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Activity of antibiotics towards extracellular and intracellular *Staphylococcus aureus*: Pharmacodynamic studies *in vitro* using a model of human THP-1 macrophages

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ABSTRACT

S. aureus is responsible for persistent and recurrent infections, where intracellular forms of the bacteria may play a central role. In the present study, we have developed an *in vitro* model of intracellular infection of THP-1 macrophages by *S. aureus* over 24 h, in an attempt to delineate the cellular pharmacokinetic and pharmacodynamic parameters governing the intracellular activity of antibiotics.

We first compared 16 antibiotics of seven different pharmacological classes in terms of intracellular accumulation and activity towards the extracellular and intracellular forms of a fully susceptible strain of *S. aureus.* We showed that there is no direct correlation between the intracellular activity of a drug and its capacity (a) to accumulate in macrophages or (b) to exert intracellular activity. Pharmacological comparisons showed that the intracellular E_{max} was systematically lower than the extracellular one, and that intracellular potencies are either similar or lower than the extracellular ones. At an extracellular concentration corresponding to their Cmax (total drug) in humans, only oxacillin, quinolones (levofloxacin, garenoxacin, moxifloxacin), and new hemi-synthetic glycopeptides (oritavancin and telavancin) had truly intracellular bactericidal effects.

On these bases, we compared the intracellular activity of telavancin against different strains of *S. aureus* with well-defined resistance mechanisms (MSSA [methicillin-susceptible *S. aureus*), MRSA [Methicillin–resistant *S. aureus*)], VISA [Vancomycin-intermediate *S. aureus*], and VRSA [Vancomycin-resistant *S. aureus*]). Telavancin showed time- and concentration-dependent bactericidal activity against both extracellular and intracellular *S. aureus* whatever their resistance phenotypes. Intracellularly, the bactericidal activity of telavancin was less intense than extracellularly. A bimodal relationship with respect to concentration was observed for MSSA and MRSA both extra- and intracellularly, which could indicate multiple mechanisms of action for this drug. In parallel, we demonstrated that telavancin accumulates to high levels in the lysosomal compartment of macrophages, probably by a mechanism of adsorptive endocytosis. This high cellular concentration, however, is not accompanied by the accumulation of phospholipids and cholesterol observed previously for another hemi-synthetic glycopeptide, oritavancin.

The model developed here therefore appears useful for the early evaluation of the activity of antibiotics towards the intracellular forms of *S. aureus* and the determination of the cellular pharmacokinetic and pharmacodynamic parameters which may affect their activity.

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AIM OF THE THESIS

S. aureus is an opportunistic and also a highly pathogenic bacterium, able to cause life-threatening infections not only in the hospital environment but also in the community. In fact, after 60 years of continuous antibiotic battling against it, this bacterium has been reestablished as a very dangerous pathogen. In general, *staphylococcal* infections share a common problem: the choice of the optimal antibiotic treatment to eradication. This is further complicated by

(a) the ability of the bacteria to develop or acquire many mechanisms of resistance, including the horizontal acquisition of foreign genetic material

(b) its ability to survive inside the host cells (both phagocytic and non phagocytic), causing persistent or recurrent infections.

The first aim of the thesis was to **develop an intracellular model of infection** by *S. aureus* using human macrophages. We selected macrophages cells because of their relevant long-life span and importance in fighting of infection.

We then used this model

- to study and compare the intracellular activity of antibiotics (molecules currently used for the treatment of *S. aureus* infections as well as molecules in development)
- to delineate the cellular pharmacokinetic and pharmacodynamic parameters governing this activity.

To be able to compare all antibiotic classes in a single model, we selected a wild-type strain of *S. aureus* characterized by the absence of resistance mechanisms. The drugs were examined in a pharmacodynamic perspective, meaning that we systematically explored the influence of the time and of the concentration on their activity.

The second aim of the thesis consisted in examining in details the **cellular pharmacodynamics and pharmacokinetics of telavancin**, a promising glycopeptide in development.

To this effect, we extended our model to multiresistant strains and examined the accumulation and distribution of this glycopeptide within eukaryotic cells (both phagocytic and non phagocytic).

INTRODUCTION

1. STAPHYLOCOCCUS AUREUS:

1.1.Taxonomy and biology

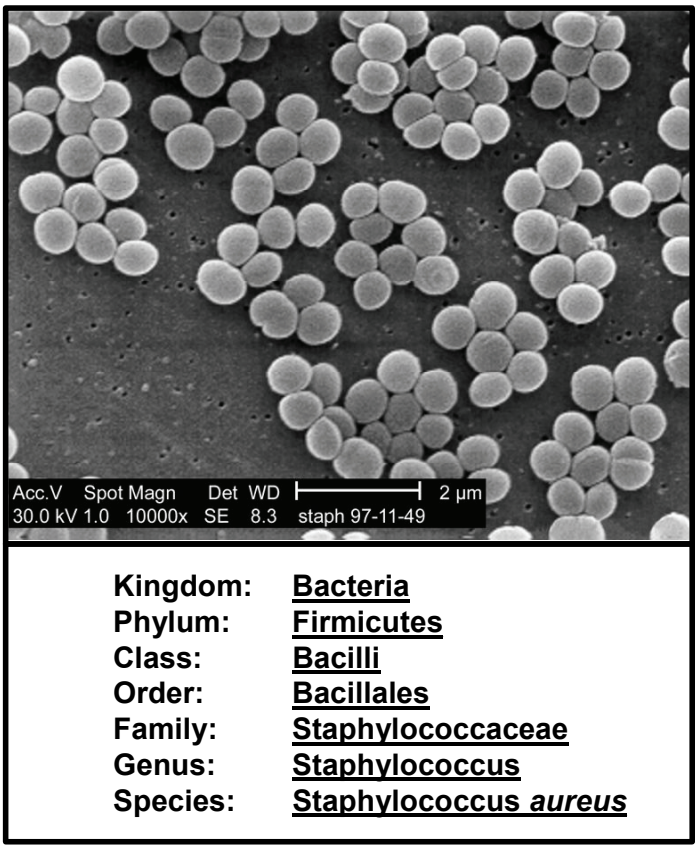

FIGURE 1. Scanning electron microscopy of *Staphylococcus aureus*
[Center for Control Disease (CDC) Public Health Image Library]

S. aureus is a potentially pathogenic bacterium found in the nasal membranes, skin, hair follicles and perineum of warm-blooded animals. Today, it is the leading cause of human infections worldwide, not only in the hospital environment but also in the community. *S. aureus* has about 2,600 genes and 2,8 millions base pairs (bp) of DNA in its chromosome. Mobile genetic elements, such as pathogenicity islands, plasmids and phages can also constitute part of the species genome. Genetic variation in *S. aureus* is very extensive, with approximately 22% of the genome comprised of dispensable genetic material.

The genus *Staphylococcus* contains 37 species, 16 of which are found in humans. The most virulent species for man in the genus include *S. aureus* (131*).*

Staphylococci were formerly classified in a common genus with *Micrococci spp*. (20) until recently when it was grouped with *Bacillus spp*. on the basis of ribosomal RNA sequences (5). Subsequently, about 50% of the *S. aureus* genome shares homology with non-

pathogenic sporulating *Bacillus subtilis,* indicating that the two organisms are quite close and have evolved from a common ancestor (149). As opposed to *Micrococci*, *Staphylococci* have low guanine/cytosine content and peptidoglycan-bound teichoic-acids in their cell wall structure (186).

1.2. Biochemical properties

S. aureus is distinguished from other species on the basis of positive results of catalase, coagulase, mannitol-fermentation, and deoxyribonuclease tests. The bacteria can use galactose and lactose as energy sources, and as such is a notorious cause of bovine and ovine mastitis (11,188); and food poisoning via contaminated food, dairy related especially.
Staphylococci together with *Streptococci* are members of a group of bacteria known as the invasive pyogenic cocci, since they can cause various suppurative or pus-forming diseases in humans and other animals (186).
On microscopic examination, *S. aureus* appear as gram-positive spherical cocci cells, between 0.5 to 1.7 μm in diameter, non-motile and non-spore forming, arranged in grape-like clusters, hence their name, from the Greek term *staphyle* and *aureus* for its distinctive "golden" colour. The bacteria form characteristic smooth and translucent yellow-gold colonies, owing to the production of triterpenoid carotenoids located in their cell membrane (115). These pigments not only give *S. aureus* its characteristic colour but also shield the bacteria from the action of toxic oxidants from polymorphonuclear cells (PMN) and phagocytes (53).
More than 90% of clinical isolates of *S. aureus* elaborate polysaccharidic capsules, among which 11 serotypes have been reported (246). Capsules type 5 and type 8 *S. aureus* have been found in up to 75% of clinical infections. However, about 20% of human isolates and up to 86% of bovine strains of *S. aureus* have been so far non-typable (57). In addition, the capsule protects the bacteria from phagocytosis.
Lysozyme, a muramidase enzyme present in various host tissues (mucous membranes, respiratory and intestinal tracts) and fluids (serum, saliva, and tears) do not kill *S. aureus.* This resistance may contribute to its ability to colonize skin and mucosal tissues such as the anterior nares (31).
Outside a host, the bacteria can withstand numerous environmental stresses, as desiccation, variable pH, osmotic stress, starvation, heat shocks or high temperatures ($\leq$ 50°C) and are considerable salt tolerant, thus able to grow in the presence of concentrations of salt that normally inhibit most other bacteria ($\leq$ 15% NaCl). Being so resistant, this bacterium can be found throughout the natural world from dust to door knobs and is common even in the most cleanest health care facilities.

The remarkable ability of *S. aureus* to acquire useful genes from various organisms has been revealed through homology alignment and phylogenetic trees. The evidence of repeated lateral and horizontal gene transfers (including plasmids) to and from distantly related organisms includes homologues in vertebrates, other bacterial species and even plants (149).

In addition, a large number of mobilizable exogenous DNA stretches, including insertion sequences, transposons, bacteriophages and pathogenicity islands (also referred to as genomic islands) that contain specific determinants responsible for disease and antibiotic resistance have been identified.

Overall, the *staphylococcal* cell wall plays an important role for the bacteria's strength and success (186). As in many gram-positive bacteria, the peptidoglycan (PDG) in *S. aureus* is a cross-linked polymer forming a layer or wall outside the plasma membrane. It consists of repeating and alternating layers units of two aminosugars N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), linked together to glycine aminoacids in a pentapeptide bridge. In this crooslinking, a peptide bond is formed between a D-alanine on one chain and the free amino end of an L-lysine residue on the other chain (Figure 2).

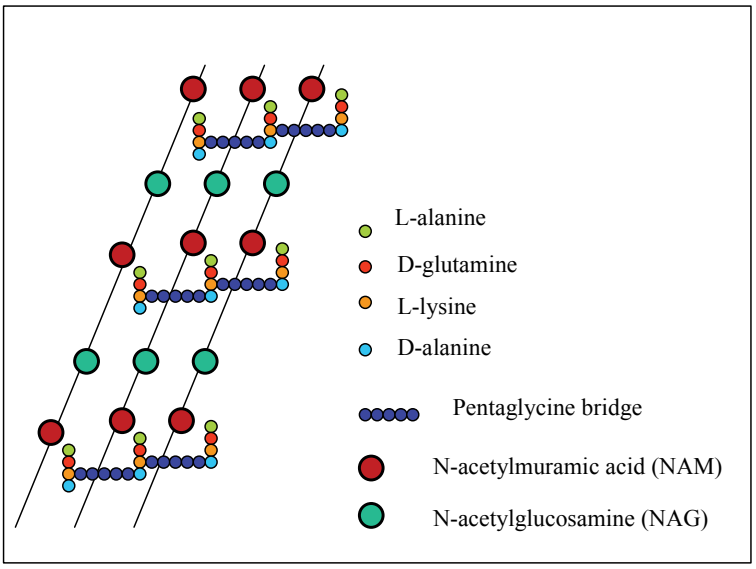

FIGURE 2. Basic structure of *S. aureus* peptidoglycan.

