that ATP depletion is as effective as the addition of a P-glycoprotein inhibitor. It also explains why macrolides with only minor chemical differences (viz. clarithromycin *vs* erythromycin) may markedly differ in their sensitivity to inhibitors. Substrate recognition by P-glycoprotein is indeed known to be highly variable even between chemically very-closely related drugs and defined pharmacophores are still far from being clearly recognized, apart from general properties related lipophilicity and size (Stouch and Gudmundsson, 2002). It is, however, intriguing that P-glycoprotein inhibitors do not markedly influence macrolide efflux. Biophysical studies suggest, actually, that P-glycoprotein binds its substrates from within the membrane, and not from the cytosol, acting as a flippase (Higgins and Gottesman, 1992) or as a 'vacuum cleaner' (Rosenberg *et al.*, 1997). In this type of model, a lower cell accumulation results more from a decreased influx than from an increased net efflux (Eytan *et al.*, 1996; Gaj *et al.*, 1998) as substrates do not need to have access to the cytosol to be extruded (Marbeuf-Gueye *et al.*, 1999). Experimental testing of this model for macrolides could be made using sublines of the J774 macrophages overexpressing the P-glycoprotein, or appropriate reconstituted membrane models.

We may therefore conclude that, even though the accumulation of macrolides (and of azithromycin in particular) in macrophages is high, it is still suboptimal, because of the presence of active transporters able to expel them out of the cells.

III.2.b.2. Pharmacodynamic studies: influence of efflux pumps on the activity of azithromycin against intracellular infections

The most immediate consequence from the previous observation concerns the potential interest of macrolides for the treatment of intracellular infections. It has for example been demonstrated that human cancer cells overexpressing P-glycoprotein show a decreased macrolide accumulation and a correspondingly reduced antimicrobial activity against intracellular forms of *Listeria monocytogenes* (Nichterlein *et al.*, 1998). We have therefore undertaken to examine the impact of P-glycoprotein on azithromycin activity against intracellular infections caused by microorganisms localizing predominantly in the cytosol (*L. monocytogenes* [Ouadrhiri *et al.*, 1999]) or in the phagolysosomes (*Staphylococcus aureus* [Seral *et al.*, 2003c]; figure 21 in chapter II), the latter compartment being the major site of accumulation for azithromycin.

For this purpose, we have examined the influence of verapamil on azithromycin activity, and have correlated these data with the changes in antibiotic accumulation in the different cell compartments (Seral *et al.*, 2003a for experimental procedures).

a) Influence of verapamil on the intracellular activity of azithromycin

We thus first examined the dose-effect relationship of the intracellular activity of azithromycin in the absence and in the presence of verapamil, based on our observations that verapamil was able to increase the drug cellular concentration (figure 25).

Considering the *L. monocytogenes* model first, azithromycin was poorly active against the intracellular form of this bacterium, becoming bacteriostatic at an extracellular concentration corresponding to 10 X its MIC only (MIC = 0.5 mg/L). However, addition of verapamil allowed for the same level of activity to be already obtained at extracellular concentrations of about 1-2 X the MIC. No increase in activity, however, was noted at larger extracellular concentrations, demonstrating that azithromycin was only bacteriostatic in this model.

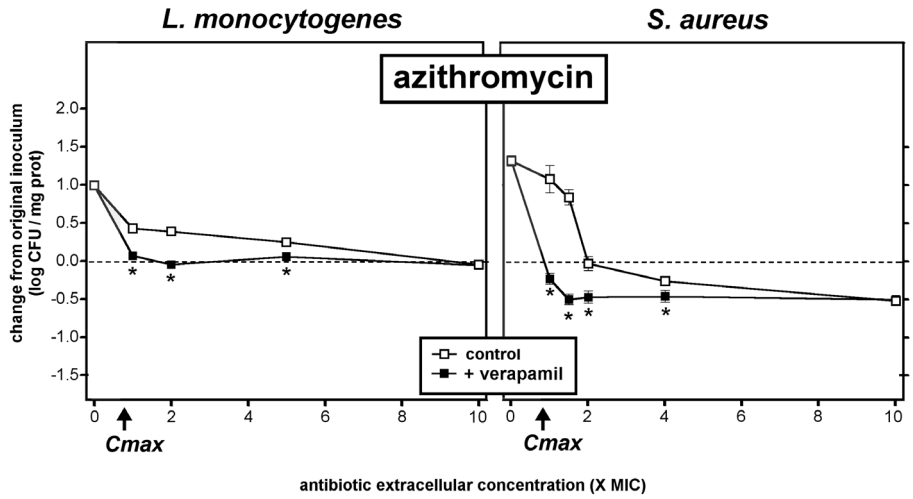


Figure 25: Influence of verapamil 20 µM on the activity of azithromycin on intracellular *L. monocytogenes* or *S. aureus*.

The ordinate shows the changes in cfu (as log cfu/mg cell protein) from the initial inoculum at the end of the incubation with the antibiotics (5 h for *L. monocytogenes*; 24 h for *S. aureus*). The dotted line corresponds to a bacteriostatic effect. The abscissa shows the extracellular concentration of antibiotic used (as multiple of the corresponding MIC [0.5 mg/L for both bacteria]). The corresponding maximal serum peak level that can be reached in the serum of patients undergoing a conventional treatment is shown by an arrow marked Cmax. Each data point is the mean ± SD of three independent determinations. The asterisks show the statistically significant differences between inhibitor-treated and control cells (paired points; $P < 0.01$).

(Seral *et al.*, 2003a)

In the *S. aureus* model also, azithromycin was, globally speaking, poorly bactericidal. Thus, it was ineffective up to an extracellular concentration of about 1 X its MIC (MIC = 0.5 mg/L), and became abruptly bacteriostatic when its extracellular concentration was brought to about twice this value. Yet, further increase of activity was very minimal and only a slight reduction in the inoculum over the post-phagocytosis value was seen at 10 X the MIC (0.5 log). Addition of verapamil shifted the effect-concentration curve of azithromycin to the left, causing the drug to achieve a similar antibacterial effect at an extracellular concentration corresponding to about 1.5 X its MIC, but with no further gain at higher extracellular concentrations.

To give a clinical connotation to these studies, we also show in the figure the values of extracellular concentrations corresponding to the C_{max} of the antibiotic (i.e. the maximal serum level observed in patients undergoing a conventional treatment with azithromycin; ~ 0.4 mg/L). Interestingly, a bacteriostatic effect of azithromycin could only be observed at this value for cells incubated with verapamil in both models.

b) Influence of verapamil on the cellular concentration and subcellular distribution of azithromycin

To correlate changes in activity with increase in drug cell content, we then determined the concentration of azithromycin in the different fractions of a cell homogenate in control conditions or in cells exposed to verapamil. Figure 26 shows the influence of the efflux pump inhibitors on the cellular accumulation and subcellular distribution of azithromycin in uninfected cells after 24 h of incubation with the antibiotic (these experiments needed the use of large extracellular concentrations of azithromycin [50 mg/L] for allowing its detection in the subcellular fractions). We checked in independent experiments that infection did not affect the antibiotic accumulation, whether in control conditions [no inhibitor added] or in cells exposed to verapamil.

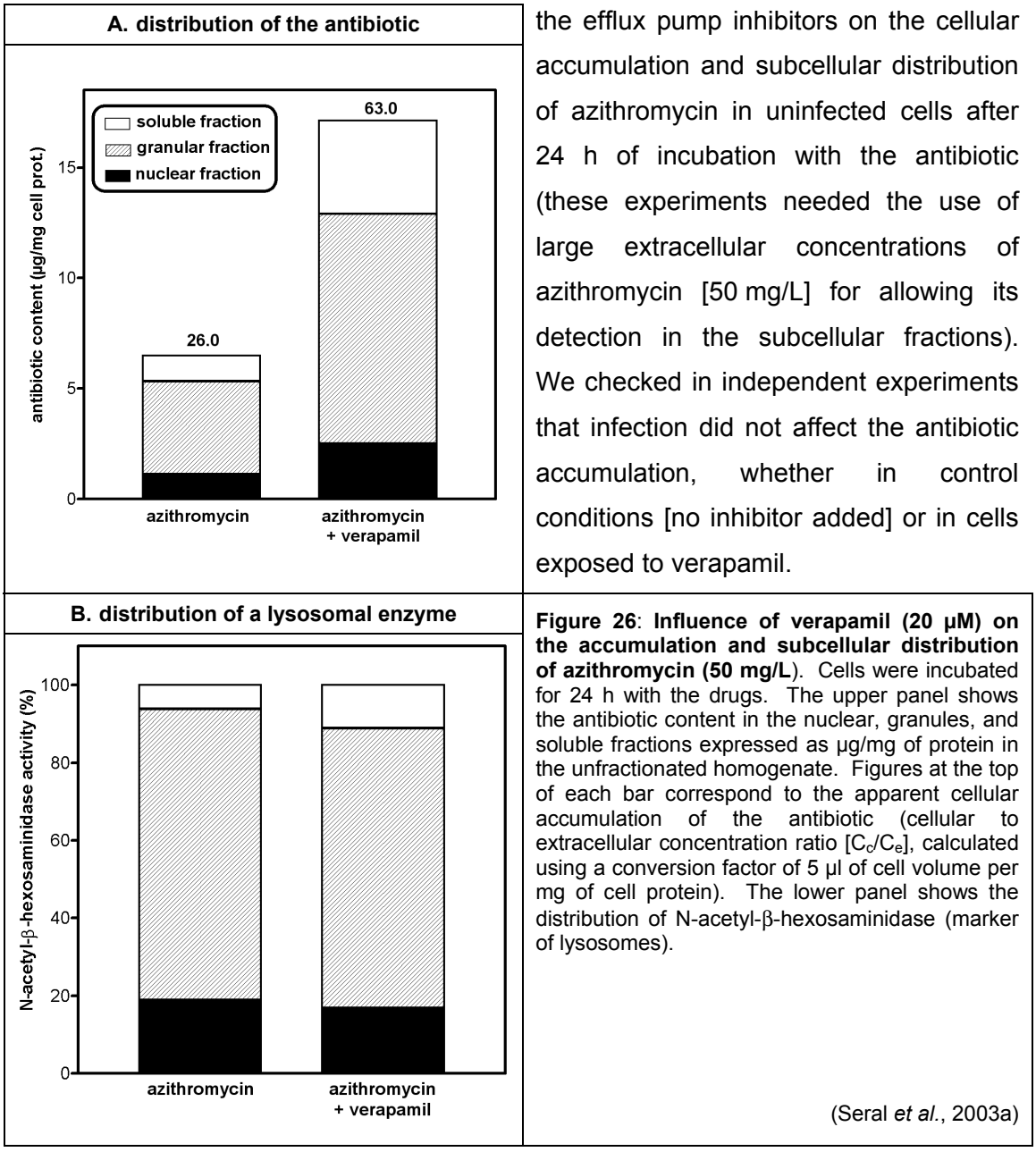


Figure 26: **Influence of verapamil (20 µM) on the accumulation and subcellular distribution of azithromycin (50 mg/L**). Cells were incubated for 24 h with the drugs. The upper panel shows the antibiotic content in the nuclear, granules, and soluble fractions expressed as µg/mg of protein in the unfractionated homogenate. Figures at the top of each bar correspond to the apparent cellular accumulation of the antibiotic (cellular to extracellular concentration ratio [C_c/C_e], calculated using a conversion factor of 5 µl of cell volume per mg of cell protein). The lower panel shows the distribution of N-acetyl-β-hexosaminidase (marker of lysosomes).

(Seral *et al.*, 2003a)

The figure also shows the distribution of N-acetyl-β-hexosaminidase (used as a marker of lysosomes). No change in the distribution of this marker was seen, demonstrating that verapamil did not grossly affect the biophysical properties and integrity of lysosomes or the distribution of soluble proteins (the distribution of lactate dehydrogenase as a marker of soluble proteins was also assessed and was neither

modified). As anticipated from the results of previous studies (Carlier *et al.*, 1994), azithromycin was accumulated to a large extent by cells, and predominantly localized in the "granules fraction". Verapamil increased the cellular accumulation of azithromycin by about 2.4-fold (this effect was similar to that seen with cells exposed to lower azithromycin concentrations [5 mg/L], see above). Interestingly, this increased in accumulation occurred without marked change in azithromycin subcellular distribution.

c) Discussion of the results and potential impact for the treatment of intracellular infections

The data presented here first of all contribute to the knowledge of the parameters governing the intracellular activity of macrolides. Intracellular infection represents indeed a complex situation in which compartmentalization (of drugs and bacteria), and local physico-chemical conditions could profoundly modulate the intrinsic pharmacodynamic properties and overall chemotherapeutic efficacy of these drugs. Actually, we show here that the intrinsic pharmacodynamic properties of azithromycin observed towards extracellular bacteria (i.e. a concentration-dependent activity within a narrow range of concentrations only, and no or little bactericidal activity) seem to also prevail against intracellular *L. monocytogenes* and *S. aureus* (Carryn *et al.*, 2002; Seral *et al.*, 2003c). Yet, this activity appears to be considerably defeated, considering the exceptionally large accumulation of azithromycin. However, we observe here that verapamil makes azithromycin active at lower extracellular concentrations against both cytosolic (*L. monocytogenes*) and phagosomal (*S. aureus*) bacteria. Although the effect is of limited amplitude, because of the intrinsically bacteriostatic activity of azithromycin (Carbon, 1998), it develops in parallel with the increase in drug content observed both in the cytosol and in the cell granules. This effect can be explained by the mechanism of accumulation of azithromycin, which, as discussed previously, freely diffuses through membranes, but is trapped under its protonated form in the acidic compartments (i.e. mainly the lysosomes and related vacuoles of the endocytic system). The data presented here confirm this behavior, as azithromycin was mainly found in the "granules fraction" which contains the bulk of those organelles, as demonstrated by the distribution of the N-acetyl-β-hexosaminidase, a typical lysosomal enzyme. Blocking P-glycoprotein at the cell surface should therefore increase azithromycin content in the cytosol. Yet, we may anticipate that the drug will then quickly re-equilibrate between cytosol and lysosomes, hence the effect seen towards intracellular *S. aureus*.

Beyond their direct pharmacological interest, the conclusions of this study may also lead to potentially useful therapeutic applications. The extracellular concentrations at which we observe an effect of efflux inhibitors on azithromycin are indeed in the range of those reached in the serum of patients treated with conventional doses of this antibiotic. We may therefore suggest that the activity of azithromycin against intracellular bacteria is actually suboptimal due to basal efflux mechanisms operating in the macrophages. Even though verapamil is clearly unusable in clinics because of its own pharmacological activity, the data suggest that more specific inhibitors (like GF120918) could prove useful to optimize antimicrobial intracellular therapy. This widely opens the door to research aimed at designing and evaluating such inhibitors. The problem is however not easy to address since we may fair the toxicological consequences of blocking physiological transporters.

III.2.c. Active efflux of quinolones by a MRP-like transporter

Introduced into our clinical armamentarium since the mid 80's, fluoroquinolones remain of very large clinical interest because of their wide spectrum, intense bactericidal activity and excellent bioavailability (Hooper, 2000; Van Bambeke *et al.*, 2005). A key feature of fluoroquinolones is also their ability to accumulate in cells (Tulkens, 1991; Michelet *et al.*, 1994), and most notably in polymorphonuclear leucocytes and macrophages where they show useful activity against several types of intracellular bacteria (Easmon and Crane, 1985; Easmon *et al.*, 1986; Vilde *et al.*, 1986; Carlier *et al.*, 1990; Michelet *et al.*, 1994; Michelet *et al.*, 1997; Carryn *et al.*, 2002). Quite significant differences in the level of accumulation reached have been observed among closely-related derivatives (Carlier *et al.*, 1990; Rispal *et al.*, 1996; Hara *et al.*, 2000; Dorian *et al.*, 2001), but have not received satisfactory explanations so far. One factor that can modulate antibiotic accumulation in eukaryotic cells is their differential recognition by active efflux transporters (Van Bambeke *et al.*, 2003b).

Early work indeed showed that probenecid increases the accumulation of norfloxacin in J774 macrophages (Cao *et al.*, 1992). Subsequent studies established that both probenecid and gemfibrozil enhance the activity of ciprofloxacin against intracellular *Listeria monocytogenes* (Rudin *et al.*, 1992). These effects have been interpreted as demonstrating the existence of an efflux mechanism for fluoroquinolones similar to that responsible for the extrusion of organic anions described in J774 macrophages (Steinberg *et al.*, 1987). However, no further characterization of this efflux and of the transporters involved has been reported up to now.

In the present study, we have therefore examined in details the influx and efflux processes of ciprofloxacin in J774 macrophages and its consequences for the intracellular activity of the drug. We used a series of conditions to demonstrate the role of specific transporters already described in eukaryotic cells (Van Bambeke *et al.*, 2000; Van Bambeke *et al.*, 2003b), namely depletion in ATP and glutathione, exposure to acid and basic pH and co-incubation with a proton ionophore, as well as co-incubation with various inhibitors. We have then extended these studies to other quinolones to gain a first insight on the structure-activity relationship of this efflux. For this purpose, we selected levofloxacin, and moxifloxacin, 2 derivatives of major clinical interest in the treatment of infections caused by Gram-positive organisms, and garenoxacin, an investigational compound of the new class of desfluoroquinolones (Takahata *et al.*, 1999; Frechette, 2001; figure 27).