The individual PDG units are produced inside the cell, but the final crosslinking is catalysed outside the cytoplasmic membrane by a group of membrane-anchored bacterial enzymes known as the cell-wall transpeptidases or penicillin binding proteins (231).

1.3. Pathogenicity

S. aureus is a major human pathogen (100), able to colonize and infect humans of all genetic backgrounds (149). In fact, humans are its natural reservoir. However, it can also colonize a variety of members of the animal kingdom, including diverse mammals, reptiles, birds and fish. Infection models in lower organisms, such as insects (*Drosophila melanogaster*) and worms (*Caenorhabditis elegans*), have been described as well (190,243). Both wild-type *S. aureus* and some mutated strains are persistent colonizers (108).
The heterogeneity of the infections caused by *S. aureus* is linked to the extreme complexity of its genome and to its unique plasticity to adapt to a great variety of environments and living creatures, including antibiotics and the human immune system (108,253).
Overall the bacteria is the leading overall cause of nosocomial infections and as more patients are treated outside the hospital setting, it is an increasing concern in the community (104,168).

1.3.1. Host colonization

In order to colonize a host, and depending on their growth phase, the bacteria are able to produce and regulate a number of pathogenic proteins, such as surface proteins to colonize host tissues, factors that probably inhibit or affect normal phagocytosis (capsule, immunoglobulin, binding protein A), invasins and toxins that damage host tissue.

1.3.1.1. Surface proteins

The synthesis of many of these proteins is controlled by regulatory genes (186). Surface proteins such as protein A and a number of adhesins serve the bacteria to colonize the host and cause disease.

1.3.1.1.1. protein A

Staphylococcal Protein A (Spa) is a major surface protein of *S. aureus* strains (99). It initially inhibits phagocytic engulfment but then it also allows the bacteria to interact with several host components, such as immunoglobulins (G, A and E), platelets adhesion factor in sites of endothelial damage (von Willebrand factor), etc.
Spa provides the bacteria with a protective coating (immunological disguise) allowing it to bind to the Fc portion of host IgG and thus be opsonised and enter phagocytes. *S. aureus* is

also able to utilize other host cell receptors, including serum factors (the subunit of the third complement system C3b), and possibly other complement receptors and coreceptors to be opsonised and internalized by phagocytes cells (270).

1.3.1.1.2. Adhesins

The bacteria can produce around 20 different potential cell wall associated proteins called adhesins or cell adhesion molecules (CAMs), with the ability to adhere to a wide range of host proteins and interact with host extracellular matrix (ECM) components. Adhesins in *S. aureus* are present in all isolates and encoded on the chromosome. They are reassembled under the acronym MSCRAMM, for microbial surface components recognizing adhesive matrix molecules. Many **MSCRAMM**s have not yet been identified or characterized. However, most of the microbial adherence factors found in the bacteria are mainly of proteinic (polypeptides) or polysaccharidic (carbohydrates or sugars) nature (206). They basically confer adherence to a variety of host proteins (79,80). Among them, cell-associated protein clumping factor (Clf) A and B and fibrinonectin-binding protein A (FnBPA), play an important role in experimental endocarditis, and collagen binding protein, in osteoarthritis (207,214,215). Coagulase, extracellular adherence protein (Eap), extracellular matrix and plasma proteins (Emp) are also important (121).
Bacterial adherence is supported by cell surface components (cell adhesion molecules: **CAM**s) and extracellular matrix (**ECM**) like several plasma proteins (34,125), fibrinogen, fibronectin (FN), prothrombin, collagen, fibrinogen/fibrin, elastin, vitronectin, laminin, von Willebrand factor, as well as plasma proteins; vitronectin, thrombospondin, bone sialoprotein, hemin, decorin and heparan sulphate-containing proteoglycans, immunoglobulins, integrins ($\alpha_5\beta_1$) cadherins (4,124,244), etc.

1.3.1.2. Secreted proteins

Once bacteria have adhered to the target host cell, they can modulate the secretion of proteins like cytotoxins or invasins. These are exoenzymes (leukocidin, hemolysins, nucleases, proteases, lipases, kinases, hyaluronidase and collagenase), able to destroy host tissues while at the same time permitting spread and conveying nutrients to the invading bacteria.
S. aureus has a minimum of four unrelated **hemolysins** referred to as alpha (α), beta (β), gamma (γ) and delta (δ)-hemolysins, in the order of their discovery (75). **Alpha-hemolysin (alpha-toxin)** is the most potent membrane-damaging toxin of the bacteria; platelets and monocytes are the most sensitive to it. Alpha-hemolysin and **delta-hemolysin** are pore-

forming toxins, able to cleave the phospholipids content of the host cell membranes. **Beta-hemolysin** is a shingomyelinase that damages membranes rich in this lipid, however most human isolates do not express this toxin. **Gamma-hemolysin** is a major cytolysin, with activity against erythrocytes from many species. A few homologues of γ-hemolysin have been described, such as the two-component toxin, reported in 1932 by Panton and Valentine (**PV leukocidin**) (138). Gamma-hemolysin is produced by virtually all strains of *S. aureus* while PV leukocidin is found in only a few strains. It is encoded by mobile phages and can be transferred among other strains (139).

PVL causes important leukocyte destruction and tissue necrosis. It is produced by less than 5% of *S. aureus* strains isolates, but epidemic clones are more and more frequently reported (129,173). They are frequently associated with abscesses, furunculosis, arthritis, or severe hemorrhagic pneumonia, including in young adults and children, and with high mortality rate (107,181).

1.3.1.3. Staphylococcal toxins

Other secreted proteins of the bacteria are the *staphylococcal* toxins. They include the exfoliative toxins that produce *staphylococcal* scalded skin syndrome (SSSS) and the *staphylococcal* enterotoxins (SE), small peptides (six serotypes so far, SEA, B, C, D E, H) involved in food poisoning and toxic shock syndrome (TSST) (186).

TSST-1 and SEs are the paradigm of a large family of pyrogenic exotoxins called **superantigens** (SAgs) (179). The bacteria can cause TSS by the release of superantigens into the blood stream. All these proteins are known to have potent effect on cells of the immune system, and other biological effects still not well understood. TSST-1 and SE are also known as pyrogenic toxin superantigens (PTSAgs), involved in the induction of the proliferation of T-lymphocytes.

Staphylococcal food poisoning is due to the absorption of *staphylococca*l enterotoxins preformed in the food.

TABLE 1. summary of the factors involved in the bacteria pathogenesis

ACTIVITY/FUNCTION	PRODUCT	GENE
Anti-immune, antiphagocytosis	Protein A	*Spa*
	Polysaccharide capsule type 5	*Cap5*
	Polysaccharide capsule type 8	*Cap8*
Collagen binding	Collagen BP	*cna*
Fibronectin binding	Fibronectin BPA	*FnbA*
	Fibronectin BPB	*fnbB*
Fibrinogen binding	Clumping factor A	*clfA*
	Clumping factor B	*clfB*
Lactoferrin binding	Lactoferrin BP	*LBP*
Hemolysins, cytotoxin	α-Hemolysin	*hla*
	β-Hemolysin	*hlb*
	δ-Hemolysin	*hld*
	γ-Hemolysin	*hlg*
Leukolysin	PVL leukocidin*	*lukS/F*
Food poisoning, TSS	Enterotoxin A	*sea*
	Enterotoxin B	*seb*
	Enterotoxin C	*sec*
	Enterotoxin D	*sed*
	Enterotoxin H	*seh*
Scalded skin syndrome	Exfoliatin A	*Eta*
	Exfoliatin B	*etb*
Toxic shock syndrome	Toxic shock toxin-1	*tst*
Putative protease	Serine protease-like	*splA-F*
Spreading factor	V8 protease	*ssp*
Spreading factor	Hyaluronidase	*Hys*
Spreading, nutrition	Staphopain (protease II)	*Scp*
	Glycerol ester hydrolase	*geh*
	Lipase (butyryl esterase)	*lip*
Nutrition	Nuclease	*Nuc*
Fatty acid esterification	FAME	*Fme*
	PI-phospholipase C	*Plc*
Clotting, clot digestion	Coagulase	*Coa*
Plasminogen activator	Staphylokinase	*Sak*
Processing enzyme?	Metalloprotease (aureolysin)	*Aur*
	Cysteine protease	*sspB*

1.3.2. Intracellular character

S. aureus has long been considered exclusively an extracellular pathogen (167). However, many studies have pointed to its remarkable ability to survive and replicate intracellularly (38). The ability of *S. aureus* to escape host immunity and then establish a cell-niche, are actually crucial steps in its pathogenesis (166,167).

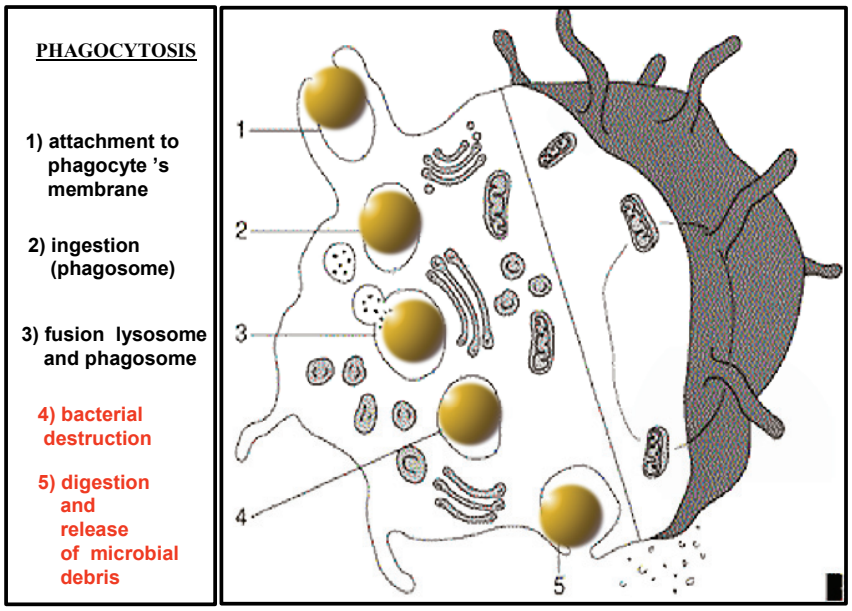

FIGURE 3. The normal process of phagocytosis: *S. aureus* is able to miss out steps 3 and 4 and reside intracellularly (45). (Adapted from www.whfreeman.com)

1.3.2.1. Internalization

Normally macrophages and other leukocytes cells in the host eliminate invading bacteria by a process called phagocytosis.

Phagocytes pull the bacteria into phagosomes, which eventually fuse with lysosomes forming a phagolysosome where the organisms are destined for destruction. There, the bacteria are exposed to a number of toxic chemicals and enzymatic agents, among which reactive oxidative intermediates such as superoxide anions and hydrogen peroxide, a low pH, and degradative proteases.

Bacteria bind to cell surface integrins, cadherins and immunoglobulin-related host cell adhesion molecules, which triggers their internalization (101,102,123).

1.3.2.2. Intracellular survival

At the present time, the mechanisms allowing for the intracellular survival of *S. aureus* have not yet been fully established (98,167). In non-phagocytic cells, with low defence mechanisms, *S. aureus* is able to escape from phagolysosomes and multiply in the cytosol. It also causes the death of these cells by apoptosis (29,183,195). In contrast, in phagocytic cells, *S. aureus* seems to remain confined within vacuoles of the phagocytic apparatus (113,216). In this compartment, however, its multiplication is slowed down but not impaired by the acidic pH (60). *S. aureus* is also known to produce enzymes degradating oxidant species (catalase, hydroperoxide reductase, superoxide dismutase [55,56]), which may contribute to protect it intracellularly.

1.3.2.3. Cell types affected

S. aureus has been shown to be internalized “in vitro” and “in vivo” by different mammalian cells including professional phagocytes and non-phagocyte cells, where it is able to survive and multiply (Table 2).

TABLE 2.
Studies pointing to the ability of *S. aureus* to survive intracellularly in different host cell types

INFECTED CELL-TYPE	REFERENCES
Epithelial cells	11,29,54,67,79,80,242
Mammary epithelial cells	29,38,281
Enterocytes	127
Keratinocytes	143,182,195
Osteoblasts	85,86,132
Fibroblasts	148
Endothelial cells	169,215,267,269,283,284
Neutrophils	38,38,83,113,204,205
Macrophages	240

1.3.2.4. Small-colony variants (SCVs)

In the intracellular milieu, *S. aureus* can switch to a phenotype known as small-colony variants (SCV’s) that provides marked advantages for survival, among which are

anaerobiosis and reduced growth rate (3,52,147,167). In fact, SCV's demonstrate a number of characteristics that are atypical to *S. aureus* and linked to interruption of electron transport (276); they also present an altered antibiotic sensitivity (52), being most notably resistant to aminoglycosides (28). SCV's are auxotroph and can revert to normal growth and morphology in the presence of menadione and hemin, thiamine, or CO_2 rich culture medium (211,212).

Based on these characteristics, SCVs should always be suspected in indolent and recurrent infections and are increasingly implicated as a cause of persistence, recurrence, and antibiotic resistance in many clinical cases, including lung, bone, skin, wound, cystic fibrosis, and foreign body infections (1,37,137,163,278). Importantly in this context, SCV's apparently allows the bacteria to remain intracellular in a dormant metabolic state for months or even years, which could actually explain the persistent character of *S. aureus* infections (272,274,275,277).

1.3.3. infections in humans

Nasal carriage of *S. aureus* is considered to be a key risk factor for development of *staphylococcal* infection (273). Three bacterial carriage patterns have been distinguished in the healthy adult population: about 20% of individuals are persistent *S. aureus* carriers; around 60% are intermittent carriers and 20% are persistent non-carriers (186). So far and based on currently available information, there are insufficient data to confirm or refute the existence of host-bacteria genotypes that predispose to carriage (47).

However, studies comparing carriage- and infecting isolates of *S. aureus* have found that individuals are usually infected with their own carriage isolate. In fact, the temporary eradication of these carriage, has been shown to reduce nosocomial infection in immunocompromised patients (145).

1.3.3.1. Skin-related infections

S. aureus is a leading cause of many skin-related infections including keratitis, atopic dermatitis, carbuncles, cellulites, impetigo, psoriasis, furuncles, follicles, mastitis, etc.

In most cases, a minor rupture of the skin or mucosal barrier may allow the internalization of the bacteria forming an abscess enclosing a central core of pus containing not only the bacteria but also neutrophils and macrophages (186).

1.3.3.2. Deep-seated infections

Once the bacteria have broken down the natural skin barrier, they can disseminate into more profound sites or migrate directly into the blood. Thus any localized infection has the potential to become the seeding ground for a more severe spread (186).
S. aureus is able to cause deep-seated related threatening systemic infections such as endocarditis, osteomyelitis, pneumonia, bacteremia and septic shock (186) in which intracellular foci are probably present. Hospital-device-associated equipment may also allow s*taphylococci* access to adjoining tissues or the bloodstream.

1.3.3.2.1. Osteomyelitis

S. aureus is responsible for 70% of human osteomyelitis, 80% of cases of joint infections in patients with rheumatoid arthritis and is a common agent in other bone associated diseases (156). It can invade and persist within osteoblasts causing rapid tissue destruction via the production of toxins (86).
Antibiotic resistant strains and their intracellular localisation make successful treatment of osteomyelitis very difficult (84,85).

1.3.3.2.2. Endocarditis

S. aureus is a virulent pathogen with a unique capacity to cause difficult-to-eradicate endovascular infections. This facility relies in part on his affinity to colonize and invade endothelial cells and endovascular tissue and its ability to resist killing by platelet microbicidal peptides (285). A number of cellular changes result from these progression including the release of proinflammatory cytokines and the expression of Fc receptors and adhesion molecules. These cellular alterations may play a crucial role in initiating and amplifying the inflammatory response, which results in a magnified endothelial cell injury (244).
The bacteria are responsible for about 70% of cases of endocarditis in intravenous drug users and more than 30% cases of native valve endocarditis (187).

1.3.3.2.3. Pneumonia

Once *staphylococci* are in the lung, they multiply and sometimes invade the epithelium of the bronchioles (67). The production and release of cytokines and chemokines, including IL-8, due to the presence of *S. aureus* elicit infiltration of PMNs and macrophages, leading to tissue damage and subsequent pneumonia. It has been found responsible for about 10% of

community-acquired pneumonia and about 30% of cases of hospital acquired-pneumonia. Incidence is high especially in patient's over-75 years at nursing homes.
A necrotizing pneumonia caused by PVL leukocidin-positive *S. aureus* in otherwise healthy children and young adults are now recognized as a new nightmare, since the lethality rate is very high. *S. aureus* is also a major pathogen in cystic fibrosis patients (112,226).

1.3.3.3. Bacteremia and septic shock

The frequency of the presence of bacteria in blood-stream has increased over the past decades. *S. aureus* bacteremia still carries a 20-25% mortality rate.
Staphylococcal sepsis and septic shock result from the overproduction of inflammatory mediators as a consequence of the interaction of the host immune system with the bacteria and its wall constituents. The increasing frequency of *S. aureus* sepsis is vastly linked to the increased use of invasive equipment on persons with a weakened immune system and the ability of the bacteria to easily colonize intravascular catheters or surgically implanted materials (259).
The cellular events leading to septic shock are similar in s*taphylococcal* infection and infections with gram-negative bacteria. *S. aureus* and *S. epidermidis* are responsible for half of cases (259).

1.3.4. Major phenotypes of antibiotic resistance

S. aureus possesses a remarkable ability to develop resistance to antibiotics. It started shortly after the introduction of penicillin, a beta-lactam antibiotic, into clinical use.

1.3.4.1. Beta-lactam resistance

All beta-lactam antibiotics have a common element in their molecule structure: a four-atom ring (cyclic amide) known as a beta-lactam. Beta-lactams antibiotics are analogous of D-Alanyl-D-Alanine, the terminal aminoacid residues of the precursor NAM/NAG-peptide subunits of the nascent PDG layer. This structural similarity facilitates their binding to the active site of the penicillin binding proteins (PBPs) and prevents the final crosslinking of the nascent PDG layer, disrupting cell wall synthesis, causing cell lysis and death. The inhibition of PBPs also leads to the activation of autolytic enzymes in the bacteria cell wall.

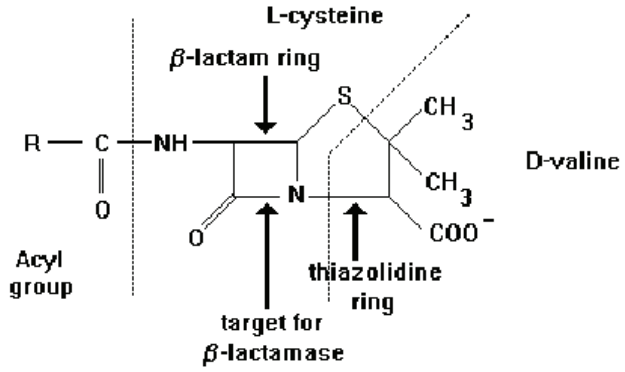

FIGURE 4. Common core of beta-lactam antibiotics (beta: to denote four-membered ring size), (lactam: grammatical blend from lactone plus amide).

Staphylococci have at least two major mechanisms for resistance to beta-lactam antibiotics. The first one is the **production of beta-lactamases** also known as penicillinases, enzymes that hydrolytically destroy beta-lactams rendering the antibiotic ineffective, and the other is the intrinsic expression of a new PBP, called penicillin binding-protein 2A or PBP2A, which has low affinity for present beta-lactam compounds and make the bacteria resistant to inhibition by all currently available beta-lactam antibiotics (162) .

Methicillin-Sensitive-*S. aureus* (**MSSA)** is the term used to denote the bacteria susceptibility to all beta-lactams including beta-lactamase inhibitors combinations, cephems and carbapenems.

A less known and understood form of penicillin resistance mechanism in *S. aureus* is **tolerance** to the bactericidal effects beta-lactam antibiotics (172,223,225). It is manifested in strains deficient in autolytic enzymes due to an excess of autolysin inhibitors and causes cross-tolerance to the killing effects of other cell wall inhibitors such as vancomycin (172). Bacteriophages have also been implicated on the conversion of non-tolerant organisms to tolerance (225).

1.3.4.1.1. BlaZ- Penicillin resistance: beta-lactamase production

The introduction of penicillin in the early 1940s dramatically improved the prognosis of patients with *staphylococcal* infections (172). In fact, penicillin G still remains one of the best choices for the fully susceptible strains (MIC ≈ 0.01 μg/ml).

However, as early as 1942, **penicillin-resistant staphylococci** were recognized, first in hospitals and subsequently in the community. These penicillin-resistant (MIC ≥ 0.25 μg/ml), oxacillin-susceptible strains (MIC ≤ 2 μg/ml) are resistant to beta-lactamase-labile penicillins

but susceptible to other beta-lactamase stable penicillins, beta-lactamase inhibitor combinations, cephems and carbapenems.

The *staphylococcal* resistance to penicillin can be found inherently present on the bacterial chromosome or may be acquired via plasmid transfer. It is mediated by the *blaZ* gene that encodes a beta-lactamase enzyme when the bacteria are exposed to beta-lactam antibiotics, opening the beta-lactam ring and forming inactive penicilloic acid and thus, rendering the beta-lactam antibiotic inactive. By the late 1960s, more than 80% of both community- and hospital-acquired *staphylococcal* isolates were resistant to penicillin. At the present time, beta-lactamase-dependent resistance is found in > 95% isolates (166).

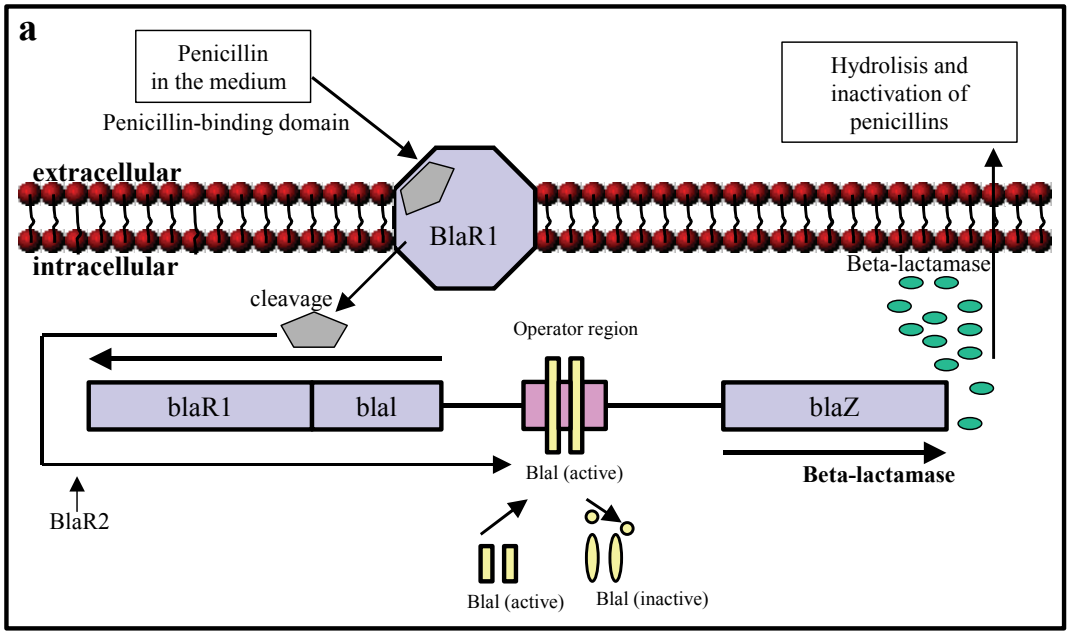

FIGURE 5. Induction of beta-lactamase synthesis in the presence of the beta-lactam penicillin: The presence of penicillin in the extracellular medium stimulates the transmembrane sensor-transducer BlaR1 to autocatalytic activation. Active BlaR1 cleaves Bla into inactive fragments, allowing transcription of both blaZ and blaR1 to commence. Beta-lactamase, the extracellular enzyme encoded by blaZ is produced and hydrolyzes the beta-lactam ring of the penicillin antibiotic rendering it inactive. (Adapted from 168).