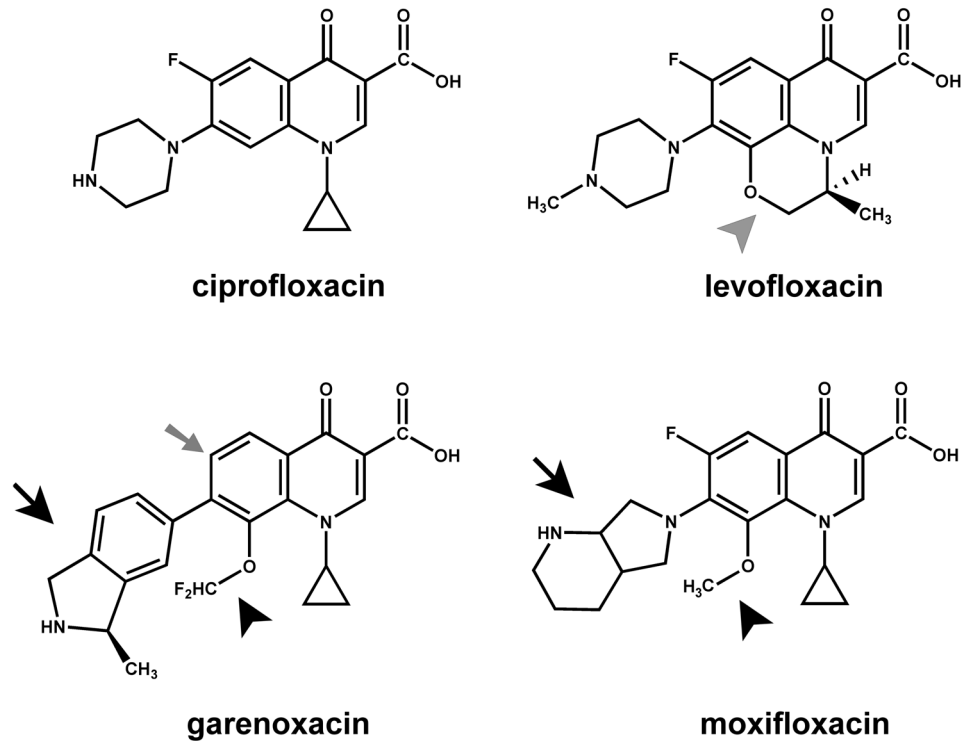


Figure 27

Structural formula of the quinolones used in this study. The figure emphasizes that (i) both garenoxacin and moxifloxacin possess bulky substituents in position 7 (denoted by the large solid arrows; garenoxacin, 2,3-dihydro-1-methyl-1H-isoindole; moxifloxacin, 4a,7a-octahydro-6H-pyrrolo[3,4-b]pyridine) that confer a more hydrophobic character to these molecules as compared to ciprofloxacin or levofloxacin (for which the corresponding substituents are a piperazine or a 4-methyl-piperazine, respectively); (ii) both garenoxacin and moxifloxacin possess free rotating substituents in position 8 (denoted by the solid arrowheads; garenoxacin, difluoromethoxy; moxifloxacin, methoxy) which contribute to an increase in lipophilicity. In levofloxacin, the substituent in position 8 is part of a more rigid structure (morpholino) which bears some degree of similarity with a methoxy; (iii) garenoxacin is a desfluoroquinolone (no F substituent in position 6 [grey arrow]). The reported log P / log $D_{pH=7}$ values (octanol/water partition coefficient) / (octanol/water partition coefficient at pH 7; log D = log P - log[1+$10^{(-charge * (pH-pKa))}$]), two accepted measures of the hydrophobic character of organic molecules) are 1.31 ± 0.81 / -1.20, 1.49 ± 0.79 / -1.35, 1.62 ± 0.93 / -0.90 , and 1.98 ± 0.82 / -0.53 for ciprofloxacin, levofloxacin, garenoxacin and moxifloxacin, respectively. The pKa of the protonable function of the C7 substituent of the 4 quinolones are 8.76 ± 0.25 (ciprofloxacin), 6.8 ± 0.3 (levofloxacin), 8.4 ± 0.4 (garenoxacin), and 10.8 ± 0.4 (moxifloxacin). This will result in larger proportion of moxifloxacin (and a lower proportion of levofloxacin) to be under a zwitterionic form at neutral pH. All log P, log D, and pKa data, calculated with the Advanced Chemistry Development (ACD) Software Solaris V4.67[©1994-2003 ACD], were obtained from Sci Finder Scholar 2004 [American Chemical Society, Washington, DC]).

Finally, we developed new tools for the study of active efflux, namely two cell lines made resistant to pump inhibitors (probenecid) or substrates (ciprofloxacin), with the aim to better characterize and possibly identify the transporter(s) involved in quinolone efflux.

III.2.c.1. Pharmacokinetic studies and phenotypic characterization of the quinolone transporter in J774 macrophages

These first studies (see Michot *et al.*, 2004 for detailed experimental procedures) were thus focused on ciprofloxacin, for which active efflux was already demonstrated to occur in J774 macrophages (Rudin *et al.*, 1992).

a) Influence of the extracellular concentration of ciprofloxacin on its cellular accumulation.

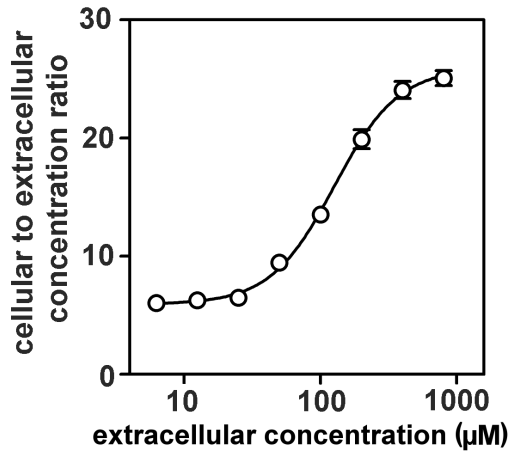


Figure 28: Influence of the extracellular concentration of ciprofloxacin on its accumulation at equilibrium (2 h incubation) in J774 macrophages. Regression parameters (sigmoid dose-response with variable slope): $R^2 = 0.999$, Hill's slope = 1.845, $EC_{50} = 126.4 \pm 1.0$ µM. All data is the mean of at least 3 independent measurements ± SD (when not visible, the SD bar is smaller than the symbol).

(Michot *et al.*, 2004)

In a first series of exploratory experiments, we noticed that the ability of cells to concentrate ciprofloxacin, rather than being impaired (as would be observed for a saturable mechanism of uptake), was actually increased upon raising the extracellular concentration. Examining this apparently paradoxical property in detail with cells incubated for 2 h with the drug (figure 28), we observed indeed that the apparent cellular to extracellular concentration ratio of ciprofloxacin rose from approx. 6 to approx. 25 in a clearly sigmoid fashion when the extracellular concentration was increased from about 8 to 250 mg/L (approx. 25 µM to approx. 800 µM). Half of this effect was obtained for an extracellular concentration of approx. 40 mg/L (approx. 125 µM).

Based on these findings, we explored the kinetics of accumulation and efflux of ciprofloxacin using both a low and a high extracellular concentration (50 µM [17 mg/L] and 200 µM [68 mg/L], respectively). Figure 29 (left panel) shows that accumulation was in both cases rapid, reaching equilibrium within 30 to 60 min. Cells incubated with the highest concentrations of ciprofloxacin showed a higher cellular to extracellular concentration ratio at all times points. In contrast, cells that had accumulated a large amount of ciprofloxacin showed a slower efflux. Figure 29 (right panel) shows indeed that the half-life of ciprofloxacin was about 4 min for cells loaded

with a high extracellular concentration of ciprofloxacin (68 mg/L; 200 µM) but only 1.7 min for cells loaded with a low concentration (17 mg/L; 50 µM) (see inset).

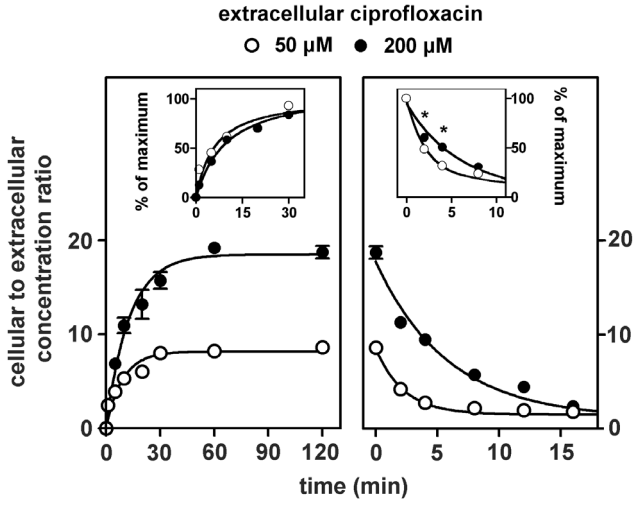


Figure 29: Kinetics of accumulation (left) and efflux (right) of ciprofloxacin in J774 macrophages at two different extracellular drug concentrations (17 mg/L [50 µM] and 68 mg/L [200 µM]). All data is the mean of at least 3 independent measurements ± SD (when not visible, the SD bar is smaller than the symbol).
Insets: data presented in relative values (% of maximum; *, significant difference between cells incubated with 17 mg/L [50 µM] and 68 mg/L [200 µM] by paired t test analysis with $p < 0.05$).

(Michot *et al.*, 2004)

This difference vanished at 8 min, after which efflux proceeded at the same fractional rate in both conditions. In view of the data presented in figure 28 and 29, all subsequent experiments were performed at a fixed ciprofloxacin extracellular concentration of 17 mg/L (50 µM) to keep below the level at which concentration-dependent effects became clearly detectable while, at the same time, allowing to assay cell-associated ciprofloxacin with sufficient accuracy.

These data therefore highly suggest that ciprofloxacin is subject to an active efflux in J774 macrophages, which can be saturated by high concentrations in substrate. In a next series of experiments, we try to further characterize this transport using a series of conditions well known to interfere with the functioning of efflux pumps. Analysis of current literature teaches us that fluoroquinolones are recognized by several eukaryotic multidrug transporters, and most notably by two main members of the ABC (ATP-Binding Cassette) superfamily of transporters, namely the MRP (Multidrug Resistance-related Proteins) and the P-glycoprotein (Gollapudi *et al.*, 1995; Ito *et al.*, 1997; Cormet-Boyaka *et al.*, 1998; Tamai *et al.*, 2000; Naruhashi *et al.*, 2001; Naruhashi *et al.*, 2002; Rodriguez-Ibanez *et al.*, 2003). We therefore used by priority conditions susceptible to impair the activity of these types of transporters.

b) Influence of modulators of the activity of efflux pumps

b.1) ATP-depletion.

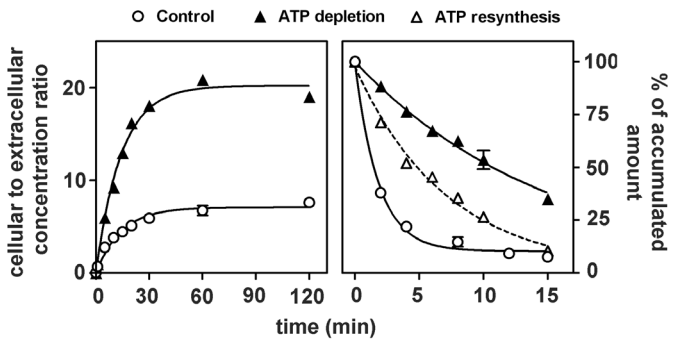
To determine whether the ciprofloxacin transporter was energized by an ATP-dependent process, we measured the accumulation of ciprofloxacin (50 µM; 2 h) in ATP-depleted cells (obtained by preincubation for 20 min with 5 mM NaN_3 and 60 mM 2-D-deoxyglucose; these conditions were also maintained during the incubation period). Figure 30A shows that ATP-depletion caused a marked increase in ciprofloxacin accumulation, which, qualitatively as well as quantitatively, was similar to that observed when comparing accumulation at low (17 mg/L; 50 µM) and high (68 mg/L; 200 µM) extracellular concentrations. Ciprofloxacin efflux was then studied with cells depleted in ATP, loaded with ciprofloxacin in the presence of NaN_3 and 2-D-deoxyglucose, and transferred to a drug-free medium in the continuing presence of these agents (so as to maintain their state of ATP-depletion). Figure 30A (right panel) shows that the rate of efflux of ciprofloxacin was very markedly decreased under these conditions compared to what was observed for control cells (no ATP-depletion treatment). In parallel, we measured the efflux of ciprofloxacin from cells loaded with the drug while in a state of ATP depletion, but which were then transferred to a drug-free and a NaN_3 and 2-D-deoxyglucose-free medium (this allowed ATP levels to be restored to 25.5 ± 6.2% of control values within 15 min). Efflux proceeded at an intermediate rate between control cells and cells maintained in a state of ATP depletion.

These experiments therefore highly suggest that the ciprofloxacin transporter indeed belongs to the ATP-binding Cassette superfamily.

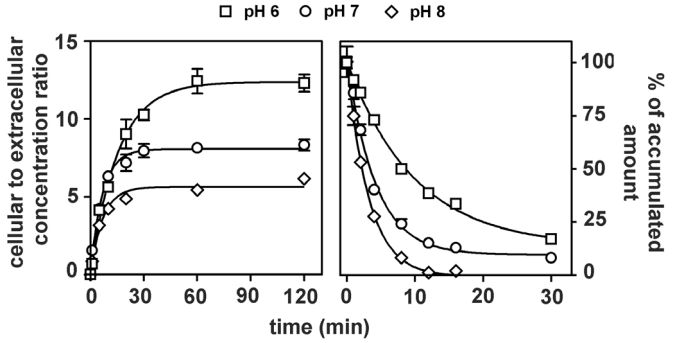
b.2) Glutathione depletion.

As stated in the introduction, glutathione is necessary for the activity of MRP transporters but not for that of P-glycoprotein. We therefore depleted the cells in glutathione (obtained by 24 h preexposure to 80 µM buthionine sulfoximine and maintain of this agent during the incubation period [residual content of sulfhydryl-containing constituents: ~ 3.6 %]). Cells depleted in glutathione showed a 47.58 ± 3.22 % increase (n =3) in ciprofloxacin cell content after 2 h incubation with 50 µM antibiotic, suggesting therefore the involvement of an MRP-like transporter in ciprofloxacin efflux.

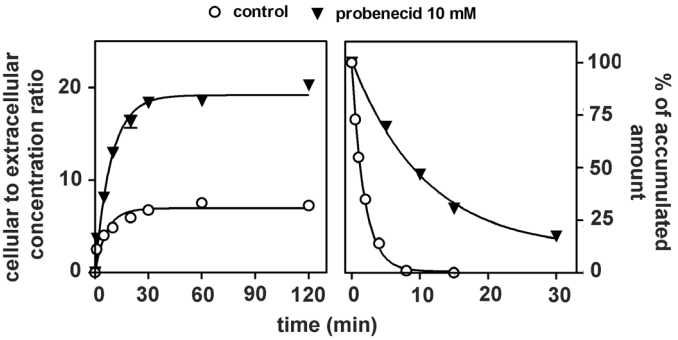
Figure 30: Kinetics of accumulation (left) and efflux (right) of ciprofloxacin (extracellular concentration, 17 mg/L [50 µM]) in J774 macrophages :



A. maintained under normal conditions (control) or in cells depleted in ATP (20 min preincubation with 2-D-deoxyglucose and NaN_3 [ATP levels were reduced to less than 3 % of the original content; influx was followed in the continuing presence of the inhibitors; for efflux, cells were either transferred to a drug-free medium in the presence of the inhibitors, or transferred to a drug-free medium without inhibitors [ATP resynthesis]).



B. maintained at three different pH using bicarbonate-free media, buffered with 5 mM phosphate (the value of the pH was checked before and after the experiment).



C. incubated in the presence or in absence of 10 mM probenecid (both drugs were added simultaneously for the accumulation experiment).

(Michot *et al.*, 2004)

b.3) Modification of extracellular pH and collapsing of pH gradients.

As explained in the introduction, proton gradients serve as energy source for secondary transporters. Independently of this role, pH and pH gradients play many other critical roles in drug uptake and efflux, not only by controlling the degree of ionization of drugs, but also by modulating cell physiology (endocytosis, proteins functioning, ..). In the next series of experiments, we have therefore studied the accumulation and efflux of ciprofloxacin (17 mg/L; 50 µM) in cells incubated with buffered media adjusted to specific pH values ranging from 6 to 8. Figure 30B (left panel) shows that an acid medium (pH 6) caused an increase in the absolute amount of drug accumulated in comparison with cells incubated at neutral pH (pH 7).

Conversely, incubation in an alkaline medium (pH 8) caused a lower accumulation of ciprofloxacin. Figure 30B (right panel) shows that the rate of ciprofloxacin efflux was inversely proportional to the acidity of the medium (but the decay remained of first order [see caption for kinetic data]). Interestingly enough, cells transferred from an acid to a neutral medium during efflux (for instance at 8 min) reacted almost immediately to this change of condition. They indeed showed a rapid loss of ciprofloxacin, after which efflux proceeded at rate similar to what was observed in cells incubated in a neutral medium throughout (data not shown). Monensin, a natural carboxylic ionophore with antifungal properties, known to dissipate transmembrane gradients in higher eukaryotes (Tartakoff, 1983), was then used here to further assess the importance of pH transmembrane gradients and intracellular pH on ciprofloxacin accumulation. Monensin did not affect ciprofloxacin accumulation (tested at pH 6, 7 and 8). As a control of an effective collapse of the transmembrane pH gradients with monensin, we checked that it entirely suppressed the accumulation of azithromycin which accumulates in J774 macrophages by proton-trapping as previously reported (Tyteca *et al.*, 2002).

b.4) Probenecid and other efflux pumps inhibitors.

Considering finally the effect of inhibitors of efflux pumps, figure 30C (left panel) shows that probenecid (10 mM) added to cells simultaneously with ciprofloxacin (17 mg/L; 50 µM) caused immediately a marked increase in the absolute amount of drug accumulated by cells in comparison to controls. Again, this effect was both qualitatively and quantitatively similar to what was observed with cells exposed to a large concentration of drug or with ATP-depleted cells. Probenecid-treated cells loaded with ciprofloxacin were then examined for ciprofloxacin efflux in the continuing presence of probenecid (10 mM), in comparison with cells in which ciprofloxacin accumulation and efflux was taking place in the absence of probenecid (controls). As shown in figure 30C (right panel), probenecid also markedly reduced the rate of efflux of ciprofloxacin from probenecid-treated cells compared to controls. In parallel, we examined the efflux of ciprofloxacin from cells incubated with ciprofloxacin (17 mg/L; 50 µM) in the absence of probenecid but which were then transferred to a ciprofloxacin-free medium containing 10 mM probenecid. Efflux was delayed almost exactly as in cells incubated in the presence of probenecid during both ciprofloxacin uptake and efflux (data not shown). This indicates that the influence of probenecid was immediate, directed towards efflux, and unrelated to the amount of ciprofloxacin accumulated by cells.

In the next series of experiments, we examined the influence exerted by gemfibrozil, another non-specific inhibitor of anionic transporters, and by MK571 which is a preferential MRP inhibitor. This was studied in comparison with two known P-glycoprotein modulators, namely verapamil and cyclosporin A (both of which, however, are not specific of the P-glycoprotein) and with GF 120918 (a preferential inhibitor of P-glycoprotein). Table 9 shows the results obtained with a fixed concentration of these inhibitors. Azithromycin (substrate of P-glycoprotein in J774 mouse macrophages; see III.2.a.1), was used as comparator.

Table 9: Influence of efflux pump inhibitors on the accumulation of ciprofloxacin [a] and azithromycin [b] by J774 macrophages

(Michot *et al.*, 2004)

modulator	concentration	increase in accumulation (% of controls)	
		ciprofloxacin	azithromycin
probenecid	2.5 mM	288 ± 4	101 ± 12
gemfibrozil	200 µM	308 ± 3	101 ± 12
MK571	200 µM	392 ± 5	n.d.
verapamil	100 µM	136 ± 4	438 ± 45
cyclosporin A	50 µM	236 ± 5	353 ± 77 *
GF 120918	1 µM	116 ± 2	372 ± 61

Statistical analysis: ciprofloxacin, all differences significant; azithromycin, differences significant for verapamil, cyclosporin A, and GF 120918 (paired *t* test compared to controls).
n.d.: not determined
[a] 17 mg/L (50 µM), 2 h incubation [time to equilibrium]
[b] 5 mg/L (6.8 µM). 3 h incubation [time to equilibrium].
* 20 µM cyclosporin A

It clearly appears that probenecid, gemfibrozil, and MK571 enhanced ciprofloxacin accumulation to essentially a similar extent (approx. 2.5 to 4 fold) whereas verapamil and GF 120918 had only minimal effects.

Cyclosporin A showed an intermediate behavior. In contrast, azithromycin accumulation was not affected by probenecid or gemfibrozil, but increased by verapamil, cyclosporin A, and GF 120918.

c) Short term kinetic studies.

To refine the analysis of the influence of ATP depletion and of the inhibitors on ciprofloxacin influx, we repeated the accumulation experiments focusing on the first 5 min after addition of ciprofloxacin. As described above, the influence of ATP depletion was assessed on cells that had been pre-exposed to 2-D-deoxyglucose and NaN_3 for 20 min in order to obtain a stable state of depletion before adding ciprofloxacin. Conversely, the other inhibitors were added at the same time as ciprofloxacin to examine whether their effect would be immediate or not. As shown in figure 31, control cells and cells incubated with cyclosporin A showed a curvilinear

kinetic of accumulation right from the first minutes of the uptake (with slight but significantly faster rate of accumulation for cyclosporin A-treated cells). In contrast, ATP-depleted cells as well as cells exposed to probenecid and cells exposed to probenecid plus cyclosporin A showed an accumulation rate that was faster (and similar for all three situations). Moreover, after 1 min of contact, the kinetics remained almost perfectly linear for at least the 4 subsequent minutes, with no meaningful differences between each condition tested.

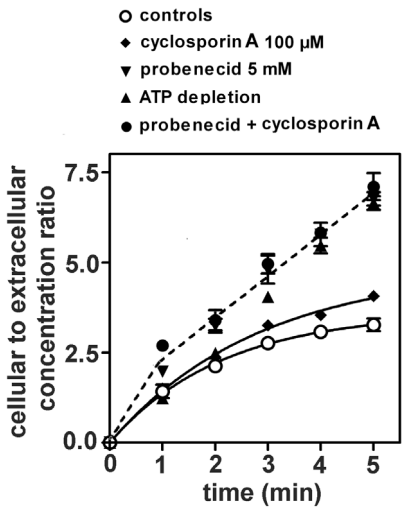


Figure 31 : Short term kinetics of the accumulation of ciprofloxacin (17 mg/L; 50 µM) in control J774 macrophages in comparison with ATP-depleted J774 macrophages, and with J774 macrophages co-incubated with transporter inhibitors (probenecid, 5 mM; cyclosporin A, 100 µM; and the combination of probenecid and cyclosporin A). For control cells and cells incubated with cyclosporin A alone, a one phase exponential association equation could be fitted to data (R^2 = 0.999 and 0.997). For all other conditions, fitting to either one phase exponential association or to a simple linear equation were both considered statistically acceptable. Examination of the data, however, suggests a first fast phase of uptake (0-1 min) followed with by a slower phase still linear with respect to time (1-5 min). Simple dotted lines illustrate these two successive linear processes with no attempt to distinguish between the conditions tested (ATP-depletion, probenecid, probenecid plus cyclosporin) since no significant difference could be noted between any of them.

(Michot *et al.*, 2004)

d) *Summary of the efflux kinetic data from wild cells.*

Table 10 shows in a summarized fashion the half-lives of ciprofloxacin recorded under the main conditions investigated in this study and having led to an increase in the cellular accumulation of the drug in wild cells.

It clearly appears that all these conditions were associated with significant increase in half-lives, which systematically reached values 3 to 6-fold larger than in control conditions.

Table 10. Half-lives of ciprofloxacin in J774 macrophages as measured during efflux studies under conditions that caused a 2.5 to 4-fold increase in drug accumulation at equilibrium

(Michot *et al.*, 2004)

Condition	half-life (min) *
ciprofloxacin (17 mg/L; 50 µM) $	1.68 ± 0.30 a
ciprofloxacin (68 mg/L; 200 µM) $	4.08 ± 0.74 b
exposure to pH 6 #	9.26 ± 0.53 c
ATP-depletion #	10.72 ± 0.24 c
addition of probenecid (10 mM) #	9.51 ± 0.53 c
addition of MK571 (100 µM) #	7.70 ± 1.19 c

* groups with the same letter are not different from one another but significantly different from those bearing a different letter (paired *t* test analysis; $p < 0.05$)
$ drug extracellular concentration during uptake only
\# ciprofloxacin extracellular concentration during uptake 50 µM; indicated treatment applied during uptake and efflux

e) *Studies with probenecid-resistant cells.*

Cells resistant to a given toxin can be obtained by cultivating them for prolonged periods of time with increasing concentrations of this agent. Indeed, in these conditions, only those cells that have acquired a mechanism of resistance will be able to withstand the presence of the toxin. This mode of selection is prone to select a so-called multifactorial resistance (Gottesman *et al.*, 1998). We have therefore applied this procedure to the selection of probenecid-resistant cells, because (i) probenecid is known to reverse multidrug resistance (Gollapudi *et al.*, 1997) and (ii) these cells have indeed been shown previously to display enhanced transport of organic anions (Cao *et al.*, 1993).

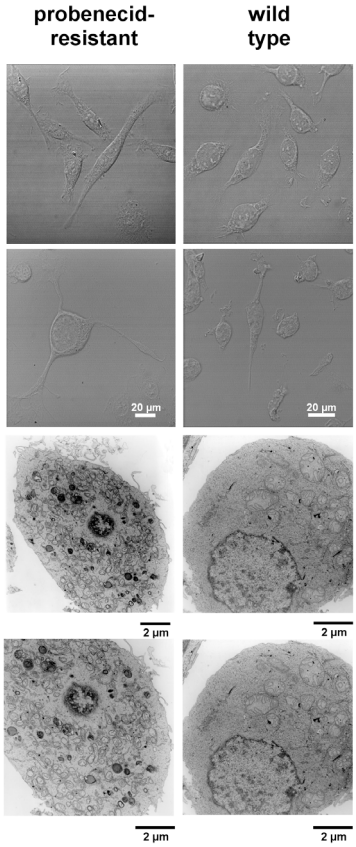
Probenecid resistant cells were obtained as previously described (Cao *et al.*, 1993) using a stepwise approach involving a succession of passages (at least 20) in media with increasing concentrations in probenecid (1, 2 and finally 3 mM). Clones of cells surviving these selection steps were then propagated for 9 months in the continuing presence of 3 mM probenecid. Cells were harvested and then frozen at - 80°C. Experiments were performed on cells thawed and cultivated in the presence of 3 mM probenecid for about 1 month, i.e. upon reaching stable growth properties.

Compared to wild cells (table 11), cells made resistant to the continuous presence of 3 mM probenecid were slightly less swollen (lower volume to protein ratio). They appeared larger and displayed conspicuous elongations extending far away from the cell body, giving sometimes a pseudodendritic aspect to the cell (this aspect could be clearly evidenced with non-confluent cells only). In the electron microscope, probenecid-resistant cells looked essentially normal except for an apparently larger content in mitochondria.

The growth rate of probenecid-resistant cells was 30 % lower than that of wild cells (based on formazan reduction; cells were, however, always compared at a same degree of confluency, i.e. after 2-3 days of culture for wild cells and 3-4 days for probenecid resistant cells). Protein synthesis (measured by ^{3}H-leucine incorporation 3 days after subculture for probenecid-resistant cells and 2 days for wild cells) was slightly more intense in probenecid-resistant cells. Other key parameters such as DNA synthesis, phospholipid or cholesterol to protein ratios, or ATP content were similar.

Table 11.
Main characteristics of probenecid-resistant J774 macrophages in comparison with wild cells.

parameter	Method (units)	probenecid-resistant cells [a]	wild cells	Microscopic appearance
volume	urea/sucrose partition (µl/mg protein)	2.68 ± 0.27 [b] (n = 6)	3.08 ± 0.16 [c] (n = 21)	
growth rate	blue tetrazolium (Δ O.D./well)	0.701 ± 0.047 [b] (n = 8)	0.751 ± 0.047 [c] (n = 8)	
DNA synthesis	[^{3}H]-thymidine incorporation (dpm/pg prot)	28.86 ± 2.18 [d,g] (n = 3)	26.29 ± 1.82 [e,g] (n = 3)	
protein synthesis	[^{3}H]-leucine incorporation (dpm/µg prot)	38.46 ± 3.57 [b,f] (n = 3)	28.51 ± 1.50 [c,f] (n = 3)	
energy status	ATP cell content (nmol/mg prot)	34.44 ± 2.37 [b] (n = 3)	32.92 ± 1.49 [c] (n = 3)	
lipid/prot. ratio	cholesterol	46.0 [b]	52.2 [c]	
	phospholipids (nmol/mg prot)	198.7 [b]	181.7 [c]	



[a] cells were cultivated in the presence of probenecid 3mM;
[b] at day 3 after subculture;
[c] at day 2 after subculture;
[d] at day 2 after subculture (day at with maximum was observed in the 0-5 day period);
[e] at day 1 after subculture (day at with maximum was observed in the 0-5 day period);
[f] TCA-insoluble material (in % of total cell radioactive material): resistant cells, 62.1 ± 5.3; wild cells, 75.2 ± 9.3.
[g] TCA-insoluble material (in % of total cell radioactive material): resistant cells, 90.2 ± 5.3; control, 92.0 ± 9.3.

In relation with the present work, probenecid-resistant cells clearly overexpressed the MRP1 transporter (figure 32), as evidenced by Western blotting, with this protein being clearly displayed at the cell surface (confocal microscopy).

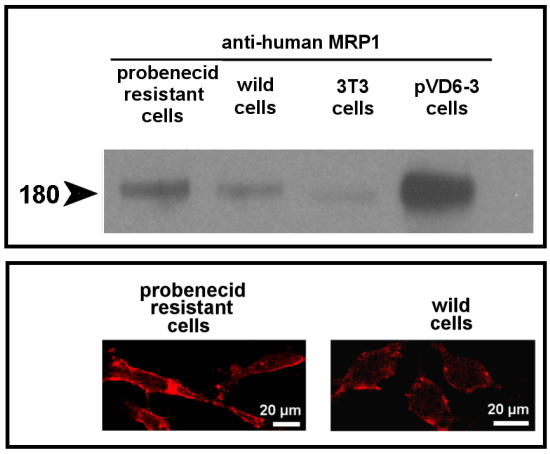


Figure 32: Top: Western blots of lysates from probenecid-resistant and wild (control) J774 macrophages (15 µg protein each), as revealed with an anti-human MRP1 antibody. The controls included 3T3 cells (which express only poorly constitutive MRP1) and 3T3 cells transfected with a multicopy plasmid containing the human *MRP*1 gene (pVD6-3 cells). Equiloading of the gels was checked by revealing actin (no difference detected). **Bottom: Confocal microscopy of probenecid-resistant and wild (control) J774 macrophages**. Cells were immunostained with the same anti-human MRP1 antibody as for the Western blot analysis.

(Michot *et al.*, 2004)

Quite intriguingly, figure 33 (left panel) shows that the accumulation of ciprofloxacin (17 mg/L; 50 µM) in probenecid-resistant cells was only marginally increased in comparison with wild cells and that the rate of release was essentially similar in both cell types in the absence of probenecid. When efflux was followed in the presence of a fixed concentration of probenecid (3 mM, i.e. the concentration at which the probenecid-resistant cells are maintained), wild cells showed a markedly reduced rate of efflux. In contrast, probenecid (3 mM) exerted much less effect on ciprofloxacin efflux in probenecid-resistant cells.

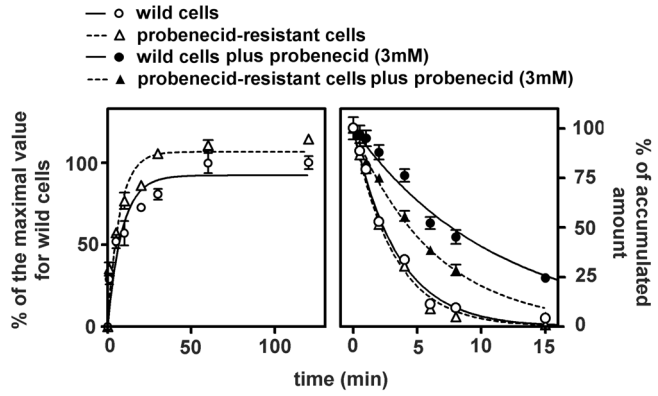


Figure 33: *Left:* kinetics of accumulation of ciprofloxacin (extracellular concentration, 17 mg/L [50 µM]) in wild (control) J774 macrophages and in probenecid resistant-J774 macrophages. *Right:* kinetic of efflux of ciprofloxacin from wild (control) and probenecid-resistant cells incubated for 2 h in the presence of ciprofloxacin (50 µM) and then transferred to a ciprofloxacin-free medium with or without probenecid (3 mM).

(Michot *et al.*, 2004)

f) Discussion: tentative identification of the ciprofloxacin transporter in J774 macrophages and therapeutic significance

Evidence for a role of fluoroquinolone transporters in macrophages, in relation to drug accumulation and efflux, has remained indirect so far and primarily based on the observation that organic anions, such as probenecid and gemfibrozil, significantly increase fluoroquinolone accumulation in J774 macrophages (Rudin *et al.*, 1992; Cao *et al.*, 1992; Rispal *et al.*, 1996). The present data confirm and significantly extend these findings in two respects. First, they provide direct evidence that probenecid and gemfibrozil impair ciprofloxacin efflux with no detectable change in its kinetics of influx. Second, they show that a similar effect on ciprofloxacin accumulation and efflux can be obtained with ATP depletion and with the selective MRP inhibitor MK571. We also show that cells exposed to a large concentration of ciprofloxacin accumulate the drug to a 3 to 4-fold larger extent than when exposed to therapeutically-meaningful concentrations. Glutathione deprivation and acid pH also increase ciprofloxacin accumulation, and this effect is mediated by a corresponding change in efflux in the case of acid pH.

Our results suggest that J774 macrophages have an MRP transporter that is (i) sensitive to organic anions, (ii) dependent on energy and of glutathione, (iii) inhibited by H^+ and by ciprofloxacin itself. The fact that a large ciprofloxacin concentration during uptake causes a change in efflux in the early phase of the washout experiments only is probably due to the fact that efflux is measured in the absence of drug in the extracellular milieu. As the intracellular drug concentration decreases, it probably falls rapidly below the level at which the transporter can be saturated.

Fluoroquinolones are zwitterionic and may therefore be recognized by both anion and cation transporters.

> Based on the Saier's classification of transport in eukaryotic cells (Saier, Jr., 2000; see introduction for details), anion transporters comprise (i) the Oat (Organic Anion Transporters) which are members of the MFS superfamily (Major Facilitator Superfamily); (ii) the OATP and OATK which are members of the OAT (Organo Anion Transporters) superfamily ; (iii) the MRP (Multidrug Resistance Protein), which are members of the ABC (ATP Binding cassette) superfamily. Oat, OATP, OATK are energized by ion gradients (most often H^+) and act as symports, uniports or antiports. MRPs are energized by ATP hydrolysis and require glutathione as co-factor. Cation transporters include (i) the Oct (Organic cation transporters) which are, like Oat, members of the MFS superfamily; (ii) the MDR (Multi Drug Resistance), also known as P-glycoprotein which, like MRP, are members of the ABC superfamily (and therefore rely on ATP for activity) but do not require glutathione for optimal activity (Davey *et al.*, 1996).

If we analyze our results globally based this classification, we may strongly suggest that we are dealing here with an MRP transporter. Besides the characteristics mentioned above, we also observe an impairment of ciprofloxacin efflux by the preferential MRP inhibitor MK571 (Gekeler *et al.*, 1995) and no marked effect exerted by cationic compounds such as verapamil and the preferential P-glycoprotein inhibitor GF 120918 (Safa, 1988; Boer *et al.*, 1996). The intermediate effect observed with cyclosporin A may result from the fact that this compound not only acts on P-glycoprotein but also inhibits the MRP (Twentyman, 1992; Leier *et al.*, 1994). Impairment by acid pH has been reported for MRP1 and may be related to the fact that ATPase activity of MRP is it-self adversely affected by acid pH (Mao *et al.*, 1999). Since monensin, a drug collapsing transmembrane pH gradients, does not markedly influence ciprofloxacin accumulation, it is also reasonable to accept that pH gradients *per se* and intracellular pH are unimportant in this context, which globally excludes the transporters energized by H^+ gradients.