1.3.4.1.2. PBP2A mediated-resistance

In the late 1950's new beta-lactam semi-synthetic compounds were developed and became available. They were stable to the action of BlaZ mediated penicillinase and hence categorized as **penicillinase-stable penicillins**. The group includes methicillin, oxacillin, nafcillin, flucloxacillin, and dicloxacillin, methicillin being the prototype (92,114).

Methicillin use in the 1960's was rapidly followed by reports of mutants being resistant to the whole new group of beta-lactams and from the 1970s to today, MRSA has become the main cause of nosocomial infection worldwide and created great clinical difficulties for treatment

on a global scale (103). *S. aureus* strains resistant to this group of antibiotics are known as methicillin-resistant isolates or **MRSA** (178), even though methicillin is no longer the agent of choice for testing or treatment.

MRSA are by definition not inhibited by less than 25 µg/ml methicillin, whereas ordinary strains are usually inhibited by 1.6 to 3.2 µg/ml (224). They are also oxacillin resistant (MIC ≥ 4 µg/ml), hence also named ORSA. Multiresistant MRSA (MDR-MRSA) have been defined as *S. aureus* isolates with a MIC for oxacillin at ≥ 4 µg/ml and with four or more additional resistances (74).

MRSA resistance is mediated by the bacterial acquisition of a novel mobile genetic element, designed SCC (Staphylococcal-Chromosomal–Cassette) or mec chromosomal **mecA gene** that codes the alternative supplementary target protein **PBP2A**, with low affinity for beta-lactams, including cephalosporins, carbapenems and beta-lactamase inhibitor combinations. MecA is a large 20- to 65-Kb mobile element in *S. aureus*. Moreover, the cassette have also been found to act as a trap for additional unrelated drug resistance genetic determinants, thereby leading to multiple resistances. SCCmec consists of the mec gene complex, containing *mecA* and the *mecA* regulatory genes, and the cassette chromosome recombinase (ccr) complex, which encodes a recombinase(s) responsible for SCCmec movement. The SCC elements are classified according to the type of recombinase they carry and their general genetic composition. SCC types I,II, III, IV and V have been described and SCC lacking mecA have also being reported (120). In addition, this mechanism of resistance seems to be shared with induction of a gene(s) in the penicillinase plasmid (257).

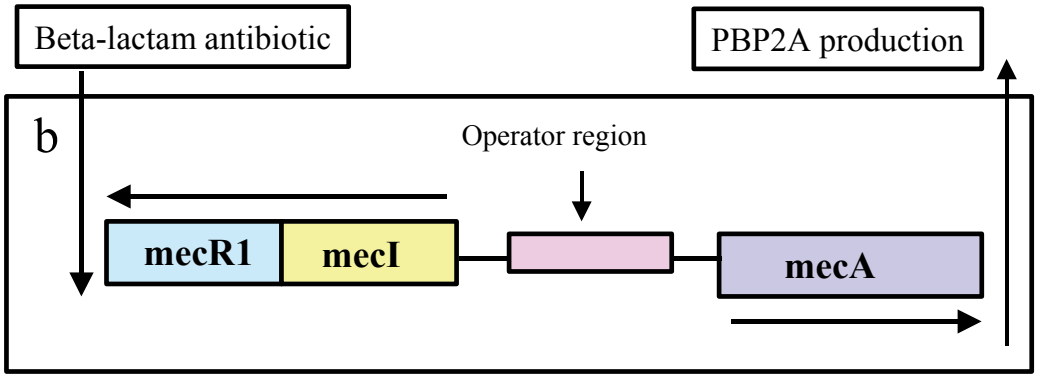

FIGURE 6. Mechanism of *S. aureus* resistance to methicillin: Synthesis of PBP2A proceeds in a fashion similar to that described for beta-lactamase. mecI and BlaI have corregulatory effects on the expression of PBP2A and beta-lactamase. Exposure of mecR1 to a beta-lactam antibiotic induces mecA activation. MecR1 inactivates mecI, allowing expression of PBP2A.

In MRSA, the PBP2A acts as a surrogate enzyme able to perform both transpeptidase and transglycosylase activities of the normal four PBPs of the bacteria (210).

The lateral and then horizontal gene transfer of *mecA*, has played a fundamental role in the evolution of *S. aureus* (94). Several clones with different types of SCCmec have been reported, such as type IV found mainly in Community-MRSA (CA-MRSA) in Australia and the United States and types I to III being prominent in the Hospital-MRSA setting (HA-MRSA) (170).

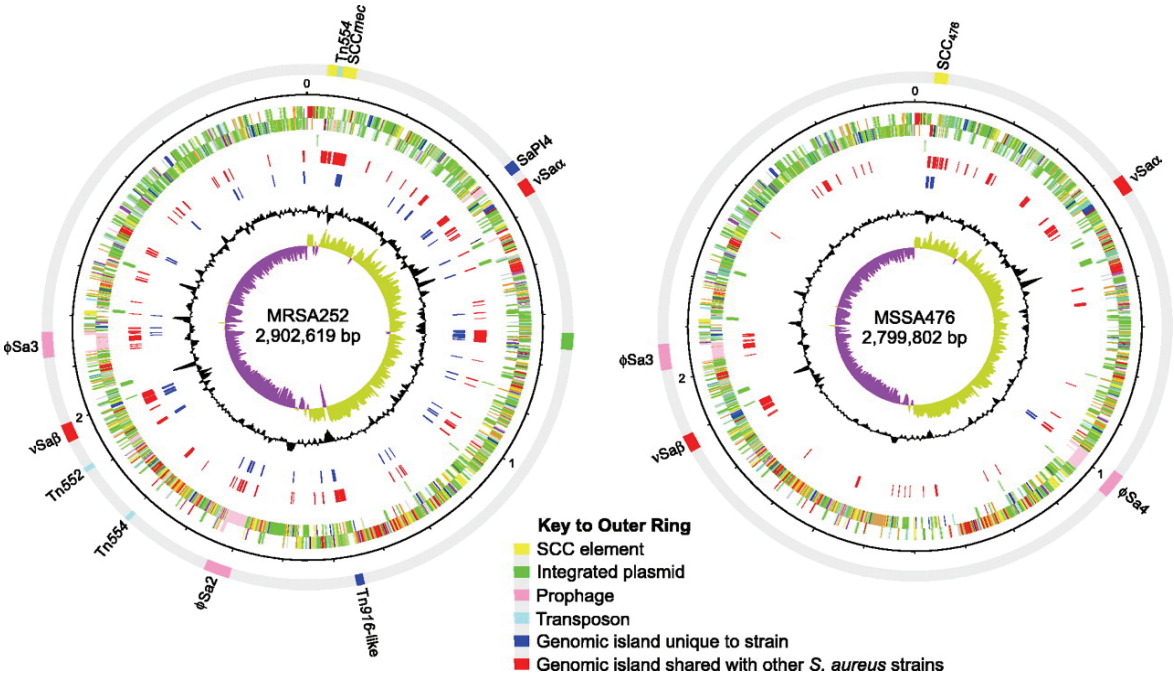

Figure 7: Schematic circular diagrams of the MRSA252 and MSSA476 chromosomes. This comparative genome approach between an epidemic MRSA clone (MRSA252) and an invasive MSSA (MSSA476) illustrates the role of accessory elements in the rapid evolution of the bacteria (130).

Hospital-acquired MRSA constitutes 25-50% of clinical isolates all over the world (74). It has reached more than 40% in Europe (166,168). In Belgium MRSA accounts for 25-30% (EARSS data, figure 8). Between 1997 and 1999 MRSA was found to be the most prevalent cause of bloodstream infection, skin and soft-tissue infection and pneumonia in hospital cases in USA, Canada, Latin America, Europe and the Western regions (73), see graph below. Today is though that MRSA kills more americans than HIV, mainly because of hospital practices of no screening for MRSA in blood donors or MRSA asynthomatic carriers entering their presmises (The New York Times, Novembre 14th 2006).

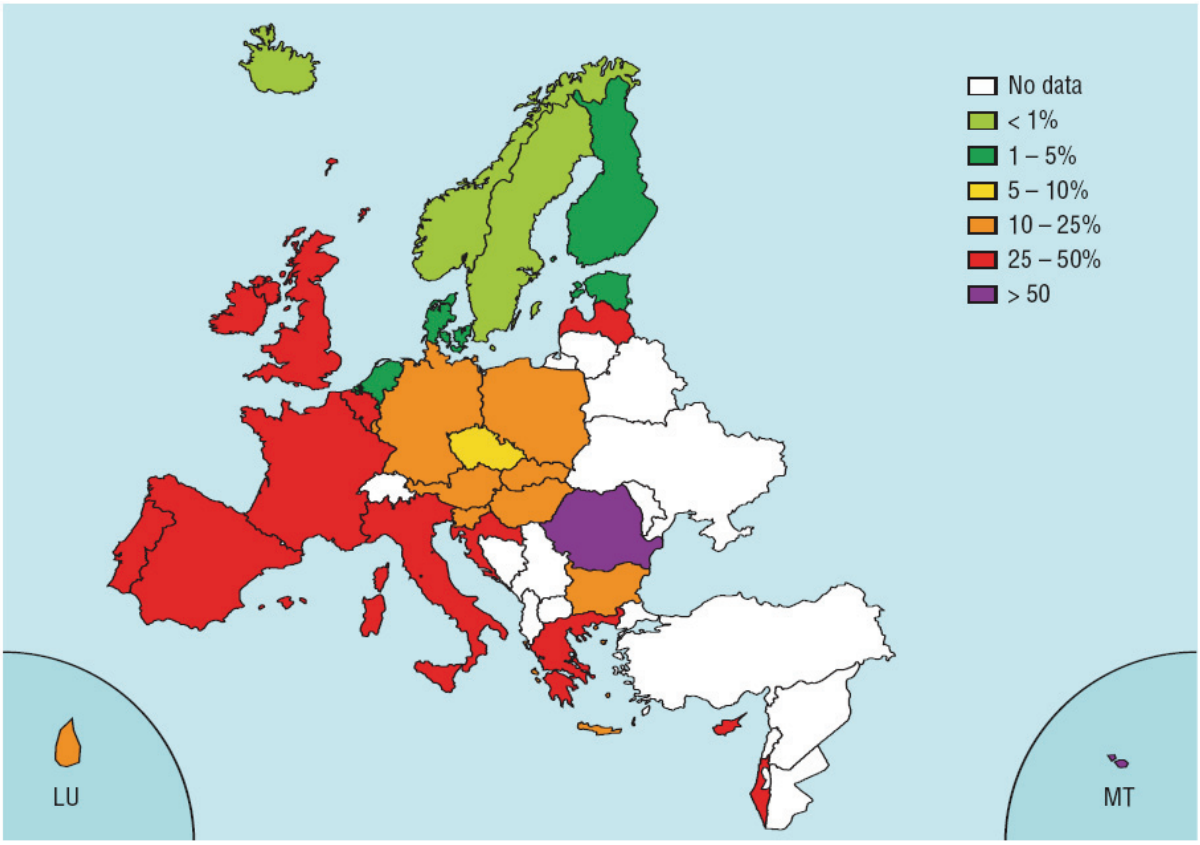

FIGURE 8. Percentage of MRSA resistance in Europe in 2004: *S. aureus* proportion of invasive isolates MRSA in 2004 (Data from the European antimicrobial resistant surveillance system, EARSS).

Strains of *S. aureus* exhibiting either beta-lactamases or PBP2A directed resistance, or both, have unfortunately already established a considerable ecological niche among humans. So far today, the frequencies of both community-acquired and hospital-acquired *staphylococcal* infections have increased steadily, with little change in overall mortality from the pre-antibiotic era. Multitude of factors that include the widespread and sometimes inappropriate use of antimicrobials, not only in humans but also extensive use of these agents as growth enhancers in animal feed and husbandry, together with the increase in regional and international travel, facilitates the relative ease with which antimicrobial-resistant bacteria cross geographic barriers (166,168).

A typical MRSA possesses several insertion sequences with antibiotic resistance genes (see Table 3 for typical examples). Thus, MRSA resistance may encompass cell wall inhibitors such as β-lactams, ribosomal inhibitors including macrolide-lincosamide-streptogramin-B-(MLS_B), aminoglycosides, tetracyclines, fusidic acid, and the new oxazolidinones, the RNA polymerase inhibitor rifampin, the DNA gyrase blocking quinolones, and also the antimetabolite trimethoprim-sulfamethoxazole (25).

TABLE 3. MRSA likely resistance genes and products (adapted from 168).

ANTIBIOTICS	RESISTANCE GENE	GENE PRODUCT
beta-lactams	*blaZ*	Beta-lactamase
	mecA	PBP2A
quinolones	*parC*	ParC (or GrlA) component of topoisomerase IV
	GyrA or *gyrB*	GyrA or GyrB comoponents of gyrase
aminoglycosides (gentamicin)	Aminoglycoside-modifying enzymes *(aac, aph, ant)*	Acetyltransferase,
		Phoshotransferase
		Nucleotidyl transferase
bleomycin	*bleO*	Beomycin resistance protein
erythromycin, pristinamycins	*ermA*	rRNA methylase
tetracyclines	*tetM*	TetM efflux protein
glycopeptides	Unknown (VISA)	Altered PDG
	VanA	D-Ala-D-Lac
ansamycin (rifampin)	*rpoB*	Mutation in beta-subunit RNA polymerase
trimethoprim-sulfamethoxazole (TMP-SMZ)	Sulphonamide*: sulA*	Dihydropteroate synthase
	TMP :dfrB	Dihydrofolate reductase (DHFR)
oxazolidinones	*rrn*	23rRNA
quinupristin-dalfopristin (Q-D)	*Q: ermA, ermB, ermC*	Ribosomal metylases
	D: vat, vatB	acetyltransferases
oxazolidinones	*rrn*	23S rRNA

1.3.4.1.2.1. MRSA-Hospital acquired: HA-MRSA

Methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for the largest outbreak of hospital-acquired infection that the world has ever seen (111). MRSA hospital isolates are multiresistant and clonal, they are associated with risk factors including recent hospitalization or surgery, living in a nursing home, and having an indwelling catheter or device. The prevalence is highly variable among geographical regions, ranging from 2 to 40 % between North and South European countries (248), with about 30 % in Belgium . Early diagnostic and strict hygiene measures appear therefore as the best strategies to control their spreading (164,249).

1.3.4.1.2.2. MRSA-Community acquired: CA-MRSA

Although originally confined to the hospital environment, MRSA have emerged in community-acquired infections (170). As compared to hospital acquired MRSA, CA-MRSA carry a distinct molecular makeup; they are less resistant but more virulent and invasive (50,77)

They are frequently found in young, healthy patients without significant health care contact, especially in populations with close physical contacts (sport teams, prisons [140,203]).

Although both HA-MRSA and CA-MRSA harbour an SCC*mec* carrying the *mecA* gene, their SCC*mec*s are of quite different sizes and most likely of different origin. Thus, the two types of organisms are not alike. Furthermore, clinical and molecular epidemiology indicates that they represent two separate evolutions of the strain. CA-MRSA may represent a hybrid between HA-MRSA, which escaped from the hospital environment, and the more easily treatable community organisms. These hybrids strains have in turn acquired new virulence factors, which make their infections after only minor cuts or scrapes, more aggressive and invasive (25). The prevalence rates of CA-MRSA vary widely among studies, in part because of the use of different definitions to distinguish between CA-MRSA and HA-MRSA, but also because of the different settings in which studies have been performed (It should be noted that relatively few studies have been conducted among randomly selected healthy members of the community, most studies being based on hospitalised patients, or patients upon admission to the hospital, which has probably resulted in an overestimation of the 'true' prevalence of CA-MRSA. [146]). Thus, prevalence of CA-MRSA was about 30 % among MRSA isolates from hospitalised patients, but only 0.2% among community members (228).

hospitalised patients, but only 0.2% among community members ().

The main features that distinguish CA-MRSA from HA-MRSA is their susceptibility to other classes of antimicrobial agents, their genetic diversion from HA-MRSA (including toxins), and the inclusion of new markers including Panton-Valentine Leukocidin (PLV) and SCC MEC type IV (See Figure 9).

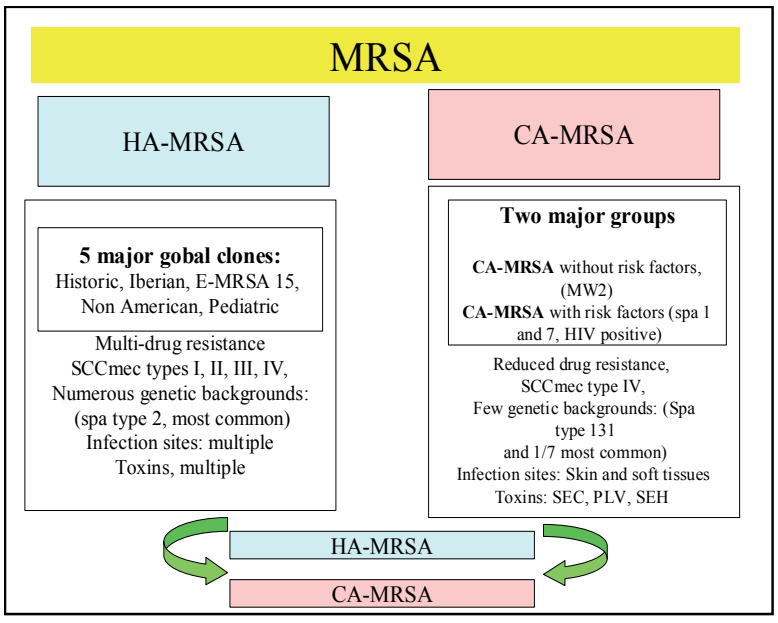

FIGURE 9: major differences between HA-MRSA and CA-MRSA (adapted from [49])

1.3.4.1.2.3. Beta-lactams anti- MRSA in development

The consensus that beta-lactam antibiotics are inherently more efficacious than vancomycin against MSSA has prompted more research on the field. MRSA resistance to beta-lactams depends upon the supplementary peptidoglycan transpeptidase PBP2A that has low affinity for these compounds. PBP2A does not lack affinity for beta-lactams but the beta-lactam compounds developed so far have low affinity. So, it is though that in principle beta-lactams can be re-engineered to bind beta-lactams strongly. On this basis, new anti-MRSA compounds with improved affinity towards PBP2A have been designed (162). Studies of MRSA strains have shown that PBP2A can be effectively blocked by beta-lactams with improved affinities for PBP2A and stability against degradation by s*taphylococcal* penicillinase (87) and hence restore beta-lactam affinity. These new compounds include carbapenems and cephalosporins (162), in particular LB11058, ceftobiprole (formerly BAL9141, Ro 63-9141), RWJ-54428 (MC-02,479) (51,279). New developmental anti-MRSA carbapenems include SM-197436, SM-232721 and SM-232724 (258). Faropenem and doripenem carbapenems also include MRSA in their spectrum (35,114,116,235).

1.3.4.2. Vancomycin-resistance

Vancomycin and teicoplanin are still the only glycopeptide antibiotics approved to treat multi-resistant bacterial strains in human use (260,264).
In sensitive *S. aureus* strains these large hydrophilic molecules are able to form a five-point hydrogen bond interactions with the terminal D-alanyl-D-alanine moieties of the N-acetylmuramic acid (NAM)/ N-acetylglucosamine (NAG)-peptide subunits preventing the incorporation of these subunits into the peptidoglycan matrix (PDG) (17,219). See Figure 10.

Teicoplanin is not used in the USA, but vancomycin has been widely used orally to treat *Clostridium spp*. and *Enterococcus spp.* infections (106,189).
S. aureus has already developed different ways to overcome the action of these antibiotics. One of them includes the tolerance of the bacteria to continue to survive in presence of glycopeptides at clinical relevant doses (16,17,61); the other depends on the acquisition of genetic material from a transposon found in the vancomycin resistant *Enterococcus spp*. (109). We therefore also get an insight into glycopeptide resistance in *Enterococcus spp*. (Table 4).

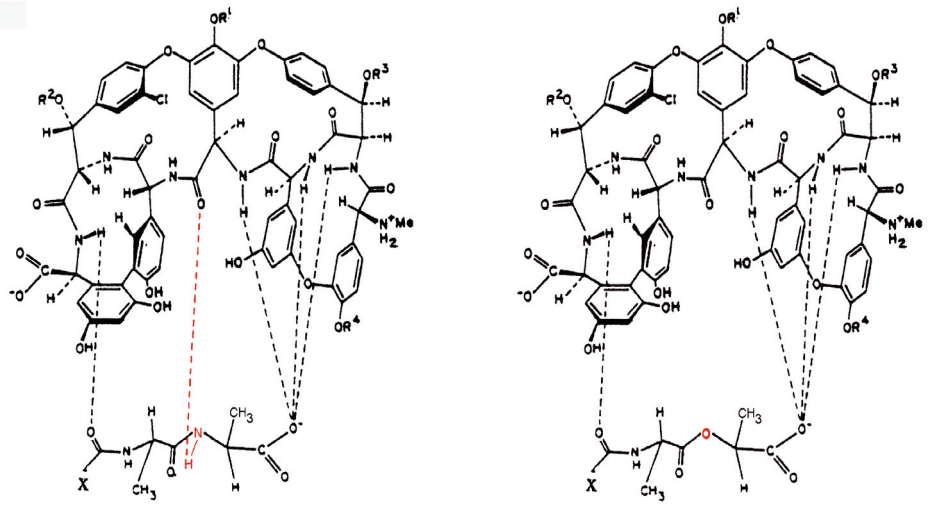

FIGURE 10: Hydrogen bonding interaction in vancomycin susceptible (left) and vancomycin resistant (right) bacteria (17).

1.3.4.2.1. Vancomycin resistance in Enterococcus spp*: VRE*

Among enterococci, six types of acquired glycopeptide resistance have been described (VanA, VanB, VanC, VanD, VanE, and VanG; Table 4); they are named based on their specific ligase genes (e.g., *vanA*, *vanB*, etc.) (61). A related gene cluster, *vanF*, has been found in *Paenibacillus popilliae* strains (formerly *Bacillus spp.*), a vancomycin-resistant biopesticide used in the United States which could be the progenitor of the resistance genes acquired by enterococci (61).

TABLE 4. Vancomycin MIC (µg/ml) ranges for vancomycin and teicoplanin resistant *enterococci* phenotypes (17,61).

Glycopeptide-resistant-enterococci VANCOMYCIN (MIC µg/ml)					
VanA	**VanB**	**VanC**	**VanD**	**VanE**	**VanG**
64 - >1000	4 - 1024	2 - 32	128	16	16
TEICOPLANIN (MIC µg/ml)					
VanA	**VanB**	**VanC**	**VanD**	**VanE**	**VanG**
16 - 512	≤ 0.5	≤ 0.5	4	0.5	05

In resistant strains a novel cell wall precursor with reduced glycopeptide affinity is synthesized, a hydrogen bond is lost in the five-hydrogen interaction pocket and the antibiotic looses 1000X times affinity. The new dipeptides of the PDG wall are not then D-ala-D-ala but D-Ala-D-Lac or D-Ala-D-Ser, depending of the genetic material (17). See Figure 10.

1.3.4.2.1.1. the *vanA* operon

The most common genotype of vancomycin and teicoplanin resistance (*vanA*) in e*nterococcus* arises as the result of the horizontal acquisition of operons (transposon Tn1546) carrying genes coding for enzymes for the synthesis of PDG with low affinity precursors (the C-terminal D-Ala residue is replaced by D-lactate), resulting in 4-point hydrogen bonding interaction and 1000 times-fold decrease in vancomycin affinity (17,61).