The complete human ATP-binding cassette (ABC) transporters subfamily (ABCC) has at least 13 identified members [ABCC1-13], among which 9 belong to the multidrug resistance-like subgroup [MRP] (Borst *et al.*, 2000; Renes *et al.*, 2000; Müller, 2003). Based on these reviews of the main properties of MRP transporters, we may probably exclude MRP2, MRP3, and MRP4. MRP2 shows the same substrate specificity as MRP1 but is mostly localized in bile canaliculi *in vivo* and has been described *in vitro* in polarized cells only. MRP3 is restricted to intestine and kidney cells and is a poor transporter of glutathione conjugates (Hirohashi *et al.*, 1999). MRP4 is localized in specific tissues and is probably transporting phosphate conjugates only. The intervention of MRP5 can be excluded since it is not inhibited by MK571 (Jedlitschky *et al.*, 2000) and transport substrates independently from glutathione (McAleer *et al.*, 1999). MRP6 is difficult to immediately exclude since it acts as a organic anion transporter, is inhibited by probenecid, and can be co-amplified with MRP1 in cells that overexpress the latter transporter because of their close location on the chromosome (Kool *et al.*, 1999; Ilias *et al.*, 2002). However, MRP6 is mainly expressed in polarized cells (Madon *et al.*, 2000; Scheffer *et al.*, 2002) and shows substrate specificities that are largely different from those of the other MRP. MRP 7 has the lowest degree of relatedness with the other MRP transporters and its RNA transcript is only expressed at very low levels in most tissues. Its capacity to transport anionic compounds is still unknown (Hopper *et al.*, 2001). MRP 8, expressed in various adult human tissues including liver, lung, and kidney, and also in several fetal tissues, is a transporter of amphipathic anions. It is able to efflux cAMP and cGMP and to function as a resistance factor for commonly employed purine and pyrimidine nucleotide analogues. MRP9 is an unusual truncated member of the ABC transporter superfamily, that is highly expressed in breast cancer and in testes but not expressed at detectable levels in essential normal tissues (Bera *et al.*, 2002).

Limiting the discussion to the evidence presented with wild J774 macrophages would make MRP1 a prime candidate for ciprofloxacin efflux. MRP1 functions indeed as a multispecific organic anion transporter, which transports drugs and other hydrophobic compounds in the presence of glutathione (Müller, 2003). Yet, when this hypothesis was tested using the probenecid-resistant cells (which overexpress MRP1 as evidenced by both Western blot analysis and confocal microscopy), no marked change in the accumulation and efflux of ciprofloxacin was noted. The overexpressed MRP1 was nevertheless functional since probenecid (and gemfibrozil and MK571; data not shown) are less effective inhibitors of ciprofloxacin efflux in these cells as compared to wild cells, which is what can be expected since anions are actively extruded in probenecid-resistant cells (Cao *et al.*, 1993). The data must, therefore, be interpreted as demonstrating that MRP1 is not the actual ciprofloxacin transporter.

Conversely, the data presented here unambiguously show that P-glycoprotein is not involved in ciprofloxacin efflux, even though J774 macrophages express a functional P-glycoprotein that acts on azithromycin (Seral *et al.*, 2003b). A similar conclusion was reached concerning the transport of ciprofloxacin in MDR1 [P-gp] - transfected MDCK-II epithelial cells (Lowes and Simmons, 2002). Interestingly enough, the closely-related fluoroquinolone, grepafloxacin was found to be a substrate of both P-glycoprotein and MRP2 in these cells, and to compete for the ciprofloxacin-sensitive pathway. This suggests that apparently minor structural differences in quinolones may play a key role in transporter recognition (as they do with respect to antibacterial spectrum). It must, however, be stressed that the situation in polarized cells may be considerably more complex than in macrophages.

Although the nature of the ciprofloxacin transporter in J774 macrophages remains undefined, the data presented here may already have important biological and therapeutic significance. We show indeed that the ciprofloxacin transporter causes a significant reduction of the intracellular concentration of this fluoroquinolone at therapeutically meaningful concentrations. If this takes place with macrophages *in vivo*, we may anticipate that it will reduce the accumulation of the antibiotic and thereby decrease its intracellular activity. The activity of fluoroquinolones is concentration-dependent including towards intracellular organisms (Rudin *et al.*, 1992; Carryn *et al.*, 2002; Seral *et al.*, 2003c), and suboptimal concentrations of are associated with insufficient eradication and potentially rapid emergence of resistance (Hooper, 2000). We therefore examine how the activity of ciprofloxacin against intracellular infections is affected by the presence of efflux pumps in J774 macrophages.

III.2.c.2. Pharmacodynamic studies: influence of efflux pumps on the activity of ciprofloxacin against intracellular infections

a) Influence of gemfibrozil on the intracellular activity of ciprofloxacin

As done for azithromycin and verapamil (see section III.2.a.2), we have also studied the influence of gemfibrozil on the activity of ciprofloxacin against intracellular bacteria in macrophages incubated with increasing extracellular concentrations (expressed as multiples of the MIC). Ciprofloxacin showed a strong, concentration-dependent activity towards intracellular *L. monocytogenes* over the whole range of concentrations examined, being bacteriostatic at an extracellular concentration equivalent to its MIC (MIC = 1.25 mg/L) and becoming markedly bactericidal at larger concentrations. As shown in figure 34, addition of gemfibrozil made the drug more effective at all concentrations examined, decreasing of about 2-fold the extracellular concentration needed to obtain a bacteriostatic effect while increasing the bactericidal effect observed at larger concentrations in comparison with controls. Interestingly, the addition of gemfibrozil confers to ciprofloxacin a bactericidal activity when cells are exposed at a concentration corresponding to its Cmax (maximal concentration observed in the serum of patients [~ 4 mg/L]).

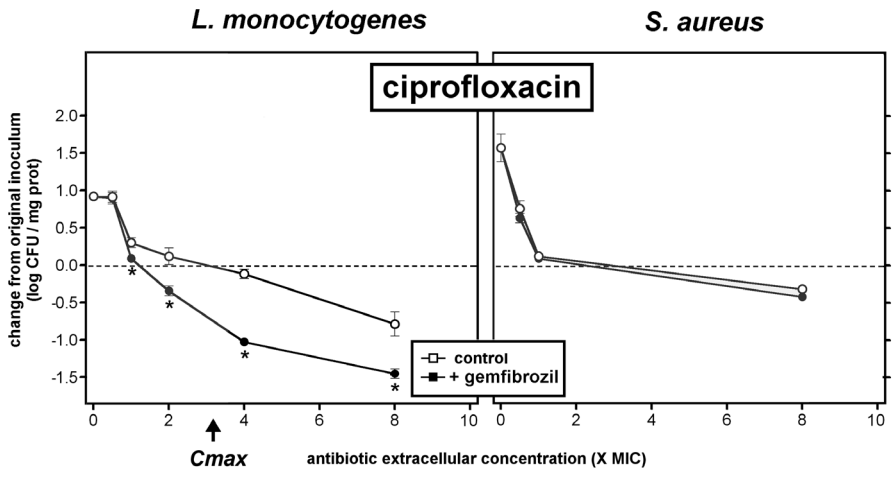


Figure 34: Influence of gemfibrozil 250 µM on the activity of ciprofloxacin on intracellular *L. monocytogenes* or *S. aureus*. The ordinate shows the changes in cfu (as log cfu/mg cell protein) from the initial inoculum at the end of the incubation with the antibiotics (5 h for *L. monocytogenes*; 24 h for *S. aureus*). The dotted line corresponds to a bacteriostatic effect. The abscissa shows the extracellular concentration of antibiotic used (as multiple of the corresponding MIC [1.25 and 0.125 mg/L]). The corresponding maximal serum peak level that can be reached in the serum of patients undergoing a conventional treatment is shown by an arrow marked Cmax (for ciprofloxacin and *S. aureus*, this value is higher than the maximal multiple of MIC examined). Each data point is the mean ± SD of three independent determinations. The asterisks show the statistically significant differences between inhibitor-treated and control cells (paired points; $P < 0.01$).

(Seral *et al.*, 2003a)

Against intracellular *S. aureus,* the activity of ciprofloxacin was quite poor, with a bacteriostatic activity obtained at an extracellular concentration corresponding to its MIC (MIC = 0.125 mg/L), and with only a slight trend towards a bactericidal activity at 8 x its MIC. In contrast to what was seen with *Listeria*, no significant effect of gemfibrozil was noted on ciprofloxacin activity against *S. aureus* at all concentrations studied (which were all below the Cmax).

b) Influence of gemfibrozil on the cellular concentration and the subcellular distribution of ciprofloxacin

We studied the influence of gemfibrozil on the cellular accumulation and subcellular distribution of ciprofloxacin in uninfected cells.

(we checked in preliminary experiments that the infection did not modify the accumulation of the drug both in the absence or in the presence of gemfibrozil). The distribution of lactate dehydrogenase (used as a marker of soluble proteins) as well as that of N-acetyl-β-hexosaminidase (used as marker of lysosomes; data not shown) was not modified by gemfibrozil (figure 35). Ciprofloxacin was mostly found in the soluble fraction of control cells. Addition of gemfibrozil increased the

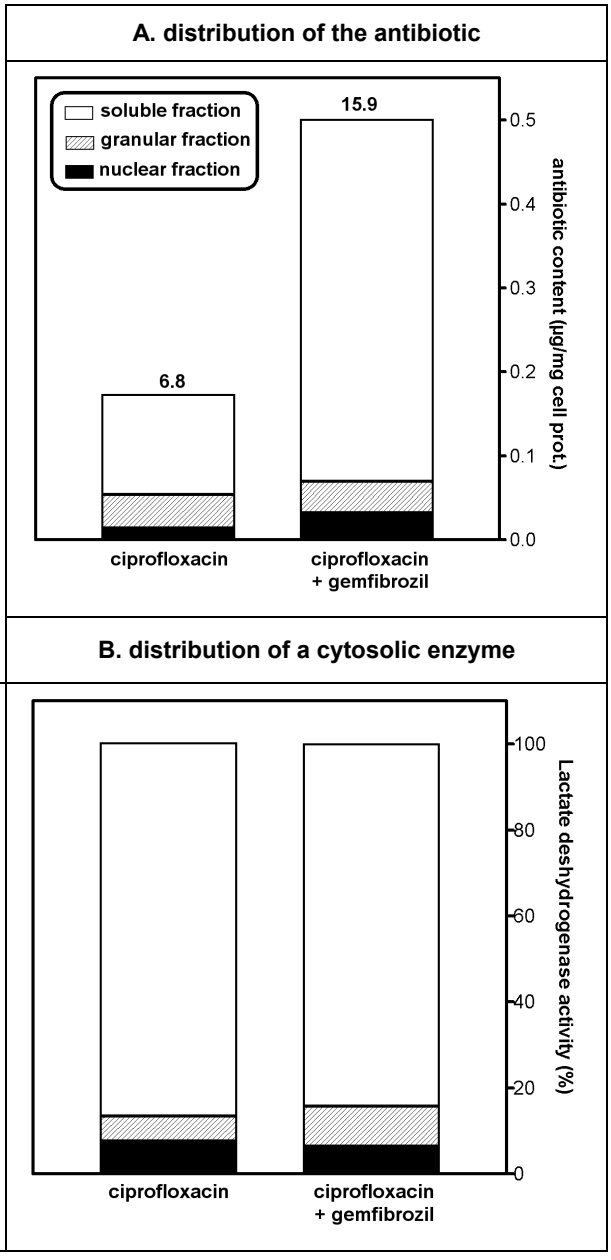


Figure 35: Influence of gemfibrozil (250 µM) on the accumulation and subcellular distribution of ciprofloxacin (5 mg/L).. Cells were incubated for 24 h with the drugs. The upper panel shows the antibiotic content in the nuclear, granules, and soluble fractions expressed as µg/mg of protein in the unfractionated homogenate. Figures at the top of each bar correspond to the apparent cellular accumulation of the antibiotic (cellular to extracellular concentration ratio [C_c/C_e], calculated using a conversion factor of 5 µl of cell volume per mg of cell protein). The lower panel shows the distribution of lactate dehydrogenase (marker of the soluble proteins).

(Seral *et al.*, 2003a)

total drug accumulation by 2.4-fold, but also modified the drug repartition between subcellular compartments, since most of the ciprofloxacin accumulated in excess in the presence of gemfibrozil was found in the soluble fraction.

c) Discussion of the results and potential impact for the treatment of intracellular infections

The data presented here unambiguously show that ciprofloxacin is a concentration-dependent antibiotic towards a cytosolic bacteria (*L. monocytogenes*), and that its intracellular activity can, accordingly, be modulated by changes in its intracellular concentration. This implies that the intracellular drug and the bacteria must come partially into direct contact with one another. This may indeed occur in the cytosol, based on the findings of the cell fractionation studies, which are entirely consistent with previous ones ([B. Scorneaux, unpublished observation for ciprofloxacin; see also [Carlier *et al.*, 1990] for other fluoroquinolones) and on the fact that actively-dividing *L. monocytogenes* are also located in this compartment. Thus, the present study extends and rationalizes the observations of Rudin *et al.*, 1992 with norfloxacin and ciprofloxacin, who showed that gemfibrozil enhances the listericidal activity of these fluoroquinolones. The present data may also offer a satisfactory explanation for the poor activity of ciprofloxacin towards intracellular *S. aureus*. They indeed suggest that only a minor proportion of intracellular ciprofloxacin will meet these phagosomal bacteria. More importantly, we also see that increasing total cell concentration of ciprofloxacin (by reducing its efflux with gemfibrozil) does not allow for a commensurate change in the amount of drug associated with the cell granules. This suggests that the cytosol (and the cytosolic constituents that probably bind ciprofloxacin) is not saturable under the conditions used here. The consequence should be that efflux pumps inhibitors will be unable to increase ciprofloxacin activity against a non-cytosolic bacteria, which is indeed what we observe with *S. aureus*.

As already concluded for azithromycin, our data indicate that the intracellular activity of ciprofloxacin is suboptimal due to its active efflux from the macrophages. We have evoked earlier the difficulty of developing clinically usable inhibitors of efflux pumps as adjuvant therapy. Moreover, this strategy would not correct for the poor activity of the drug against phagolysosomal infections. Another strategy, which would certainly make more sense here, would be to use quinolone derivatives endowed with a higher capacity of accumulation in the cells and/or a lowered affinity for efflux pumps. This conclusion prompted us to compare the pharmacokinetic behavior of 4 quinolones in the same cellular model.

III.2.c.3. Comparative studies : influence of efflux pumps on the accumulation and efflux of 4 quinolones in macrophages

Levofloxacin and moxifloxacin were chosen for this study, on the basis their increasingly lipophilic character and potential clinical interest, together with garenoxacin (Takahata *et al.*, 1999), as a typical member of the new class of desfluoroquinolones, in order to gain more information on the potential structure-activity relationships governing quinolone accumulation and efflux in macrophages (see figure 27 for structure and physiochemical characteristics). The methodologies of the experiments described here are detailed in the corresponding publication (Michot *et al.*, 2005).

a) Kinetics of accumulation of quinolones

In the first series of experiments, the kinetics of accumulation of the 4 quinolones were examined using a fixed extracellular concentration of 5 mg/L.

Figure 36 shows that all 4 drugs accumulated quickly in cells, reaching an apparent steady-state level after 30 to 60 min. These levels, however, were markedly different among quinolones, ciprofloxacin reaching the lowest value (around 3-fold), followed by levofloxacin (about 5-fold), garenoxacin (about 10-fold), and moxifloxacin reaching the highest value (about 16-fold). Because this type of experiment did not allow to measure and compare the early phases of uptake, short-term kinetics studies concentrating on the first 5 min of accumulation were ran independently. These experiments were made at two different extracellular concentrations (5 mg/L and 17 mg/L) since we knew from previous studies (figures 28 and 29) that the extent of ciprofloxacin accumulation is influenced by its concentration. Results

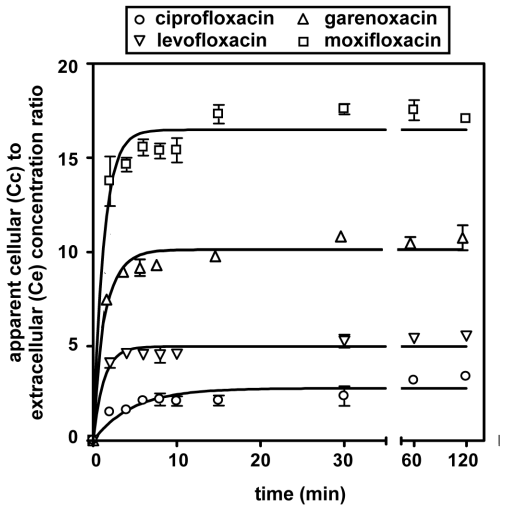


Figure 36: Comparative kinetics of the accumulation of the 4 quinolones at an extracellular concentration of 5 mg/L and at 37°C in J774 murine macrophages. All data are the means ± SD of three experiments (for symbols without bars, the SD is smaller than the symbol size). Results are expressed as the ratio of the apparent cellular concentration of each quinolone to its extracellular concentration. One-phase exponential association equations were fitted to data obtained for each drug (regression parameters: ciprofloxacin, R^2 = 0.817, y_{max} = 2.775 [c.i. 2.259-3.289]; levofloxacin, R^2 = 0.950, y_{max} = 4.987 [c.i. 4.600-5.373]; garenoxacin, R^2 = 0.983, y_{max} = 9.50 [c.i. 9.95-10.06]; moxifloxacin, R^2 = 0.967, y_{max} = 16.48 [c.i. 15.61-17.35].

(Michot *et al.*, 2005)

for ciprofloxacin and moxifloxacin are presented in figure 37. They show that the uptake of quinolones proceeded largely at a similar fractional rate within the first minute of the experiment. In absolute value, however, fluxes were about 5 to 6-fold higher for moxifloxacin when compared to ciprofloxacin at the same extracellular concentration. After this first phase, a slower uptake process became apparent, especially for ciprofloxacin.

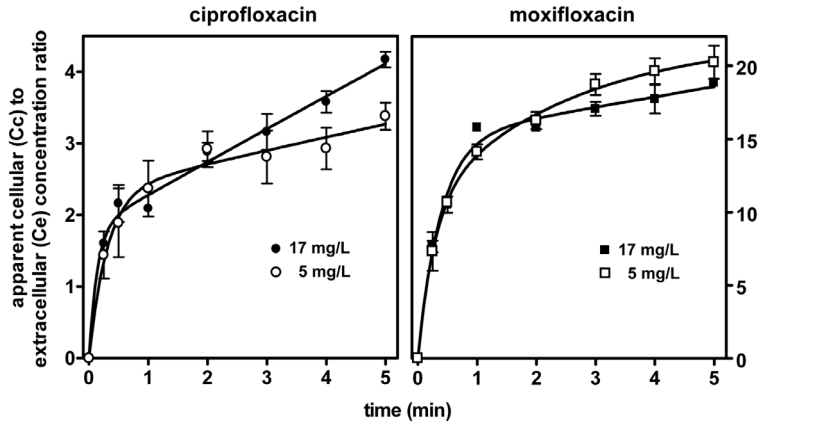


Figure 37: Comparative short-term kinetics of the accumulation of ciprofloxacin (left panel) and moxifloxacin (right panel) at two different extracellular concentrations. All data are the mean ± SD of three experiments (for symbols without bars, the SD is smaller than the symbol size). Results are expressed as the ratio of the apparent cellular concentration of each quinolone to its extracellular concentration. A two-phase exponential association was fitted to data obtained for each drug (regression parameters: ciprofloxacin 5 mg/L, R^2 = 0.987; ciprofloxacin, 17 mg/L, R^2 = 0.992; moxifloxacin 5 mg/L, R^2 = 0.999; moxifloxacin 17 mg/L, R^2 = 0.991), which best describe the biphasic character of the uptake of these two quinolones.