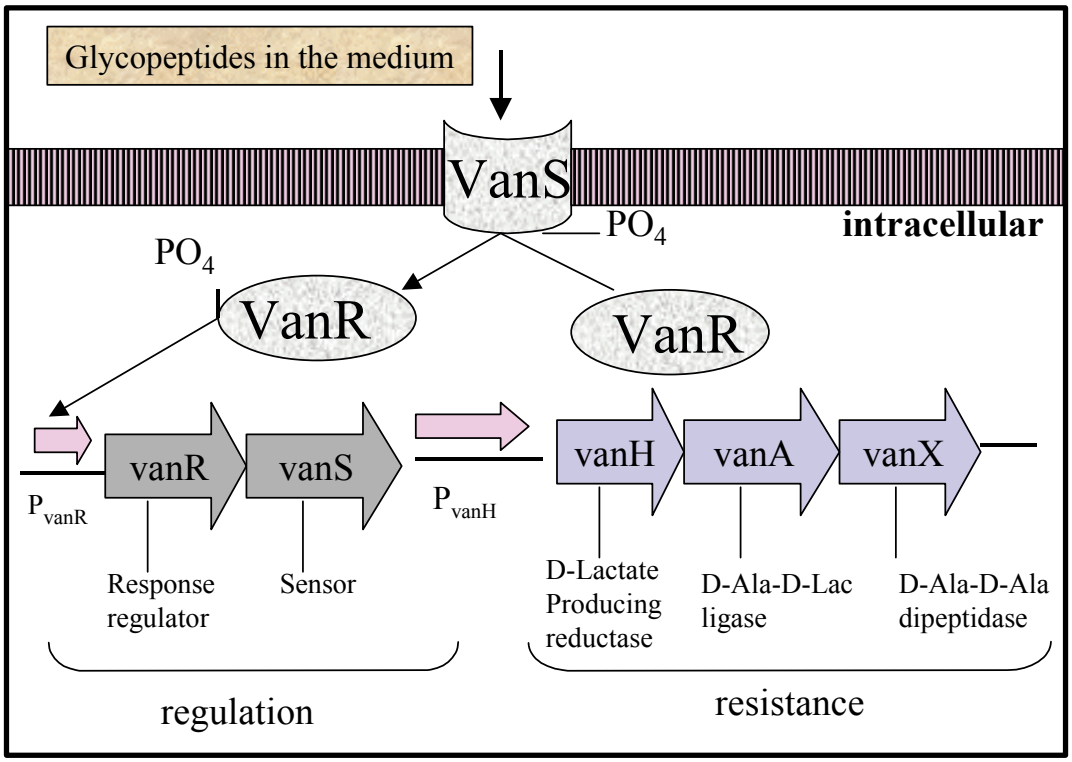

FIGURE 11. VanA-type resistance in *enterococcus* (109). In the presence of glycopeptides in the medium, a two-component regulatory system is activated. Phosphorylation of the sensor VanS, activates the response regulator gene VanR for the transcription of the corresponding enzymes vanH, vanA and VanX. Consequently the bacteria form a modified PDG with reduced affinity for vancomycin and survive.

This vanA phenotype requires a five-gene-resistance-cassette (Figure 11) to be assembled in a transposon (embedded in a plasmid) for a re-programming of the enzymes that make the D-ALa-D-Ala terminus of the peptidoglycan precursor. What is more, one of the enzymes in

the genetic package (VanX) also eliminates the high-affinity precursors (D-Ala) that are normally produced by the bacteria and thus removes the vancomycin-binding target.

1.3.4.2.2. Resistance in S. aureus

There are at present two known phenotypes of resistance, which confer to the bacteria the ability to survive to the action of the conventional glycopeptides.

1.3.4.2.2.1. Vancomycin intermediate resistance: VISA

MRSA strains with four- to eightfold increase in the MICs of vancomycin, decreased susceptibility to teicoplanin and significant thickening of the cell wall have started to appear in last decade. They are known as vancomycin intermediate *S. aureus* or **VISA**. The exact mechanism and genetic basis underlying the decreased susceptibility to vancomycin of VISA isolates is a subject of active investigation (234). Furthermore, they can already be generated in the laboratory by serial passage of the MRSA strains in the presence of vancomycin (15,16,168).

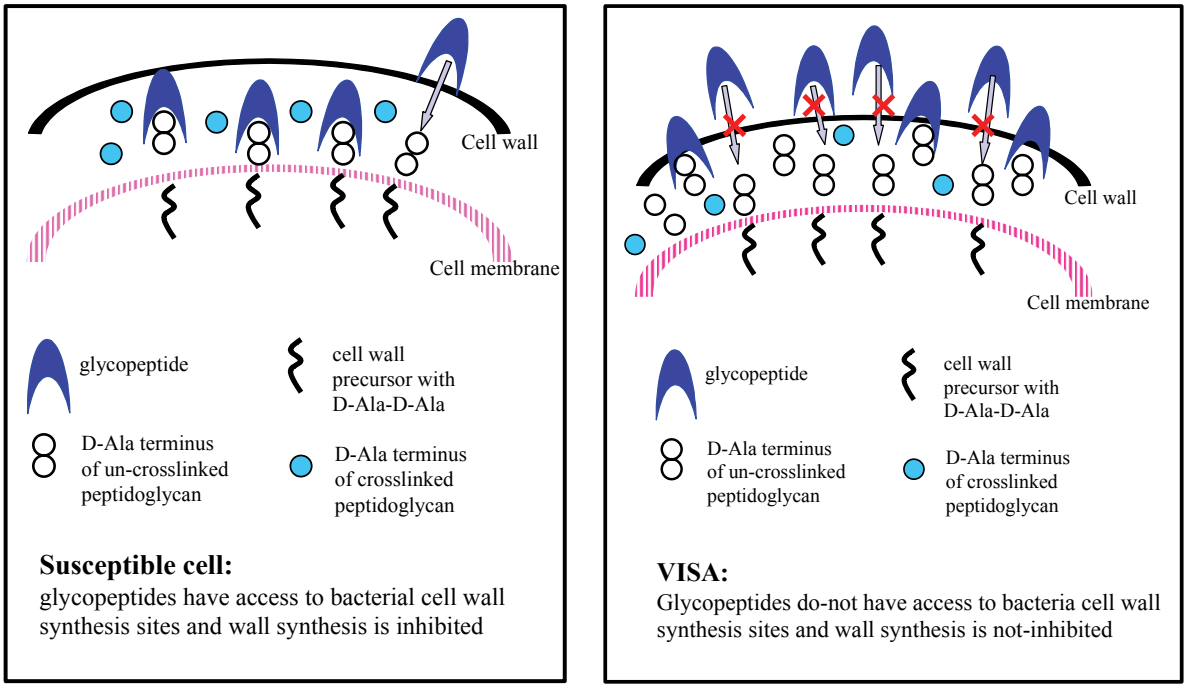

FIGURE 12. Vancomycin susceptible bacteria (left panel). Proposed VISA mechanism (right panel). The bacteria produce an increased number of precursors with thickening of PDG. Vancomycin does not fully reach its target. (adapted from 166,168).

Potential mechanisms of resistance in VISA include increases in cell wall precursors that lead to an increase of non-cross-linked D-alanyl-D-alanine side chains. These extra residues

appear to trap glycopeptides, keeping them from reaching their actual target sites near the cytoplasmic membrane. (Figure 12).

1.3.4.2.2.2. Heteroresistant- VISA : hetero- VISA, hVISA

Another form of decreased susceptibility to vancomycin and teicoplanin in *S. aureus* is a phenomenon called **heteroresistance**. These heteroresistant strains are considered precursors of VISA isolates (16,97,247).

They are defined as strains of *S. aureus* that contain subpopulations of vancomycin-resistant daughter cells but for which the MICs of vancomycin for the parent strain are 4 µg/ml.

While infection with VISA remains a rare event, data suggests that heteroresistance (hVISA) may be a more common that has been overestimated because of the lack of reliable methods of detecting it (159). Detection of VISA is difficult in the laboratory, so that their prevalence is probably underestimated (prevalence ranging between ~ 50 % to more than 20 % in Japan or in special populations like liver transplant recipients [222]). Breakpoints are given in Table 5.

TABLE 5

Interpretative criteria (µg/ml) for MIC testing of *S. aureus* and **vancomycin**			
	Susceptible	Intermediate	Resistant
BSAC	≤ 4	***	≥ 8
CLSL (2004)	≤ 2	4 - 8	≥ 16
SFM	≤ 4	8 - 16	≥ 32
SRGA	≤ 4	***	≥ 8
CDC (2006)	≤ 2	4 - 8	≥ 16
Interpretative criteria (µg/ml) for MIC testing of *S. aureus* and **teicoplanin**			
	Susceptible	Intermediate	Resistant
BSAC	≤ 4	***	≥ 8
CLSI	≤ 8	16	≥ 32
SFM	≤ 4	8 - 16	≥ 32
SRGA	≤ 4	***	≥ 8

BSAC, British Society for Antimicrobial Chemotherapy
CLSI, Clinical and Laboratory Standards Institute
SFM, Société Française de Microbiologie
SRGA, Swedish Reference Group for Antibiotics

So far, none of the VISA strains has been found to carry the vancomycin-resistant genes found in *enterococci*. However, both VISA and VRSA have a delayed growth and coagulase reaction. Morphologically their colonies often look smaller than susceptible counterparts (247).

1.3.4.2.2.3. Vancomycin resistant *S. aureus* : VRSA

In contrast to the chromosomally mediated resistance for VISA strains, the VRSA strains acquire resistance by conjugal transfer of the *vanA* operon from an *Enterococcus spp.* bacteria (168). See Figure 13.

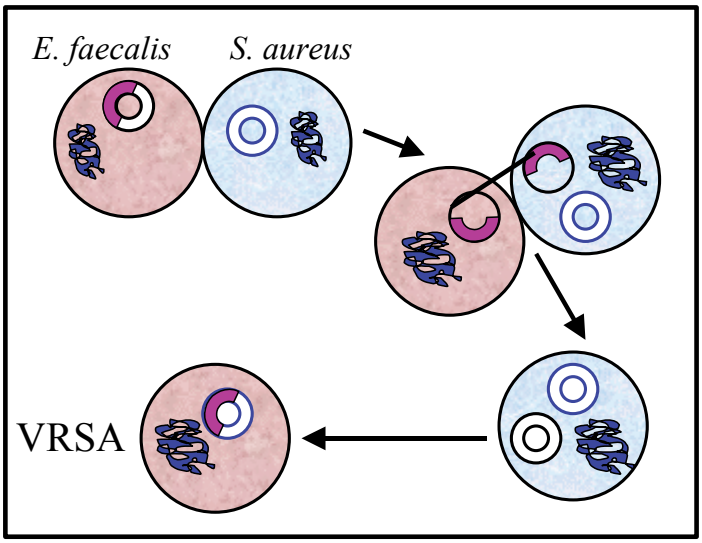

FIGURE 13. Scheme illustrating the conjugal transfer of *Enterococcus faecalis* genes to *S. aureus*.

The reports of infections caused by vancomycin-resistant *S. aureus* (VRSA) are of great concern because they reflect both complete vancomycin resistance and a very different and efficient mechanism for its dissemination. At the present time, however, only a very small number of these strains have been isolated in the clinics (16).

1.4. Conclusion

This first chapter has highlighted that the treatment of infections caused by *S. aureus* need to take into account many issues, among which the multiplicity of the resistance mechanisms it can harbour and its capacity to persist in the organism, probably as a consequence of its ability to survive within eucaryotic cells.

The next chapters will therefore focus on

(a) the current knowledge we have of the cellular pharmacokinetics and pharmacodynamics of antibiotics as predictors of their potential efficacy towards intracellular bacteria, and

(b) the properties of anti-staphylococcal antibiotics pertinent of their potential activity towards intracellular bacteria.

2. ANTISTAPHYLOCOCCAL ANTIBIOTICS

2.1. Anti-*staphylococcal* breakpoints

Table 6 summarizes the CLSI breakpoints of susceptibility of a selected group of antibiotics frequently used to treat *staphylococcal* infections and the percentage of resistance so far reported.