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Of interest also was the fact that the capacity of the cells to concentrate ciprofloxacin at 5 minutes was larger at an extracellular concentration of 17 mg/L than at 5 mg/L, whereas no significant difference (and actually a trend to a decrease) was seen for moxifloxacin. Similar experiments were made with levofloxacin and garenoxacin and showed (i) that these quinolones also displayed a fast first phase of influx, followed by a slower phase; (ii) that the accumulation of levofloxacin at 5 minutes but not that of garenoxacin was larger at 17 mg/L than at 5 mg/L.

b) Influence of extracellular concentration and of ATP depletion

Our previous experiments (figures 28 and 30) had shown that the accumulation of ciprofloxacin at equilibrium (defined by the ratio of its apparent cellular to its extracellular concentration) was not only influenced by the drug

extracellular concentration, but also by ATP-depletion of the cells. These conditions were therefore systematically explored here for the 3 other quinolones in comparison with ciprofloxacin. Figure 38 shows that, as anticipated, raising the extracellular concentration of ciprofloxacin from 2 to 200 mg/L caused its accumulation in control cells to increase about 5-fold, with a change becoming noticeable as from an extracellular concentration of approx. 15 mg/L. In ATP-depleted cells, the accumulation of ciprofloxacin was already markedly increased at the lowest extracellular concentration tested (2 mg/L), reaching values approx. 2.5-fold those of control cells). It still increased when the extracellular concentration was raised, but in less marked fashion than in control cells. At 200 mg/L, the accumulation levels observed for ATP-depleted and control cells were essentially similar.

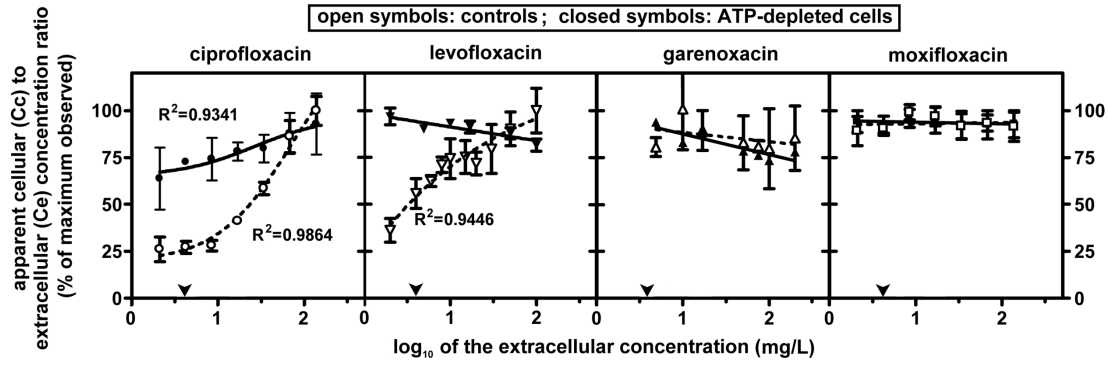

Figure 38: Influence of the extracellular concentration of quinolones on their accumulation at equilibrium (2 h) in J774 murine macrophages in control conditions or in conditions of ATP-depletion. All data are expressed at the percentage of the maximal apparent cellular to extracellular concentration ratio observed for the corresponding drug in these experiments (ciprofloxacin, 23.05 ± 0.71; levofloxacin, 9.33 ± 0.45; garenoxacin, 15.00 ± 0.50; moxifloxacin, 16.73 ± 0.31) and are shown as means ± the 95 % confidence interval (n=3). For ciprofloxacin and levofloxacin (controls), data were fitted to a variable slope sigmoid dose-response equation. For all other conditions, the figure shows straight lines corresponding to arbitrary linear regression equations. The arrow on each panel points to the human Cmax.

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Levofloxacin also showed an increase in its accumulation in control cells (no ATP depletion) when its extracellular concentration was raised, but this occurred at lower values (with levels of about 75 % of the maximal value already observed at an extracellular concentration of 17 mg/L). No significant influence of the extracellular concentration was noted for garenoxacin or moxifloxacin in control cells (no ATP depletion) throughout the whole range of concentrations investigated. With ATP-depleted cells, increasing the levofloxacin or garenoxacin concentration reduced slightly but nevertheless significantly ($P < 0.02$ for slope deviation from zero) the corresponding accumulation levels, whereas no effect was seen for moxifloxacin.

c) Influence of preferential MRP and P-glycoprotein inhibitors

c.1) Effect on quinolone accumulation

We showed previously (figure 19) that J774 macrophages express at least two antibiotic transporters, one belonging to the family of the MRP and the second being identified as the P-glycoprotein. These transporters are responsible for a decreased accumulation of ciprofloxacin and azithromycin, respectively. Preferential inhibitors of each of these transporters were therefore used in the present study to examine their influence on the cellular accumulation of levofloxacin, garenoxacin, and moxifloxacin in comparison with ciprofloxacin.

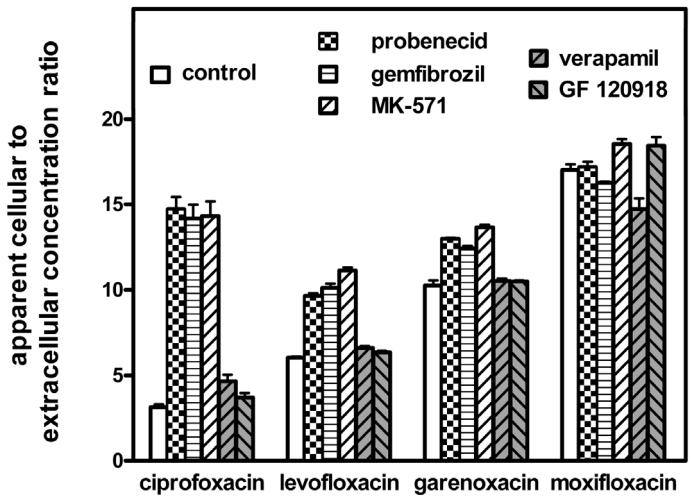


Figure 39: Influence of MRP inhibitors (probenecid [5 mM], gemfibrozil [500 µM], MK-571 [100 µM]) and P-glycoprotein inhibitors (verapamil [100 µM], GF 120918 [2 µM]) on the accumulation of quinolones. Cells were incubated for 2 h in the presence of 5 mg/L of each quinolone in the absence (control) or in the presence of the corresponding inhibitors. All values are the means of 3 independent determinations ± SD. Statistical analysis (one-way ANOVA): $P < 0.01$ for ciprofloxacin, levofloxacin, or garenoxacin alone vs. these antibiotics in the presence of probenecid, gemfibrozil, or MK 571, respectively; $P < 0.05$ for moxifloxacin alone vs. moxifloxacin in the presence of verapamil; all differences between each antibiotic alone and the same antibiotic in the presence of other inhibitors are not significant [$P > 0.05$]).

(Michot *et al.*, 2005)

Figure 39 shows that probenecid, gemfibrozil (two inhibitors of organic anion transporters including MRP) and MK-571 (a preferential inhibitor of the MRP transporters) increased the accumulation of ciprofloxacin (about 5-fold) as anticipated, but had no effect on the accumulation of moxifloxacin. Their effect on the accumulation of levofloxacin and garenoxacin was noticeable and significant but less

intense than for ciprofloxacin. As a result, and quite interestingly, the levels of accumulation of ciprofloxacin and garenoxacin became quite similar in the presence of the MRP inhibitors and only slightly lower than those of moxifloxacin. Levofloxacin and garenoxacin accumulation increased in the presence of MRP-inhibitors, but to a lower extent than that of ciprofloxacin. In contrast, GF 120918 (a preferential inhibitor of the P-glycoprotein) had no statistically significant effect on the accumulation of the quinolones. Verapamil, which also inhibits the P-glycoprotein but is far less specific, was without significant effect on the accumulation of ciprofloxacin, levofloxacin, or garenoxacin. Quite intriguingly, however, a slight but statistically significant decrease of the accumulation of moxifloxacin was observed. Globally, however, neither GF 120918 nor verapamil modified to the ranking of accumulation seen for controls (*i.e.* ciprofloxacin < levofloxacin < garenoxacin < moxifloxacin) and differences between quinolones remained essentially unchanged.

c.2) Effect on quinolone efflux

Since the previous experiments had disclosed differential effects of MRP inhibitors on the accumulation of quinolones, experiments were conducted with probenecid to examine whether this behavior could be related to differences in drug efflux. For this purpose, we used cells loaded for 2 h with 17 mg/L of quinolones and examined the drug efflux in short term kinetic studies (this high concentration of quinolone was needed for sake of sensitivity). Probenecid was added to the treated cells during both the loading time and the efflux period to ensure a maximal inhibition of the transporter. Results are presented in figure 40 for ciprofloxacin and moxifloxacin. As anticipated, the efflux of ciprofloxacin was markedly slowed down by probenecid (with a apparent half-life increase of about 4.5-fold). But quite surprisingly, we observed that moxifloxacin was released from cells at a much faster rate than ciprofloxacin, and that probenecid (5 mM) did not influence this behavior (these data, obtained using the fluorimetric assay, were independently confirmed using [^{14}C]-labeled moxifloxacin). We then extended those studies to levofloxacin and garenoxacin and saw (i) that their rate of release from control cells was in the same range as that of ciprofloxacin (half-lives [c.i.]: 2.13 [1.87-2.46] and 1.64 [1.27-2.31] vs. 1.62 [1.18-2.59]), and (ii) that probenecid (5 mM) less slightly slowed down their efflux compared to ciprofloxacin (half-lives [c.i.]: 3.28 [2.88-3.81] and 3.43 [2.31-3.49] vs. 7.20 [4.83-14.20]).

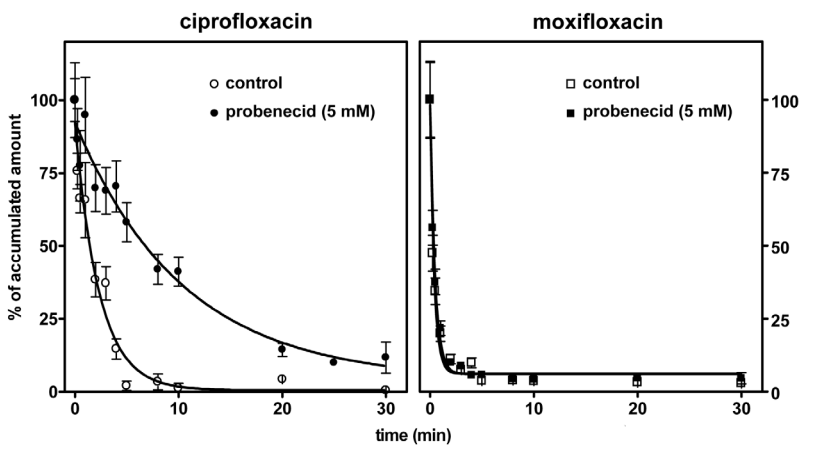


Figure 40: Influence of probenecid on the efflux of ciprofloxacin and moxifloxacin from J774 macrophages. Control (open symbols), cells were loaded by incubation at 37°C for 2 h in the presence of 17 mg/L of the corresponding quinolone and then transferred to a quinolone-free medium for measuring efflux (controls). Probenecid-treated cells (closed symbols), loading and efflux were performed in the presence of 5 mM probenecid (we showed previously (figure 30) that probenecid does not alter the kinetics of influx of ciprofloxacin). Data are expressed as percent of the amount accumulated by the cells at the end of the loading phase (apparent cellular to extracellular concentration ratios: ciprofloxacin, 3.92 ± 0.29, ciprofloxacin plus probenecid, 15.83± 0.2.03; moxifloxacin, 17.09 ± 2.26; moxifloxacin plus probenecid, 17.94 ± 2.30). One-phase exponential decay curves were fitted to the data (goodness of fit [R^2] and half-lives: ciprofloxacin, 0.967 and 1.62 ± 0.25 min; ciprofloxacin plus probenecid, 0.963 and 7.20 ± 0.59 min; moxifloxacin, 0.980 and 0.27 ± 0.03 min; moxifloxacin plus probenecid, 0.993 0.33 ± 0.22 min).

(Michot *et al.*, 2005)

d) Influence of moxifloxacin on the accumulation of ciprofloxacin

In these experiments, we wished to specifically address the question of a potential interaction of moxifloxacin with the ciprofloxacin efflux transporter. This was assessed by examining to what extent moxifloxacin could increase the cellular accumulation of ciprofloxacin as does ciprofloxacin it-self (see figure 28), an effect that we interpreted as a self-induced impairment of its efflux (see discussion of chapter III.2.b.1.). For this purpose, cells were incubated with a fixed concentration of [^{14}C]-labeled ciprofloxacin in the presence of either (i) increasing amounts of unlabeled ciprofloxacin (in a 5-195 mg/L [15.1-596 µM] range [the latter value being close to the solubility limit of ciprofloxacin in the medium]), or (ii) a fixed concentration of 5 mg/L of unlabeled ciprofloxacin plus increasing concentrations of moxifloxacin (5-700 mg/L [12.5-1746 µM]). Results presented in figure 41 show that moxifloxacin could increase the accumulation of [^{14}C]-labeled ciprofloxacin but was about globally 2-times less potent than ciprofloxacin it-self (based on equimolar comparisons).

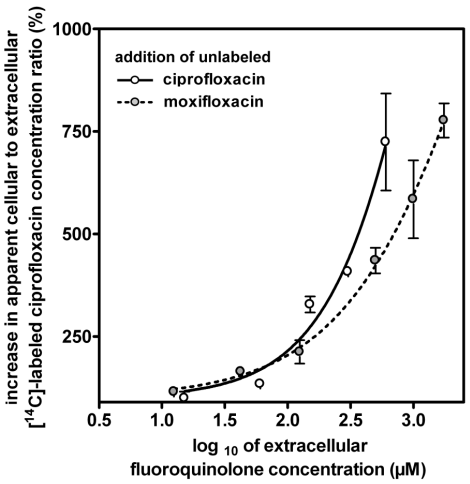


Figure 41: Influence of the addition of unlabelled ciprofloxacin on the accumulation of [^{14}C]-labeled ciprofloxacin. Cells were maintained for 2 h at 37°C with a constant concentration of 940 nCi (34.8 kBq) of [^{14}C]-ciprofloxacin per ml (corresponding to a weight concentration of 5 mg/L and a molar concentration of 15.1 µM). Open symbols and solid line: the medium contained increasing amounts of unlabeled ciprofloxacin, starting from a concentration of 0 mg/L (1st point on the graph) up to a maximum of 195 mg/L (590 µM, close to the limit of solubility in the culture medium). Grey symbols and dotted line: the medium contained 5 mg/L of unlabeled ciprofloxacin plus increasing amounts of moxifloxacin, starting from a concentration of 5 mg/L [12.5 µM]). Sigmoid dose response curves were fitted to the data (R^2 = 0.979 and 0.997, respectively) according to a previously described model of the co-operative effect of ciprofloxacin on ciprofloxacin accumulation by J774 macrophages (figure 28).

(Michot *et al.*, 2005)

e) Accumulation at 4°C and after transient exposure of the cells to 56°C.

Early investigations had revealed that certain quinolones are accumulated to some extent by macrophages even when these are maintained at 4°C (Carlier *et al.*, 1990; Hara *et al.*, 2000), and that ciprofloxacin accumulation is enhanced in cells subjected to transient heat shock (Easmon and Crane, 1985). This intriguing behavior was systematically reexamined here for all 4 quinolones studied.

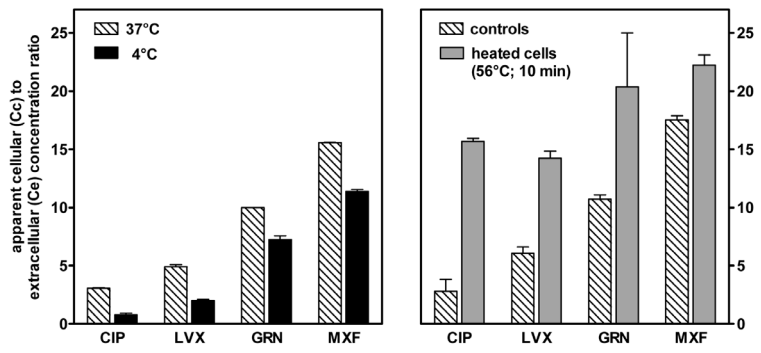


Figure 42: Cellular accumulation of quinolones after 2 h incubation at an extracellular drug concentration of 5 mg/L. *Left panel:* hatched bars, controls (incubated at 37°C); Closed black bars, cells were incubated at 4°C. *Right panel*: hatched bars, controls (incubated at 37°C); closed grey bars, cells were exposed to 56°C for 10 min in the absence of drug, cooled to 37°C, and then incubated at 37°C in the presence of drug for 2 h. All values are the means of 3 independent determinations ± SD.

(Michot *et al.*, 2005)

As shown in figure 42, and concentrating first on the comparison between 37°C and 4°C, it clearly appears that cells maintained at 4°C in the presence of ciprofloxacin accumulated much less drug than at 37°C (2 h in both cases). Percentage-wise, the difference was much smaller for cells incubated with moxifloxacin. In absolute values, however, it appears that incubation at 4°C reduced the accumulation of all four drugs of about the same amount. Levofloxacin and garenoxacin showed an intermediate behavior. Moving now to the results obtained with cells exposed transiently to 56°C (10 min) and then incubated with the quinolones at 37°C (2 h), all four 4 quinolones showed a high accumulation level (approx. 15-fold over the extracellular concentration for ciprofloxacin and levofloxacin, and up to about 22-fold for garenoxacin and moxifloxacin). Interestingly enough, this common level of accumulation (15 to 23-fold) was of the order of magnitude of that observed in ATP-depleted cells or in cells exposed to large drug concentrations (figure 38), or in cells exposed to preferential MRP inhibitors (figure 39). If cells were maintained at 56°C for 20 min, accumulation of quinolones dropped to values around 2-fold, but cells examined by phase contrast microscopy showed manifest signs of loss of integrity (data not shown).

f) Discussion – contrasting behavior of quinolones

The data presented here, together with those of previous publications (Carlier *et al.*, 1990; Hara *et al.*, 2000; Dorian *et al.*, 2001; Carryn *et al.*, 2002) show that quinolones are accumulated by macrophages but to quite different extents. Many studies also report that quinolones accumulated by cells are active against intracellular bacteria sojourning in different subcellular compartments (see Carryn *et al.*, 2003 for a recent review). This implies that part of the cell-associated quinolones must be truly intracellular, and, therefore, able to cross to some extent the pericellular membrane. In this context, differences in apparent cellular accumulation could result from differences in influx, in susceptibilities to cellular sequestration, or in efflux. We show here that moxifloxacin (i) is accumulated more than ciprofloxacin, (ii) is apparently not subject to efflux by the MRP-like ciprofloxacin transporter (based on marked differential effects of ATP depletion and addition of MRP inhibitors, (iii) is not subject to efflux through the P-glycoprotein transporter either (based on the lack of effect of the preferential Pg-glycoprotein inhibitors which increase the accumulation of azithromycin in these cells [table 9]). These data, therefore, suggest that the differences in accumulation seen between ciprofloxacin and moxifloxacin may actually result from their differential susceptibility to efflux by the ciprofloxacin transporter.

In a first analysis, our data seem compatible with a model in which (i) ciprofloxacin would penetrate J774 macrophages by passive diffusion through membrane bilayers (as also suggested by others [Rispal *et al.*, 1996; Sun *et al.*, 2002]), and binds loosely to so far unidentified intracellular constituents, while being simultaneously subject to active efflux, and (ii) moxifloxacin would simply avoid recognition by the transporter and, therefore, reach maximal accumulation. Levofloxacin and garenoxacin would be partially subject to efflux by ciprofloxacin transporter, and therefore consistently show an intermediate behavior. The ranking of susceptibility to transport to which this model would arrive to (ciprofloxacin < levofloxacin < garenoxacin < moxifloxacin) is actually quite similar to what has been concluded from the study of the bacterial transporter NorA (a member of the Major Facilitator Superfamily [MFS; Van Bambeke *et al.*, 2000]). For this transporter, recognition was shown, indeed, not to depend so much of the hydrophobic character of the quinolones studied, but of the bulkiness of the C-7 substituent and of the bulkiness and hydrophobicity of the C-8 substituent (Takenouchi *et al.*, 1996). As shown in figure 27, it is interesting to see that moxifloxacin and garenoxacin, which are the least effluxed by J774 macrophages, possess both substituents, which are absent from ciprofloxacin. Levofloxacin does not possess a bulky C-7 substituent, but displays a C-8 substituent. The latter, however, is not fully mobile and may not be bulky enough. Further studies may address directly this question by running systematic structure-activity relationships with homogenous series of derivatives. A direct comparison of the quinolone-binding sites of NorA and of the J774 ciprofloxacin transporter could also be very instructive.

This first model, however, is not entirely compatible with several observations presented here. We see indeed that moxifloxacin is able to increase the accumulation of ciprofloxacin, even though it is less effective than ciprofloxacin it-self. Based on all data assembled so far, we interpret this effect as demonstrating an impairment of ciprofloxacin efflux by moxifloxacin, which implies that moxifloxacin can be recognized by the ciprofloxacin efflux transporter (we, unfortunately, could not document directly the impairment of ciprofloxacin efflux in the presence of moxifloxacin because of insufficient supply of labeled ciprofloxacin). We need, therefore, to envisage a second model in which moxifloxacin would interact with the ciprofloxacin transporter, and be partially extruded from cells. However, moxifloxacin would then be able to immediately penetrate again in cells thanks to its greater lipophilicity. This is what has been observed in cells overexpressing MRP transporters and challenged with a series of anthracyclines of increasing lipophilicities: a larger cellular accumulation was seen

for derivatives with the faster influx rate (Marbeuf-Gueye *et al.*, 1999). This would explain the apparently puzzling data presented in figure 40, where we see that moxifloxacin leaks out of cells faster than ciprofloxacin and yet accumulates to a larger extent. This model is also in agreement with the observation that the transmembrane flux of these drugs is determined by their lipophilicity (Bermejo *et al.*, 1999; Sun *et al.*, 2002; Fresta *et al.*, 2002). The faster transmembrane movement of moxifloxacin could also be facilitated by the fact that the higher pK_a value of its protonable substituent in position 7 (4a,7a-octahydro-6H-pyrrolo[3,4-b]pyridine) will increase the proportion of the molecule being zwitterionic at neutral pH in comparison with the other quinolones studied here. We know, indeed, that it is the zwitterionic form of quinolones which is the most diffusible one (Furet *et al.*, 1992).

Our results obtained with cells maintained at 4°C also need to be critically examined. Transporter-mediated efflux, indeed, is likely to be impaired at that low temperature since it is a strictly energy-dependent process, as observed here with ATP-depleted cells and in other models of MRP-driven efflux of quinolones (Naruhashi *et al.*, 2003). A higher lipophilicity is probably the main reason for the apparently larger accumulation of garenoxacin and moxifloxacin. Conversely, moxifloxacin and garenoxacin having higher log P and log D values than levofloxacin and ciprofloxacin, are expected to more easily interact and bind to cell membranes. This process is not temperature-dependent, as shown in J774 macrophages with basic, lipophilic derivatives of ampicillin (Chanteux *et al.*, 2003). In this situation, however, the molecules associated to cells are unlikely to be intracellular, and the similarity of accumulation levels seen at 4°C and 37°C for moxifloxacin and garenoxacin should be viewed as coincidental. More detailed studies using membrane models, and membranes isolated from various cell types are, however, needed before more definitive conclusions can be drawn. It must indeed be reminded that the behavior of quinolones at 4°C with respect to cell accumulation seems to be cell-dependent (see, for instance, [Pascual *et al.*, 1999] for diverging results with PMN, in which moxifloxacin accumulation is reduced to 20 % of control values at 4°C).

Moving now to our results obtained with cells exposed to thermal shock (10 min at 56°C), we know from previous studies that the accumulation of quinolones by macrophages is unaffected or even enhanced by loss of cell viability (Easmon and Crane, 1985; Pascual *et al.*, 1999). The new finding made here is that thermal shock largely abolishes the differences in accumulation observed between quinolones. One tentative interpretation about this intriguing phenomenon is that thermal shock has

caused thermal denaturation or unfolding of the transporter protein(s) resulting in their inactivation. This would let cells accumulate a maximal amount of those quinolones that are normally effluxed, and reveal what their maximal accumulation level could be. It is indeed striking that heat-shocked cells accumulate each of the quinolones almost to the same level as seen in ATP-depleted cells or cells exposed to preferential MRP inhibitors. This hypothesis will need to be substantiated by specific studies concentrating on direct measurements of the inactivation of the ciprofloxacin transporter, and the identification of the cell binding sites for quinolones in heat-shocked and ATP-depleted cells.

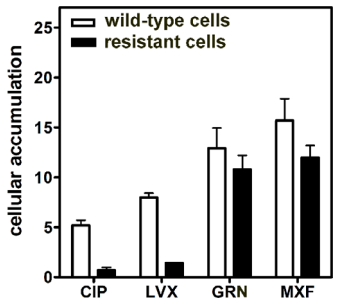
The data presented here have also implications for drug development and evaluation. The fact that the cellular accumulation of ciprofloxacin is suboptimal at 4 mg/L (a clinically meaningful concentration for most quinolones) suggests indeed possible improvements through the development of transport inhibitors. These, indeed, may allow for an enhanced activity towards intracellular bacteria which we know to be directly related to the level of drug accumulation (Carryn *et al.*, 2002; Seral *et al.*, 2003c). This approach has been successfully followed with conventional MRP inhibitors (Rudin *et al.*, 1992; Rispal *et al.*, 1996; Seral *et al.*, 2003a) but will need more specific ones. A limitation, however, could be imposed by the potential toxicities associated with higher cellular and tissular accumulation. Conversely, it may be possible to better screen for derivatives with low or no susceptibility to transport. It is interesting, in this context, to see that while neither garenoxacin nor moxifloxacin are effluxed whatever the extracellular concentration used, levofloxacin is transported but only at low concentrations. It is, therefore, possible that large doses of levofloxacin, creating typically concentrations of 6-7 mg/L or above at which efflux is largely impaired (see figure 38) will result in a greater than anticipated efficacy against intracellular bacteria. It must, however, be reminded that J774 macrophages are only one type of phagocytic cells. PMN, for instance, show no influence of probenecid on the accumulation of levofloxacin (Vazifeh *et al.*, 1999), which is a clear indication that they may behave differently.

III.2.c.4. Additional data: studies with ciprofloxacin-resistant macrophages

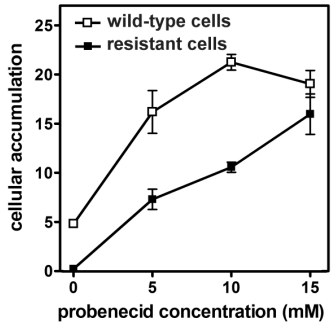
A useful tool for progressing in the identification of the quinolone transporter as well as in the elucidation of its mechanism of action would consist in obtaining a cell line overexpressing this transporter. Transfecting cells with appropriate genes is difficult here because we do not know which MRP-like transporter is involved in the transport. We have therefore applied the same approach as that successfully used previously to select probenecid-resistant cells, and consisting in exposing J774 macrophages for prolonged periods of time to high drug concentrations. After several months of culture in the continuous presence of gradually increasing concentrations of ciprofloxacin, we eventually obtained a stable cell line growing normally in the presence of 68 mg/l of ciprofloxacin.

A phenotypic characterization of these cells suggests that they indeed overexpress the ciprofloxacin transporter (Heremans *et al.*, 2004).

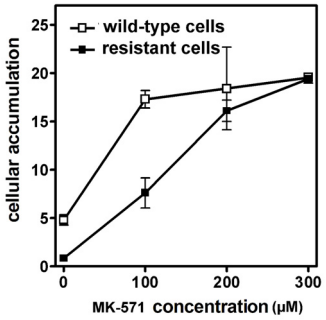
Figure 43: phenotypic characterization of ciprofloxacin-resistant cells
(Heremans *et al.*, 2004)



(a) comparison of the cellular accumulation of quinolones in wild-type and resistant J774 macrophages, after 2 h of incubation with 20 mg/L of each drug.



(b) influence of probenecid on the cellular accumulation of ciprofloxacin in wild-type and resistant J774 macrophages, after 2 h of incubation in the presence of 20 mg/L of ciprofloxacin and increasing concentrations of the inhibitor.



(c) influence of MK-571 on the cellular accumulation of ciprofloxacin in wild-type and resistant J774 macrophages, after 2 h of incubation in the presence of 20 mg/L of ciprofloxacin and increasing concentrations of the inhibitor.

Thus, as illustrated in a summarized fashion in figure 43,

(a) ciprofloxacin accumulation in resistant cells fell to almost zero, while that of levofloxacin was reduced, and that of garenoxacin and moxifloxacin was only barely affected, as compared to accumulation levels reached in wild-type cells.

(b) higher concentrations in probenecid than in wild-type cells were needed to restore a maximal level of accumulation for ciprofloxacin. Thus, a ~16-fold

accumulation factor was reached upon incubation with a probenecid concentration of 5 mM in wild-type cells but of 15 mM in resistant cells.

(c) a similar behavior was observed with the preferential MRP inhibitor MK-571, since three times higher concentrations in inhibitor were needed to obtain a maximal accumulation for ciprofloxacin in resistant cells as compared to wild-type cells.

We then evaluated the consequences of this increased efflux for ciprofloxacin intracellular activity. To this effect, we compared dose-effect relationships in wild-type and resistant macrophages infected by *Listeria monocytogenes* (Heremans *et al.*, 2005). Figure 44 shows that ciprofloxacin alone was unable to control the intracellular infection in resistant cells in a clinically-relevant range of concentrations (upper panel). A static effect was indeed obtained for extracellular concentrations corresponding to 10 X the MIC (20 mg/L) in resistant cells as compared to 2 X MIC (4 mg/L) in wild type cells.

However, as illustrated in the lower panel, ciprofloxacin became almost as active in both cells types when probenecid was added at concentrations selected to bring the antibiotic cellular accumulation to a similar level (5 mM for wild-type cells and 10 mM for resistant cells, based on data from figure 43). In these conditions, a static effect was observed in both cell types when the extracellular concentration was 1 X MIC (2 mg/L).

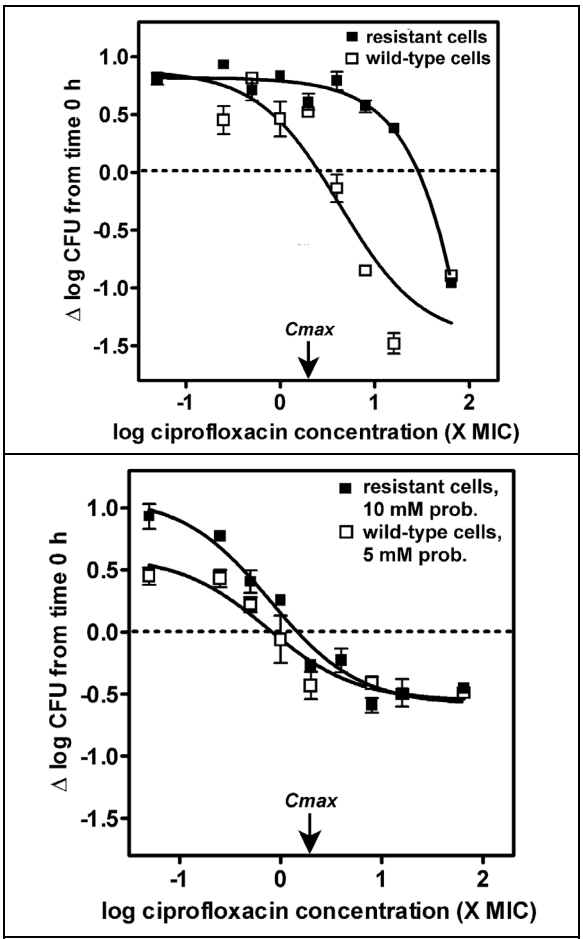


Figure 44: activity of ciprofloxacin against intracellular *L. monocytogenes* after 5 h of incubation of wild-type or ciprofloxacin- resistant J774 macrophages, with increasing concentrations of ciprofloxacin (expressed in multiples of the MIC [with MIC = 2 mg/L]). The arrow points to the human Cmax.

Upper panel:
control conditions (ciprofloxacin alone)

Lower panel:
ciprofloxacin, in combination with probenecid 5 mM (wild-type cells) or 10 mM (resistant cells), so as to obtain similar level of ciprofloxacin accumulation (see figure 43)

(Heremans *et al.*, 2005)

We then evaluated the stability of this resistant phenotype by re-cultivating the resistant cells in the absence of ciprofloxacin, and measuring ciprofloxacin accumulation in the presence or in the absence of probenecid (figure 45).

Examining first ciprofloxacin accumulation in the absence of probenecid, the figure shows that it progressively increased from 0.9-fold to 1.9-fold after 60 passages (this value remaining however significantly lower than that measured in wild-type cells [4.1-fold]).

In the presence of 5 or 10 mM probenecid, ciprofloxacin accumulation became similar to that measured in wild-type cells after 60 and 40 passages, respectively.

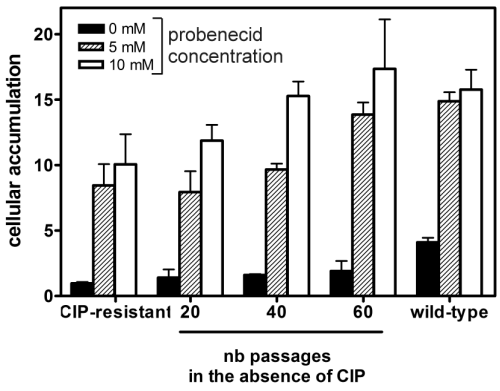


Figure 45: Evaluation of the reversibility of the resistant phenotype. The figure compares the accumulation of ciprofloxacin (extracellular concentration, 20 mg/l; time, 2 h) in control conditions or in the presence of probenecid, for wild-type and ciprofloxacin-resistant macrophages reincubated for 0, 20, 40, or 60 passages in the absence of ciprofloxacin.

(Heremans *et al.*, 2004)

These results therefore suggest that the phenotype is slowly reversible.

III.3. General discussion: molecular characterization of the interaction between antibiotic and efflux pumps.

III.3.a. Comparison between antibiotics

The data presented here show that the behaviors of azithromycin, ciprofloxacin and moxifloxacin are markedly different, as far as their interactions with efflux pumps is concerned. We found indeed that:

(a) **P-glycoprotein** inhibitors increase **azithromycin** accumulation, without affecting its efflux;

(b) **MRP inhibitors** enhance **ciprofloxacin** accumulation and also markedly slow down its efflux; and

(c) **MRP inhibitors** do not influence **moxifloxacin** accumulation or efflux, even though this drug seems capable of interacting with the ciprofloxacin transporter (see figure 41).

To try to explain this set of observations, and elaborate a comprehensive and unifying model for the transport of each of these antibiotics, we will first summarize here the key experimental findings that need to be considered.

a) Transporter involved

Whereas azithromycin specifically interacts with the P-glycoprotein, and quinolones, with MRP, both types of transporters belong to the same ABC superfamily. All these transporters are thought to extrude their substrates more probably from the membrane than from the cytosol (see chapter III.1.b. and figure 46).

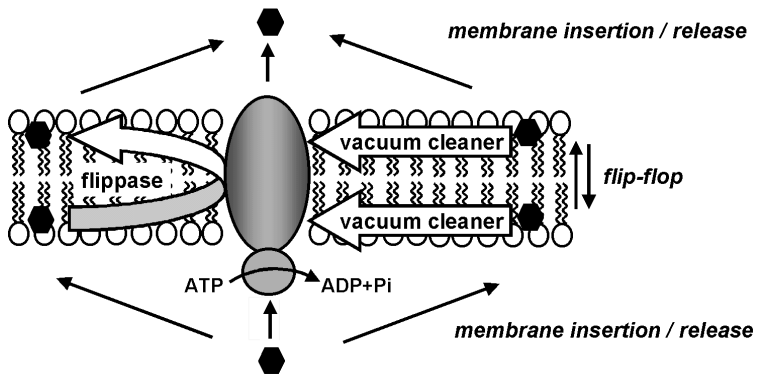


Figure 46: possible routes of multidrug transport.