TABLE 6. Antibiotic breakpoints (CLSI) and % resistance in *S. aureus.*

ANTIBIOTICS (target)	molecules	MIC (µg/ml) sensitive	MIC (µg/ml) resistant
BETA-LACTAMS (PDG synthesis)	penicillin G	≤0.12	≥0.25
	penicillin V	≤0.12	≥0.25
	ampicillin	≤0.25	≥0.5
	oxacillin	≤2	≥4
	nafcillin	≤2	≥4
GLYCOPEPTIDES (PDG synthesis)	vancomycin	≤2	≥16
	teicoplanin	≤8	≥32
	oritavancin	≤1	≥8
	telavancin	≤0.5	≥8
MACROLIDES (Protein synthesis)	azithromycin	≤2	≥8
	telithromycin	≤1	≥4
AMINOGLYCOSIDES (protein synthesis)	gentamicin	≤4	≥16
OXAZOLIDINONES (protein synthesis)	linezolid	≤4	≥8
ANSAMYCIN (RNA polymerase)	rifampin	≤1	≥4
QUINOLONES (DNA synthesis)	ciprofloxacin	≤1	≥4
	levofloxacin	≤1	≥4
	moxifloxacin	≤0.5	≥2
	garenoxacin	≤1	≥4

Data from CLSI, 2000 and 2006, MIC testing, Supplemental tables, M100-S10 (M7) and (264)

2.2. Epidemiology of resistance in *S. aureus*

Table 7 shows a global view of the *S. aureus* resistance to the main classes of antibiotics in Europe. It is based on a surveillance study run from 1997 to 1999 in 25 university hospitals from different countries, which examined 3051 isolates and classified their resistance to different antibiotic classes according to their methicillin–resistance phenotype. In this study, the percentage of MRSA span from 2 % in the Netherlands to 58 % in an Italian hospital, a

value of 25 % was recorded in the Belgian hospital participating to the survey. More recent global European data are unfortunately not available, but the global trend should be the same at the present time. This table shows that resistance in MSSA is mainly directed towards beta-lactams and is related to the production of beta-lactamases, but that it does not markedly affect the other classes of drugs, except macrolides to some extent. In MRSA, the picture is very different, with extremely high percentage of resistance to macrolides, fluoroquinolones, lincosamides, aminoglycosides, and to a lower extent, rifampicin.

TABLE 7: Percentage of susceptibility to antibiotics in MSSA and MRSA from European hospitals (95)

class	**drug**	**% susceptible MSSA**	**% susceptible MRSA**
Beta-lactams	penicillin	15.4	not applicable
	ampicillin	16.1	not applicable
	amoxicillin-clavulanate	94.8	not applicable
glycopeptides	vancomycin	100	100
macrolides	erythromycin	77.5	4.8
lincosamides	clindamycin	93.7	23.3
aminoglycosides	gentamicin	94.6	22.8
tetracyclines	doxycycline	97.7	85.2
synergistins	synercid	95.3	99.5
ansamycins	rifampicin	97.4	46.1
fluoroquinolones	ciprofloxacin	90.6	9.2

2.3. Antibiotic choice

Table 8 shows the suggested antibiotic choice for *S. aureus* infections according to the phenotype of resistance.

The main pharmacokinetic and pharmacodynamic properties of these drugs, which are pertinent for our experimental studies, will be developed in the third chapter of the introduction.

TABLE 8. Antimicrobial therapy for *S. aureus* infections.

PHENOTYPE OF RESISTANCE	ANTIBIOTICS OF CHOICE (including drugs in clinical development)
Penicillin-labile sensitive *S. aureus*	Narrow spectrum penicillins (pen G, pen V)
Penicillin-resistant, oxacillin/methicillin sensitive *S. aureus*	Narrow-spectrum penicillinase-resistant penicillins (methicillin, oxacillin, nafcillin, dicloxacillin; ampicillin, amoxicillin plus beta-lactamase inhibitors (clavulanic acid, tazobactam or sulbactam), cephalosporins, carbapenems
Penicillin or oxacillin sensitive, but patient with Beta-lactam allergy	Macrolides (azithromycin, telithromycin, etc)
	Quinolones (ciprofloxacin, levofloxacin, moxifloxacin, garenoxacin, etc)
MRSA	Vancomycin, teicoplanin, linezolid, pristinamycin; or synergistic combinations: vancomycin plus rifampicin plus gentamicin ; rifampicin plus fusidic acid ; trimethoprim plus sulfamethoxazole ; fluoroquinolones plus rifampicin
VISA	Linezolid, tigecycline, quinupristin/dalfopristin, new investigational drugs: telavancin, oritavancin, dalbavancin, daptomycin, (investigational cephems (i.e: ceftobiprole) and carbapenems)
VRSA	As for VISA, except for dalbavancin (135)

data from www.ppidonline.com

2.4. Hemi-synthetic glycopeptides in development as promising compounds for multiresistant *S. aureus* infections.

The many traits of the evolved antibiotic-resistance phenotypes of *S. aureus* have re-established the bacteria as a major human pathogen and highlight the importance of finding novel therapeutic approaches (26,262,264).

Over the past 20 years, extensive research has been performed on the chemical modification of glycopeptides. There have been over a hundred different glycopeptide antibiotics identified in the fermentation broth of various bacteria. Virtually all have a heptapeptide core of seven amino acid residues with sugar substituents at various positions.

Telavancin and oritavancin are the two hemi-synthetic derivatives of vancomycin, and dalbavancin is a teicoplanin derivative currently in clinical development. They are considered as promising on the basis of their improved activity and safety profiles (26,158,199-201).

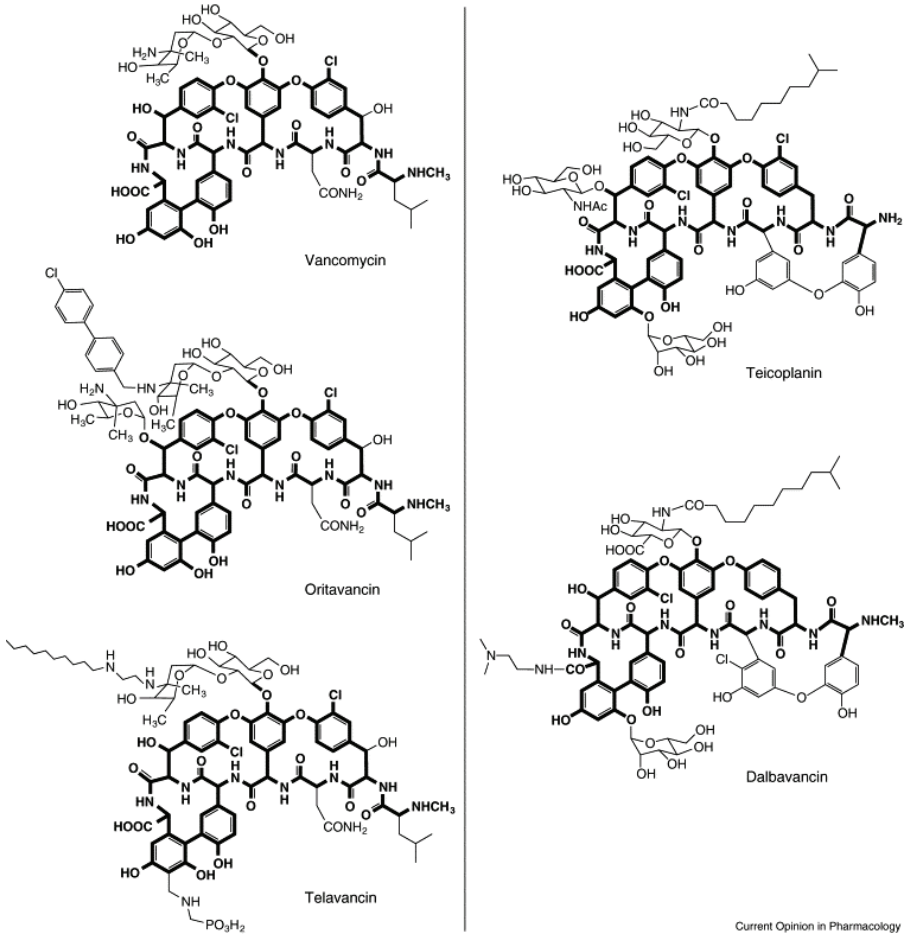

FIGURE 14. chemical structure of conventional glycopeptides, and of their hemi-synthetic derivatives in clinical development (260)

The most salient feature of these hemisynthetic derivatives is the presence of a lipophilic chain (as already present in teicoplanin). Structure-activity relationships suggest that this lipophilic chain confers to glycopeptides additional modes of action (10,264), in particular a capacity to anchor in the bacterial membrane (30), which could contribute to confer to them a bactericidal character, but also to maintain their activity against strains resistant to conventional glycopeptides (264). Table 9 compares the in vitro activity of conventional and new glycopeptides.

This lipophilic chain also increases the binding of the drugs to serum proteins, prolonging their half-life to the point that they can be administered once-a-day (oritavancin, telavancin, or even once-a-week (dalbavancin). Table 10 compares the pharmacokinetics of conventional and new glycopeptides.

TABLE 9: in vitro activity of conventional and new glycopeptides (260).

In vitro activity (MIC $_{90}$) of glycopeptides against selected target Gram-positive bacteria [32••,48–51].

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 to 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[b]		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

[a]Range based on MIC$_{90}$ values reported in different studies. [b]MIC $_{50}$
GRSA, glycopeptide-resistant *S. aureus*; MRSE, methicillin-resistant *S. epidemidis*; MSSA, methicillin-susceptible *S. aureus*; MSSE, methicillin-susceptible *S. epidermidis*; PenR, penicillin resistant; PenS, penicillin susceptible; *spp*, species.

TABLE 10: pharmacokinetic properties of conventional and new glycopeptides (260).

Pharmacokinetic parameters for glycopeptides at doses pertinent of their use in humans (or the foreseen doses for molecules in development).

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
Protein 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

[a]This breakpoint corresponds to the higher MIC for which a free AUC/MIC ratio of 125 or a free Cmax/MIC (for concentration-dependent drugs) of 10 can be reached based on a conventional daily dose (note that for new glycopeptides, these values may be underestimated because the presence of serum proteins does probably not fully impair their activity). AUC, area under the curve; Cmax, maximal concentration in the serum (peak level); Vd, distribution volume.

2.4.1. oritavancin

Oritavancin (LY333328) is the first clinical candidate of this "second generation glycopeptides". It results from a N-alkyl modification on one of the sugars of the heptapeptide core of the naturally occurring glycopeptide chloreremomycin (LY264826), a chlorobiphenyl substitution (59). This modification confers remarkably good activity against vancomycin-resistant *Enterococci* (VRE), VRSA and PRP.

The spectrum of action of the molecule includes multi-resistant strains of *S. aureus*, *S. epidermidis*, *S. pneumoniae* and *S. pyogenes*, VanA *E. faecalis* and *E. faecium* and also the intrinsically vancomycin-resistant species of *E. gallinarum* and *E. casseliflavus* (33).

The most remarkable characteristic of oritavancin is its rapid and persistent concentration-dependent bactericidal activity, a property that distinguishes it from vancomycin, linezolid and quinupristin-dalfopristin, and its insensitivity to widespread resistance in *Enterococcus spp*.

Oritavancin prolonged half-life allows for daily administration (32). Noteworthy, it reaches an exceptional cellular accumulation in the lysosomes of eucaryotic cells, in which it penetrates

by a process of endocytosis (262). In these cells, it also causes a mixed storage disorder, characterized by the deposition of heterogeneous material and the accumulation of polar lipids (263). Whether these observations may affect the safety of the molecule is unknown.
The molecule is now in Phase III clinical trials for the treatment of skin and skin structures infections.