Drugs may be extruded directly from the membrane (inner or outer leaflet into the extracellular space (vacuum cleaner model) or flipped from the inner to the outer leaflet (flippase model). Direct extrusion from the cytosol cannot be entirely excluded.
Transport is energized by ATP hydrolysis.

adapted from (Kolaczkowski and Goffeau, 1997)

This mechanism implies that each drug interacts with the membrane, and that its presence in the vicinity of the transporter in the lipidic environment is critical. All three drugs fulfill this requirement, based on their amphiphilic character.

b) Subcellular localization of the drugs

While quinolones are systematically recovered in the soluble fraction of cell homogenates, azithromycin is located for 1/3 of its total cell content in the cytosol, with the remaining being sequestered in lysosomes. This means that only a small proportion of the cell-associated azithromycin will be at any time in close contact with the pericellular membrane, and therefore, with the P-glycoprotein.

c) Accumulation at 4°C

Low temperature markedly impairs diffusion of drugs through the membrane and also blocks active transport. As shown in table 12, lowering the incubation temperature makes azithromycin accumulation undetectable, severely reduces that of ciprofloxacin, but only slightly affected that of moxifloxacin.

Table 12: comparison of residual accumulation at 4°C for three antibiotics interacting in different ways with efflux pumps

data from (Chanteux *et al.*, 2003; Michot *et al.*, 2005)

antibiotic	Residual accumulation at 4°C (% of value measured at 37°C at equilibrium
azithromycin	1 %
ciprofloxacin	26 %
moxifloxacin	73 %

This may suggest that moxifloxacin either is very diffusible, so that it can cross a rigid membrane. One could also argue that moxifloxacin actually never truly penetrates cells, but remains associated with the cell surface, as proposed for prodrugs of β-lactams (Chanteux *et al.*, 2003). The latter interpretation is, however, rather inconsistent with the fact that moxifloxacin is active against intracellular *L. monocytogenes* and *S. aureus* (Carryn *et al.*, 2002; Seral *et al.*, 2003c). This indicates that part of the drug at least must be truly intracellular. We do not know however whether moxifloxacin intracellular disposition is the same at 37°C (i.e. when examining its activity against intracellular bacteria) and 4°C.

d) Kinetics of influx and efflux

The most critical point of comparison comes from the analysis of the kinetic parameters of uptake and release for the three drugs, and the determination of their respective in-out or out-in fluxes, and of their half-lives in the absence or in the presence of appropriate efflux pump inhibitors. These data are presented in table 13.

Table 13: kinetic parameters for influx and efflux of three antibiotics interacting in different ways with efflux pumps

calculated from data of M. Heremans, J.M.Michot, and C. Seral

drug	influx			efflux		
	flux (pmol/mg prot/min)[a]	half-life (min)[b]		flux (pmol/mg prot/min)[a]	half-life (min)[b]	
		control	inhibitor[c]		control	inhibitor[c]
azithromycin	1	44	71	1	49	53
ciprofloxacin	5	8	6	6	1.2	7.2
moxifloxacin	68	0.2	0.2	66	0.6	0.6

[a] calculated from data obtained after 60 min for azithromycin and 1 min for ciprofloxacin and moxifloxacin, and normalized to an extracellular of 1 µM for all drugs (for ciprofloxacin, influx value therefore corresponds to the first, rapid phase of uptake; see figure 37).

[b] calculated from data obtained on kinetic experiments performed over 2 h for quinolones and 24 h for azithromycin, and considering a one-phase association process)

[c] inhibitor is verapamil for azithromycin, and probenecid for quinolones.

- For azithromycin, we observe that the half-lives are, in all circumstances, rather long (close to 1 h), and that the flux of the drug through the membrane is the same in both directions (these calculations refer to short-term kinetic studies, i.e. under conditions where there is no phospholipid accumulation, which would have slowed down drug efflux).
- For ciprofloxacin, we see that (i) the rate of accumulation is much faster than that of azithromycin, (ii) efflux is quicker than influx (but the addition of probenecid brings the half-life of efflux close to that of influx), and (iii) the molar fluxes are 6-fold higher than for azithromycin.
- For moxifloxacin, we notice that half-lives are considerably much shorter (< 1 min) than for ciprofloxacin or azithromycin. The molar fluxes of moxifloxacin are, therefore, much higher. Moxifloxacin efflux is not influenced by probenecid. Quite interestingly, and as for azithromycin, fluxes are equal in both directions.

III.3.b. Tentative model for the interaction between antibiotics and efflux pumps

On these bases, we propose the following models to explain the contrasting behaviors of the three antibiotics, while at the same time, keeping a unified view (figure 47).

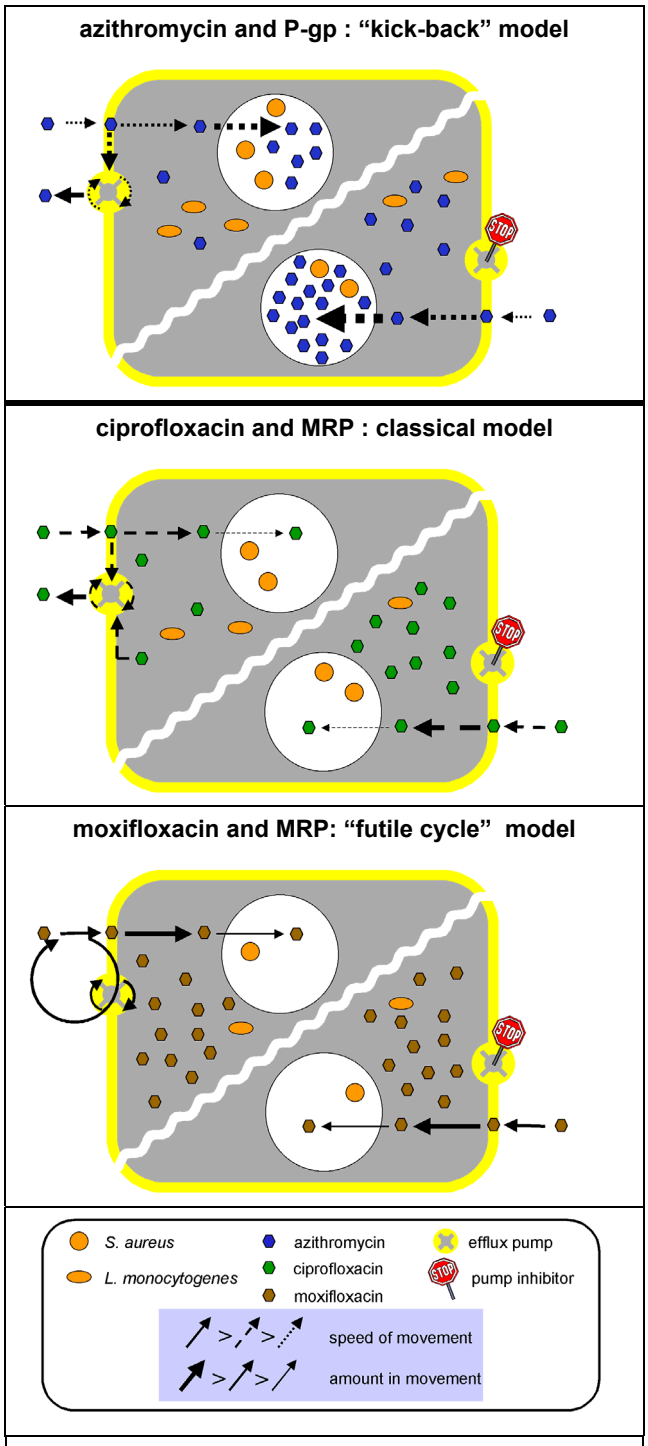


Figure 47: tentative model of the interaction of azithromycin, ciprofloxacin, and moxifloxacin with efflux pumps. The left and right parts of each cartoon reflect the situation in the absence and in the presence of inhibitor of efflux pump.

- **Azithromycin** probably crosses slowly the membrane, suggesting that it may be present in the membrane environment for a non-negligible time. It could, therefore, easily be sucked by the P-glycoprotein from the membrane (as proposed in the vacuum cleaner model), before reaching the cytosol. This would explain why the pump affects azithromycin accumulation but not its efflux, in accordance with the "kick-back" model proposed by Gaj and coworkers for vincristine in carcinoma cells (Gaj *et al.*, 1998).
- The **ciprofloxacin** kinetic parameters suggest that this drug would cross the membrane more quickly than azithromycin. In this case, the drug could access the pump both from the membrane and from the immediately adjacent zone in the cytosol. This would correspond to a classical mode of transport (Kolaczkowski and Goffeau, 1997), where both accumulation and efflux are affected by inhibitors.
- **Moxifloxacin** is characterized by an exceptionally rapid association and release from the cells. A first hypothesis is that it does not interact with the transporter at all, but simply diffuses in and out of the cells thanks to its greater lipophilicity. This hypothesis, however, is inconsistent with the fact that logP and logD values of moxifloxacin are higher than those of azithromycin (see figures 20 and 27), and that it inhibits the transport of ciprofloxacin (figure 41). We propose that moxifloxacin actually behaves like pump inhibitors, which are swept along in futile cycles without suffering any perturbation of their own cellular accumulation or efflux kinetics. This model has been proposed for P-glycoprotein by Eytan *et al.*, 1996, who compared the kinetics of transbilayer movement of substrates and inhibitors and concluded that molecules with a sufficiently prolonged half-life in the membrane ($\geq$ 3 min) are transported by the pump (and could therefore be considered as substrates), whereas those with a shorter half-life will re-enter cells faster than the pump can act, and will not be influenced by inhibitors. These compounds will nevertheless occupy the pump, so that they will decrease the efflux of substrates.

III.3.c. Perspectives for future research

The data obtained so far in the field of antibiotic efflux pumps raise numerous questions, which could orient our future research in the following three main directions.

III.3.c.1. Identifying the quinolone transporter

Based on phenotypic characters, we have tentatively identified the ciprofloxacin-transporter as belonging to the MRP family. This family counts at least 9 members (Haimeur *et al.*, 2004), so that a systematic search of the protein involved using antibodies would be fastidious and uncertain (because we cannot exclude that the transporter involved is none of these 9 proteins). More global approaches need therefore to be considered, like proteomics or microarrays, which have proven useful to unravel the mechanism of chemoresistance in cancer cells (Hutter and Sinha, 2001; Sinha *et al.*, 2003; Annereau *et al.*, 2004).

At the present time, we are planning to compare the profile of protein expression in wild-type and ciprofloxacin-resistant macrophages by means on two-dimensional electrophoresis, and are currently developing protocols for enriching samples in transmembrane proteins (Caceres *et al.*, 2005). Identification of the protein(s) of interest would be facilitated using photoaffinity-labeling with a photoactivable derivative of ciprofloxacin. The photolabeling of MRP by quinolines has indeed already proven successful (Daoud *et al.*, 2000).

III.3.c.2. Understanding the mechanisms of transport

The model proposed for explaining antibiotic transport by efflux pumps insists on the importance of their capacity to interact with the pericellular membrane. Further studies could therefore examine the molecular interaction of these antibiotics with the pump and with the membrane.

a) Studies with planar bilayers

Our models point to probable differences in the speed at which the antibiotics cross the membrane. Comparing drug fluxes through planar bilayers (Steinem *et al.*, 1996) would be informative in this respect (Amblard *et al.*, 1985; Cuello *et al.*, 1998).

b) Studies on proteoliposomes

Reconstituted vesicles are probably the most widely used model for studying drug transport mechanisms involving efflux pumps (see [Munoz-Martinez *et al.*, 2004; Rotem and Schuldiner, 2004; Pochini *et al.*, 2004] for a few recent examples). Applying the procedure described for producing proteoliposomes from membranes of cells overexpressing MRP1 (Muller *et al.*, 1994), we have therefore started to prepare vesicles from membranes of wild-type macrophages as well as from ciprofloxacin-resistant cells (N. Caceres, FNRS post-doctoral fellow). We plan to use these models in the future for:

- studying the binding of antibiotics to membranes (Montenez *et al.*, 1999; Mingeot-Leclercq *et al.*, 2001; Chanteux *et al.*, 2003 for examples) ;
- measuring antibiotic transport, as described for vesicles enriched in P-glycoprotein or MRP (Bebawy *et al.*, 1999; Mao *et al.*, 2000) ;
- determining substrate selectivity of transporters, by means of competition experiments (Mao *et al.*, 2000);
- analyzing biophysical membrane properties: the activity of membrane-embedded proteins like efflux pumps is modulated by their environment, and in particular by the biophysical properties of the membrane (see for reviews [Pallares-Trujillo *et al.*, 2000; Hendrich and Michalak, 2003]). It is very well known today that many drugs are able to disturb membrane properties that are critical for their function of physiological barrier (see [Seydel and Wiese, 2002] for review). In the context of the present study, we could therefore examine whether the penetration of antibiotic substrates in the membrane is capable of perturbing the physiological functions of the pumps. In particular, their translocase activity (flip-flop of lipid from one leaflet of the membrane to the other) is involved in the maintenance of membrane asymmetry (unequal composition of the two leaflets; [Kamp and Haest, 1998; Romsicki and Sharom, 2001]) as well as in the regulation of cholesterol trafficking (Garrigues *et al.*, 2002a).

c) Studies "in silico"

Obtaining structural data for membrane-embedded proteins is a difficult task, explaining why there is no detailed three-dimensional structure of multidrug ABC transporters available so far (only low resolution electron microscopic structures for P-glycoprotein reconstituted in a lipid bilayer is available [Lee *et al.*, 2002]). More detailed structure would be a significant advance to help elucidating the molecular mechanism for substrate binding and release, as well as to explore the drug binding

sites. With our coworkers at the center for *Bioinformatique génomique et structurale at* the *Université libre de Bruxelles* (M. Prévost), we have been constructing a three-dimensional structure model of P-glycoprotein (figure 48), by combining computational techniques including comparative modeling and rigid body dynamics simulations (Vandevuer *et al.*, 2005).

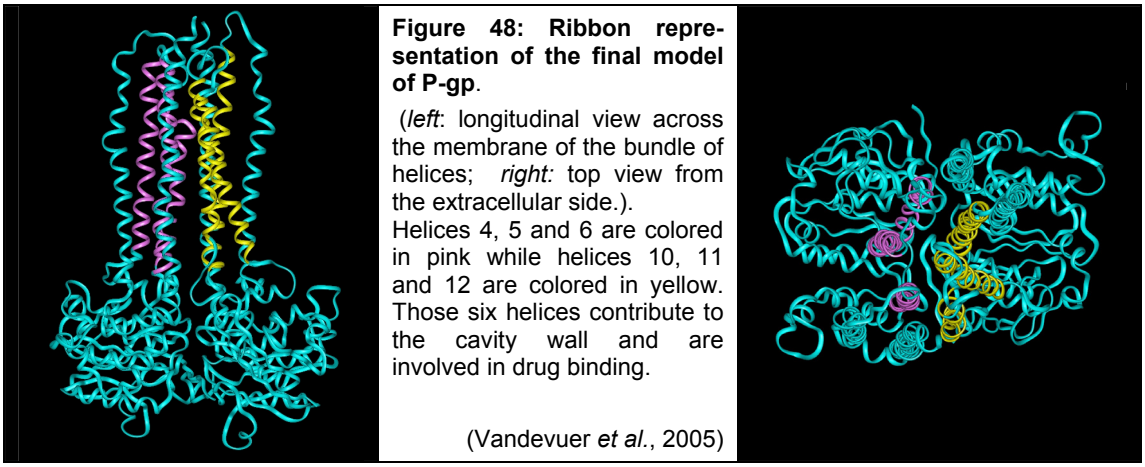


Figure 48: Ribbon representation of the final model of P-gp.

(*left*: longitudinal view across the membrane of the bundle of helices; *right:* top view from the extracellular side.).
Helices 4, 5 and 6 are colored in pink while helices 10, 11 and 12 are colored in yellow. Those six helices contribute to the cavity wall and are involved in drug binding.

(Vandevuer *et al.*, 2005)

The model is in good agreement with available structural experimental data and will now be used for docking of antibiotic substrates. The same approach will now be applied to MRP.

III.3.c.3. Evaluating the pharmacological consequences of active efflux

a) Models of infection

We have shown that the intracellular activity of azithromycin (against *S. aureus* and *L. monocytogenes*) and of ciprofloxacin (against *L. monocytogenes*) was suboptimal in wild-type J774 macrophages due to the constitutive activity of efflux pumps (Seral *et al.*, 2003a), and that the efficacy of ciprofloxacin was drastically reduced in ciprofloxacin-resistant macrophages (Heremans *et al.*, 2005). But we also know that efflux pumps expressed by bacteria reduce antibiotic efficacy, by limiting the drug concentration coming in contact with the pharmacological target (see chapter III.1.d.3 for further details).

We do not know, however, whether these bacterial pumps are expressed inside eukaryotic cells, nor whether they could cooperate with the pumps expressed by macrophages. This question could be answered by developing models of intracellular infections by *L. monocytogenes* or *S. aureus* using susceptible strains or strains resistant by efflux mechanisms (Yoshida *et al.*, 1990; Godreuil *et al.*, 2003),

and testing in these models the activity of antibiotics in combination with inhibitors of eukaryotic or prokaryotic pumps.

b) Drug interactions

Antibiotics have been shown to be substrates of efflux pumps or to inhibit the efflux of chemotherapeutic agents in resistant cancer cells (Gollapudi *et al.*, 1995; Courtois *et al.*, 1999; Terashi *et al.*, 2000). We want therefore to use our resistant cells and ask the reverse question: are these cells capable of transporting drugs from other classes ? The selection of a resistance phenotype upon exposure to antibiotics can indeed not be excluded *in vivo*, principally in patients receiving prolonged or frequent treatments for chronic infections (tuberculosis, e.g. [Veziris *et al.*, 2003]) or because of deficiency of their immune system (patients suffering from AIDS or cancer or receiving immunosuppressant drugs [Desimone, Jr. *et al.*, 2003; Vidal *et al.*, 2004]). In this respect, it is worthwhile to mention that several drugs administered to this type of patients are known to be substrates for efflux pumps (anticancer agents like anthracyclines [Marbeuf-Gueye *et al.*, 1998], anti-HIV like protease or reverse transcriptase inhibitors [Jorajuria *et al.*, 2004]).

c) Metabolic alterations

The mode of selection used to obtain ciprofloxacin-resistant cells is prone to cause multiple changes in cellular physiology, as usually observed with the mode of selection applied here (see [Gottesman *et al.*, 1998] for review, and [Larsen *et al.*, 2000; Hutter and Sinha, 2001; Leonessa and Clarke, 2003 for examples in cells exposed to anti-cancer agents).

The proteomic approach we are currently developing could be useful to identify such modifications in protein expression and try to establish links with the resistant phenotype. Potential targets include enzymes involved in glutathione synthesis or in conjugation reactions (Liu *et al.*, 2001; Annereau *et al.*, 2004) or proteins involved in cell death by apoptosis (Leonessa and Clarke, 2003), for which alterations in expression levels are detected in resistant cell lines, as well as topoisomerases (Marchal *et al.*, 1999) or proteins involved in stress response (Eriksson *et al.*, 2003), which have been found affected upon exposure to quinolones.

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IV. General conclusion: Studying the cellular fate of antibiotics does really matter

In this Thesis, we have used three antibiotic classes to study their cellular pharmacokinetics and to examine consequences for activity. These classes were selected on the basis of (i) their typical properties and characteristics as far as interactions with eukaryotic cells is concerned; (ii) their potential clinical interest in a world of increased bacterial resistance to antibiotics. We therefore hope that our work may bring important pieces of information, not only for the understanding of the mechanisms governing the specific properties of these drugs, but also for contributing to the progress of anti-infective pharmacology in a broad context.

IV.1. Implications for PK/PD studies

IV.1.a. Pharmacokinetics

In the present work, we have focused our efforts on the deciphering of the fate of antibiotics in phagocytic cells, which are primarily involved in the defense of the organism against infective agents. We have shown the usefulness of cultured cells for studying the molecular mechanisms responsible for drug uptake, distribution, or efflux, as well as for correlating pharmacokinetic to pharmacodynamic data and delineating potential relationships between the cellular disposition and the intracellular activity of antibiotics. But the interest of knowing antibiotic pharmacokinetics in phagocytic cells goes beyond the study of their activity in the infected cells them-selves. Phagocytic cells, which are abundant in infected foci, and in charge of destroying bacteria extracellularly by secreting bactericidal molecules, or intracellularly after their phagocytosis ([Dieffenbach and Tramont, 2005] for a recent review), may also act as a reservoir of antibiotics (Schentag and Ballow, 1991). In that sense, intracellular drugs are only part of the whole armamentarium that can be used against invading organisms.

Epithelial cells constitute another relevant model, which has not been explored in this Thesis. However, by their location at the surface of the main barriers in the body, they undoubtedly play a major role in the regulation of the general pharmacocokinetics of the drugs.

Combining data get in both polarized and non-polarized cells would therefore provide clues for the understanding of the molecular mechanisms involved in the following processes, as examined here.

- Drug absorption: it results from a combination of drug-related factors (diffusibility through the membranes), milieu-related factors (influence of extracellular pH, or presence of degradative enzymes), and cell-related factors (presence of active transporters or of cellular metabolic enzymes), which can be easily assessed in cell culture models. For instance (figure 1), the interplay between these factors has been recently exemplified in our laboratory by elucidating, in a model of intestinal cells, how pivampicillin, a prodrug of ampicillin, can deliver the active drug to the general circulation (Chanteux *et al.*, 2003; Chanteux *et al.*, 2005).

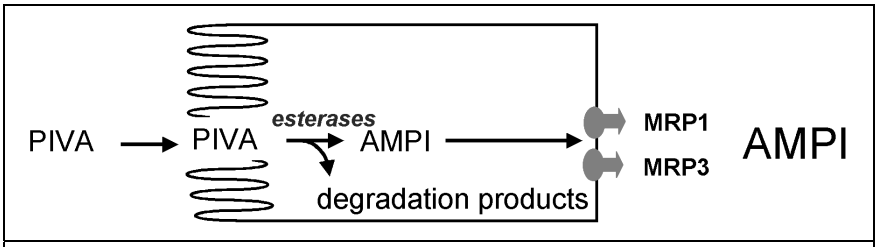

Figure 1: current view of the mechanism by which pivampicillin (PIVA) delivers ampicillin (AMPI) to the blood, as assessed in a cellular model of the intestinal barrier. PIVA is trapped in the apical membrane of the cells, from where is slowly releases AMPI (thanks to the presence of cytosolic esterases). AMPI is poorly diffusible, but is preferentially transported through the basolateral pole of the cells thanks to the presence of active transporters.

constructed from the data of (Chanteux *et al.*, 2005)

- Drug distribution: the demonstration of a marked cellular retention can predict, to some extent, general pharmacokinetic parameters, like V_d (distribution volume) or $t_{1/2}$ (half-life). The extrapolation is, however, not immediate, since other parameters, like protein binding, need also to be taken into account. Table 1 illustrates this correlation for typical representatives of the main antibiotic classes.

Table 1: comparison of the cellular accumulation and of key pharmacokinetic properties for antibiotics representative of the main classes

class	antibiotic	cellular accumulation [a]	pharmacokinetic parameters [b]		
			V_d (L/kg)	$t_{½}$ (h)	protein binding (%)
β-lactams	ampicillin	< 1	0.15	1	17
aminoglycosides	gentamicin	2-4	0.25	2	3
quinolones	moxifloxacin	10	2	12	40
macrolides	azithromycin	40-300	31	68	20
glycopeptides	oritavancin	150-300	10 [c,d]	360	90

[a] data from table 2 in chapter I

[b] data from package inserts of the corresponding drug or from table 3 in chapter III for oritavancin

[c] value not available; calculated as Cl/k, with Cl = dosis / AUC and k = ln2 / $t_{½}$), see table 3 in chapter III for these values

[d] this value should be 10 times higher if considering that probably only the free fraction can largely distribute; see discussion III.2.a.3)

- Drug elimination: it is now recognized as resulting from the chemical (metabolism; phases I and II) and physical (efflux; phase III) mechanisms of clearance and from the interplay between them (see figure 18 of chapter III), which occur at both the levels of the intestine and the liver. Such a cooperation can be envisaged only in models of intact cells, which should progressively replace microsomes in the *ex vivo* assessment of drug elimination processes (see [Li, 2004; Benet *et al.*, 2004] for general concepts and [Lan *et al.*, 2000] for an example with erythromycin).

IV.1.b. Pharmacodynamics

Studying in parallel the extracellular and intracellular activity of antibiotics probably offers a more complete view of what could be their efficacy *in vivo*.

Although most infections are extracellular, we cannot ignore the clinical importance of some obligate or facultative intracellular bacteria, which are the causative agents of frequent or life-threatening human pathologies (table 2).

Table 2: main intracellular bacteria with predominant target cells in humans and known subcellular localization of virulent forms

(Carryn *et al.*, 2003)

organism	type of parasite	target cells	subcellular localization
Brucella spp	facultative	macrophages	phagosomes
Chlamydia spp.	obligate	lung parenchyma cells	inclusions
Coxiella brunetii	obligate	macrophages, lung parenchyma cells	phagosomes, phagolysosomes
Francisella tularensis	facultative	macrophages	phagosomes
Legionella pneumophila	facultative	macrophages	endoplasmic reticulum, lysosomes
Listeria monocytogenes	facultative	macrophages, hepatocytes	cytosol
Mycobacterium tuberculosis	facultative	macrophages	early endosomes
Rickettsia spp.	obligate	endothelial cells	cytosol
Salmonella spp.	facultative	macrophages	phagosomes
Shigella flexeneri	facultative	macrophages	cytosol

But the issue of intracellular infection is probably even broader. Typical extracellular bacteria, which are normally destroyed after phagocytosis by macrophages or polymorphonuclear neutrophils, may, actually, survive in these cells. This has been demonstrated for example with common pathogens like *S. aureus* (Hamill *et al.*, 1986; Lowy, 2000; von Eiff *et al.*, 2001; Ellington *et al.*, 2003), streptococci (Segura *et al.*, 1998; Cornacchione *et al.*, 1998), *E. coli* (Anderson *et al.*,

2003; Anderson *et al.*, 2004), or enteroccci (Gentry-Weeks *et al.*, 1999; Verneuil *et al.*, 2004), and associated to the recurrent character of many of the infections they cause.

A combined evaluation of the extra- and intracellular activity of antibiotics may therefore appear as an appropriate screening tool for optimizing antibiotic choices. This is illustrated for *S. aureus* in figure 2*,* which suggests that quinolones, oritavancin, and oxacillin would be bactericidal against both extra- and intracellular bacteria, while macrolides and linezolid would be always static, and the other drugs markedly cidal against the extracellular forms only. Such models, which could be generalized to other pathogens of interest, pave the way for designing studies in *in vivo* models of intracellular infections.

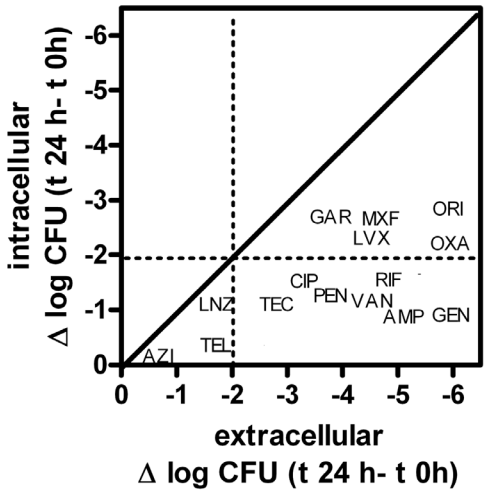

Figure 2: plot of the intracellular *vs* intracellular activity of a series of antibiotics against *S. aureus.*

The diagonal corresponds to equivalence of activity against both forms of infection. The dotted lines point to a bactericidal effect (at least 2 log reduction in the inoculum over a 24 h exposure at concentrations mimicking the human Cmax).

constructed from data of (Barcia-Macay *et al.*, 2005)

These could then lead to the more rational design of clinical studies aiming at selecting the most appropriate drug or the most effective mode and scheme of administration. It would also help the clinician to gain a more objective view of which properties of the antibiotics are important for acting against intracellular bacteria.

IV.1.c. Toxicodynamics

Safety issues are probably more of concern today that in the past, because we have a large drug armamentarium already available, and can therefore select for future development only those molecules that prove superior to the previous ones. Evidencing very early on toxic effects is therefore an advantage. Animal models are however laborious and expensive, so that cellular models would be welcome. Such models may indeed serve as “alarm flags”, orientating further *in vivo* studies. This reasoning would certainly hold true for oritavancin, based on the alterations observed here. Likewise, if it could have been applied in the past, it could have helped in better avoiding human toxicity, which was recognized as early as in the mid-60's (Greenberg and Momary, 1965), *i.e*. before it was studied in animals (Gross *et al.*, 1969), and characterized in cultured cells (Aubert-Tulkens *et al.*, 1979).

A key feature of cellular models is that they lend themselves to the study of the mechanisms of toxicity (as clearly illustrated earlier in our laboratory for the renal toxicity of aminoglycosides [Mingeot-Leclercq and Tulkens, 1999 for review]). It is therefore not a surprise that 25 years after the first world workshop on the application of tissue culture in toxicology (Zucco, 1980), the interest of these models has become indubitable (Zucco *et al.*, 2004) and has led to the development of up-to-date techniques for the early screening of common drug-associated cellular toxicities like phospholipidosis (Sawada *et al.*, 2005) or apoptosis (Nguyen and Daugherty, 2005). Here again, these studies may help in designing appropriate animal and clinical studies aiming at delineating safer conditions for clinical usage. The example of aminoglycosides illustrates well this approach, but our present work shows that it is not specific to this class of drugs and that it may be useful and even essential for all antibiotics with significant cellular accumulation.

IV.2. Perspectives for the development of new drugs

The data generated in this Thesis may also highlight some critical drug properties that would need to be taken into account in the development of new drugs.

IV.2.a. Cellular accumulation

Cellular accumulation can be the best and the worse of the properties for an antibiotic. We show indeed, with oritavancin, that positive and negative effects are not easily dissociated. This eventually may lead to selecting drugs with moderate accumulation and retention (as exemplified by the decision of the inventors of ketolides who selected telithromycin for clinical development and application to registration, rather than other derivatives in this series that showed much larger cell uptake and intracellular half-lives [Vazifeh *et al.*, 1997; Vazifeh *et al.*, 1998]). Evaluating the toxicological consequences of a high cellular accumulation in cultured cells at an early stage would certainly reduce times and costs while increasing the number of compounds that could be examined and, therefore, improving the overall discovery process. This should encourage pharmaceutical companies to introduce these models in their routine procedures, but would need standardization, optimization and, whenever possible, automation and miniaturization (Zucco *et al.*, 2004).

On the other hand, we have seen that cellular accumulation is not necessarily predictive of intracellular activity, suggesting the importance of testing intracellular activity systematically, and without *a priori,* for antibiotics showing high as well as low levels of cellular accumulation. This also calls for the use and further development of appropriate models as those we have presented here. The challenge will be to assess drugs under conditions that will be predictive of what really takes place *in vivo*. This may seem obvious and rather easy for intracellular parasites, but surprises may nevertheless occur (as demonstrated in our laboratory (i) for ertapenem, which, contrary to all expectations, proved useless against intraphagocytic *Listeria monocytogenes* under conditions in which other penems are active [Lemaire *et al.*, 2005], and (ii) for moxifloxacin, which proved to be one of the best agent against the intracellular forms of this bacteria [Carryn *et al.*, 2002]). The situation may be more complex for opportunist organisms, since intracellular survival is more linked to persistence and potential emergence of resistance that to the pathogenic process it-self. This clearly calls for further developments in this area, and these could in turn lead to a more rational clinical evaluation of the real interest of new drugs in this context.

IV.2.b. Active efflux

Active efflux by multidrug transporters should nowadays be considered as one of the major modulators of drug pharmacokinetics, and therefore be examined in details for new drugs.

At the level of the whole organism, it contributes to unexpected pharmacokinetic profiles or drug interactions (Tsuji, 2002; Chan *et al.*, 2004; Fischer *et al.*, 2005). At the level of a single cell, it lowers drug cellular accumulation, with the consequences we have seen for antibiotic intracellular activity. In a broader context, we may also fear other deleterious consequences of the presence of these multidrug transporters, in particular

- the risk of facilitating selection of resistance in intracellular pathogens by exposing them to suboptimal concentrations (as demonstrated for extracellular pathogens [Lomovskaya *et al.*, 1999]);
- the possibility of competition for transport with other substrates of efflux pumps acting on intracellular targets, like antiviral or anticancer agents (Marbeuf-Gueye *et al.*, 1998; Ford *et al.*, 2004; Jorajuria *et al.*, 2004).

Another issue regarding these multidrug transporters concerns inter-individual genetic variation, which can alter their level of expression and their functionality, as demonstrated for P-glycoprotein or MRP (Saito *et al.*, 2002; Pauli-Magnus and Kroetz, 2004).

For all these reasons, the large scale implementation of *in vitro* and cell culture models would be helpful for the high throughput screening of drug active transport, the determination of the localization of transporters, the examination of their substrate specificity, or the influence of genetic polymorphism on their activity (Zhang *et al.*, 2003).

IV.3. Concluding remarks

Coming now to the end, I hope that this Thesis would have brought a modest contribution to the elucidation of the role that host cells might play in the journey of antibiotics to their targets.

"lock and key" principle outlined on a cupboard by Ehrlich's own hand

Like this draft of the "magic bullet" theory, may it offer some "food for thought" for the evaluation of drug PK-PD properties, and contribute in that way in a more efficient development of new molecules, as well as in the more rational and effective use of the drugs we already have. Beyond the Ehrlich's concept, indeed, the magic bullet must be a weapon that keeps and expresses its activity. This is probably the most difficult challenge that antibacterial chemotherapy will face in this XXIst century.

IV.4.References

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The "magic bullet" theory stated by Paul Ehrlich more than one century ago foresees that drugs need to reach their target to exert their pharmacological activity. When dealing with antibiotics, this implies not only a capacity to interact with bacteria, but also to rejoin them in the infected compartment. The latter property is directly linked to pharmacokinetics.

In this Thesis, we have focused our interest on cellular pharmacokinetics of antibiotics in macrophages, reasoning that (i) this model would be relevant for predicting the access of antibiotics to intracellular bacteria, and (ii) deciphering mechanisms of drug entry, distribution, and efflux in single cells would provide pieces of information applicable to the understanding of general pharmaco-kinetics.

Accumulation and distribution were studied for oritavancin, a semi-synthetic glycopeptide currently in phase III of clinical development. This molecule is characterized by a highly bactericidal activity against staphylococci, including multiresistant strains, which would be of interest for the eradication of the intracellular forms of these bacteria. We show that oritavancin enters macrophages by adsorptive endocytosis and accumulates to very large amounts in lysosomes, with cellular concentrations as high as 300-fold the extracellular ones. Accordingly, it exerts a concentration-dependent bactericidal activity against *Staphylococcus aureus* multiplying in phagolysosomes. In parallel, however, it also causes morphological and biochemical alterations, characterized by the deposition of material of heterogeneous aspect and the accumulation of phospholipids and cholesterol. These data point to the difficulty of dissociating high cellular accumulation and cellular toxicity and plead for the interest of *in vitro* models in the evaluation of the intracellular activity of antibiotics, the early assessment of drug safety profile and the orientation of further *in vivo* studies.

Active efflux was evaluated for macrolides and quinolones, two antibiotic classes accumulating in macrophages and therefore usually considered as useful in the treatment intracellular infections. Macrophages indeed express multidrug transporters in their pericellular membrane, which can extrude a large variety of drugs presenting as common feature an amphiphatic character. We show that macrolides (and azithromycin in particular) are substrates for P-glycoprotein, while quinolones (and ciprofloxacin in particular) are substrates for an MRP-like transporter. Inhibiting these pumps increases the intracellular activity of these antibiotics by enhancing their cellular accumulation. We also describe different mode of transport for these antibiotics, which essentially reflect their variable fluxes through the membrane. These data underline the major role constitutive efflux plays in the modulation of the pharmacokinetics of drugs, and, hence, of their pharmacodynamics. They encourage the setting-up of large-scale screenings aimed at evaluating drug-transporter interactions in lead optimization processes.

We may therefore conclude to the usefulness of cellular models in the study of the pharmacokinetics of drugs and of its consequences for pharmacodynamics and toxicity, and suggest the implementation of such models in the early development of new drugs.