2.4.2. dalbavancin

Dalbavancin (BI397) is a semi-synthetic derivative derivative of the teicoplanin related glycopeptide A40926 (176).
The amide appendage and the alteration of the hydrophobic acylglucosamine substituent of the heptapeptide core confers the molecule with a spectrum of activity that covers many multi-resistant bacterial species, being particularly effective against *S. epidermidis* but opposed to oritavancin, marginally against *Enterococcus spp* (41).
Its high-level serum protein binding (98%) adversely affects the antibacterial activity of the molecule but prolongs its half-life and its retention in the tissues, so that it can be administered on a once-a-week basis in 2-week treatment course (76).
The company has now been granted priority review status by the FDA for the treatment of MRSA complicated skin and soft tissue infections.

2.4.3. telavancin

Telavancin (TD-6424) is another semi-synthetic derivative of vancomycin, characterized by an hydrophobic side chain on the vancosamine sugar (decylaminoethyl) and a phosphonomethylaminomethyl substituent on the cyclic peptidic core (150). The latter substituent counterbalances to some extent the hydrophobicity brought by the lipophilic side chain, explaining why the half-life of the drug is shorter than that of oritavancin despite a similar protein binding (150,241).
The mechanism of action of telavancin has been explored in details. The drug was found capable of inhibiting the synthesis of fatty acids and phospholipids required for integrity of the bacterial, but also of directly depolarizing and permeabilizing the bacterial membrane (128), which may help to explain its highly concentration-dependent and rapid bactericidal activity, including against strains resistant to conventional glycopeptides (142). Its spectrum of activity is thus globally comparable to that of oritavancin. Telavancin has also been shown to have a long postantibiotic effect (PAE) of about 4 h against *S. aureus* MSSA, MRSA and

VISA (150,199,200). In addition, studies exploring the effect of pH, serum and inoculum size on telavancin's activity showed that all these parameters only poorly affect the *in vitro* activity of the drug (154,200).

In animal models of thigh or subcutaneous infection, meningitis, and endocarditis caused by MRSA or even by VISA, telavancin confirmed its highly concentration-dependent bacterial activity (126,175,251).
In phase II randomized double-blind clinical trials of complicated skin and skin structure infections, telavancin 10 mg/kg once daily showed higher cure and eradication rates than vancomycin when MRSA was the causative organism (250). In 2005, telavancin was granted fast track designation by the FDA for the treatment of hospitally-acquired pneumonia caused by MRSA or multiresistant *Streptococcus pneumoniae*, as well as of MRSA-associated complicated skin and skin structure infection (199), based on results from Phase III studies (completed in September 2006).

Telavancin as well as oritavancin, therefore appear as promising molecules for the treatment of multi-resistant *S. aureus* infections. Examining whether they could also be active against intracellular bacteria is one of the goals of the present work.

3. CELLULAR PHARMACODYNAMICS AND PHARMACOKINETICS OF ANTIBIOTICS

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Cellular pharmacodynamics and pharmacokinetics of antibiotics: Current views and perspectives

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The treatment of intracellular infections requires the use of antibiotics presenting appropriate cellular pharmacokinetic and pharmacodynamic properties. These properties, however, cannot be predicted on the simple basis of cellular drug accumulation and minimum inhibitory concentration in broth. In most cases, intracellular activity is actually lower than extracellular activity, despite the fact that all antibiotics reach intracellular concentrations that are at least equal to, and more often higher than the extracellular concentrations. This discrepancy may result from impairment of the expression of antibiotic activity or a change in bacterial responsiveness inside the cells. It therefore appears important to evaluate the intracellular activity of antibiotics in appropriate models.

Keywords Antibiotics, cellular accumulation, cellular pharmacodynamics, cellular pharmacokinetics, intracellular infection

Abbreviations

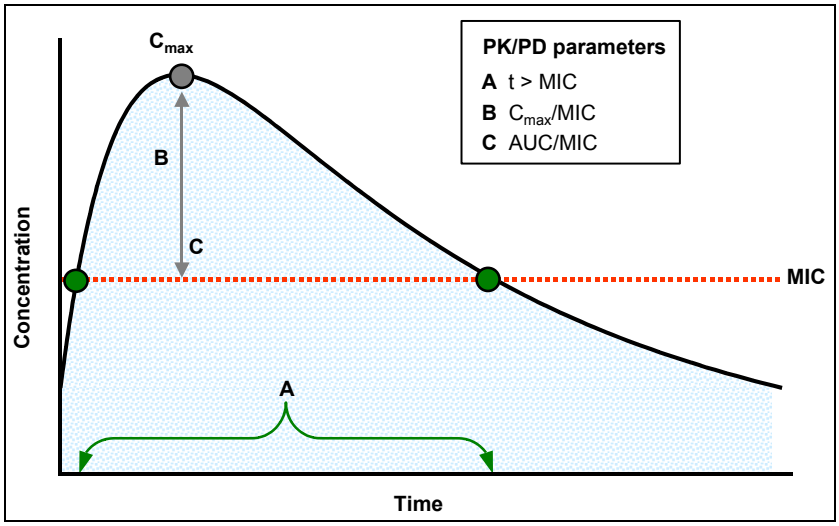
AUC	Area under the concentration-time curve
C_{max}	Peak plasma concentration
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
MRP	Multiple drug-resistance protein
PK/PD	Pharmacokinetics/pharmacodynamics

Introduction

Over the last few years, much concern has been raised regarding the optimization of antibiotic use, owing to the worrying increase of bacterial resistance and to the scarcity of new antibiotic classes under development [1]. In this context, progress in the field of anti-infective pharmacology has led to the emergence of a new discipline, referred to as pharmacokinetics/pharmacodynamics (PK/PD) of antibiotics, which is defined as the 'discipline that strives to understand the relationships between drug concentrations and effects, both desirable (eg, bacterial killing) and undesirable (eg, side effects)' [2]. Over the past 15 years, three key PK/PD parameters have been elaborated (Figure 1; for reviews, see references [3] to [6] or [7••]), which examine how antibiotic concentrations reached in body fluids over time (as predicted from the pharmacokinetic profile of the drug) compare with potentially effective antibiotic concentrations (as deduced from the minimal inhibitory concentration (MIC) or minimal bactericidal concentration (MBC) of antibiotics *in vitro*). The first parameter, time at which concentration is > MIC (t > MIC), links bactericidal effects to time and is critically dependent on the half-life of the drug, dosage and frequency of administration over a given time period. The second parameter, peak plasma concentration (C_{max})/MIC, relates bactericidal effects to concentration, and is primarily dependent on the unit dose and the volume of distribution of the drug. The third parameter, area under the concentration-time curve (AUC)/MIC, combines both types of effects, since it corresponds to the total amount of drug to which bacteria are exposed over the time period, and is directly related to the total dose given during that period and inversely proportional to the drug clearance. These parameters appear to be critical in predicting antibiotic activity and, therefore, in establishing dosages on a rational basis [8,9]. The application of these parameters, however, has so far been limited to extracellular infections in well-vascularized tissues, because they are all based on serum antibiotic levels.

The situation is, therefore, likely to be more complex when attempting to predict active antibiotic concentrations for infections developing in less accessible compartments, as is the case for intracellular infections. Some bacteria have adapted themselves to survive, and even multiply, within eukaryotic cells [10••,11]. Table 1 lists the most common pathogens responsible for intracellular infections. Besides well-known obligate or facultative intracellular organisms, several extremely common bacteria are now recognized as being able to survive intracellularly under certain circumstances. Such infections are considered as 'opportunistic', because no specific mechanism of adaptation to intracellular survival has been highlighted so far, and this survival is not an essential determinant in the life cycle of the bacteria. In the intracellular environment these bacteria become protected from humoral defenses, and probably also from antibiotic action. This may, therefore, contribute to the chronic or recurrent nature of infections in which intracellular foci are present [12,13], as classically observed for *Mycobacterium* or *Chlamydia* (for reviews, see references [14] and [15]), and also more recently demonstrated for *Staphylococcus aureus* [16-19], streptococci [20,21••], *Helicobacter pylori* [22] and *Escherichia coli* [23,24]. Thus, the selection of antibiotics endowed with intracellular activity or, preferably, with mixed extracellular and intracellular activity, appears critical in the management of such infections. For a discussion on the definition of cellular PK/PD parameters that are predictive of intracellular activity, see reference [25]. As well as considering the influence of drug concentration or the time

Figure 1. Illustration of the main PK/PD parameters that correlate with efficacy against extracellular infections.



A Time (t) during which the concentration remains above the minimum inhibitory concentration (MIC) of the antibiotic against the pathogen, **B** ratio between the peak plasma concentration (C_{max}) of the antibiotic reached in the serum and the MIC, **C** ratio between the 24-h area under the concentration-time curve (AUC) and the MIC.

Table 1. The main human pathogenic bacteria capable of intracellular survival.

Type of intracellular life	Bacterial species	Subcellular localization	Associated pathologies
Obligate	*Chlamydia pneumoniae*	Inclusions	Pneumonia
	Chlamydia trachomatis	Inclusions	Trachoma, sexually transmitted diseases
	Coxiella burnetii	(Phago)lysosomes	Q Fever, pneumonia, encephalitis, endocarditis
	Mycoplasma pneumoniae	Cytosol	Pneumonia
	Rickettsia spp	Cytosol	Fever, cat scratch, etc
Facultative	*Brucella*	Phagosomes	Brucellosis
	Francisella tularensis	Phagosomes	Tularemia
	Legionella pneumophila	Endoplasmic reticulum, lysosomes	Pneumonia
	Listeria monocytogenes	Cytosol	Meningitis, abortion
	Mycobacterium tuberculosis	Phagosomes	Tuberculosis
	Salmonella spp	Phagosomes	Digestive infections
	Shigella flexneri	Cytosol	Digestive infections
Opportunistic	*Bacillus anthracis*		Anthrax
	Borrelia burgdorferi		Lyme disease
	Campylobacter jejuni		Digestive infections
	Escherichia coli		Urinary and digestive infections
	Helicobacter pylori		Peptic ulcer
	Staphylococcus aureus	Phagolysosomes, cytosol	Skin and soft tissues infections, osteomyelitis, endocarditis, pneumonia, etc
	Streptococcus pneumoniae		Upper and lower respiratory tract infections
	Streptococcus pyogenes		Pharyngitis
	Yersinia pestis		Plague, digestive infections

of exposure on the chemotherapeutic effect at the site of infection, other parameters must be examined that will specifically modulate responses in the intracellular environment [10••]. This will result in a modulation of the MIC and MBC values within the cells, a factor which is almost never taken into account in the context of pharmacodynamics [26,27] and which may lead to inappropriate therapeutic choices and a risk of persistent infection [28,29•,30].

The objectives of this review are to present and discuss the current knowledge of the PK/PD parameters governing the intracellular activity of antibiotics, and to propose strategies for optimizing this activity.

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Cellular pharmacokinetics of antibiotics

While general pharmacokinetics relate to the absorption, distribution, metabolism and elimination of drugs in the body, cellular pharmacokinetics are centered on evaluation of the penetration, distribution, degradation and efflux of drugs in individual cells [21••,31,32]. These two fields are closely related because the cellular disposition of a drug (eg, its capacity to cross biological membranes, response to enzymatic modification or transport through epithelial cells) governs its general fate (absorption, distribution and elimination) in the body. Studying the pharmacokinetics of antibiotics in eukaryotic cells is therefore of prime importance because it defines the access of the drug to the site of infection.

Mechanisms of antibiotic uptake, distribution and efflux in eukaryotic cells

To gain access to extracellular targets or to the cellular medium within the body, drugs often use non-specific routes of entry [31], such as diffusion or endocytosis, depending on their physicochemical properties. Some drugs can also take advantage of the presence of transporters that recognize them because they share some structural similarities with endogenous molecules or nutriments.

Accumulation and distribution

Diffusion

Diffusion is the most common way for molecules of a sufficiently small size (usually molecular weight < 700 Da) and with good lipid solubility (for a review on these general concepts, see reference [33]) to cross cell membranes. Among the factors that dramatically affect membrane permeation, the ionization status of the drug appears to be of prime importance, with charged species being characterized by low lipid solubility and almost no ability to cross membranes in the absence of a specific transport mechanism. The actual rate of diffusion of a drug will thus vary according to the environmental pH, with weak bases diffusing faster at basic pH than at acidic pH and weak acids exhibiting the opposite behavior. As a result, weak bases tend to accumulate in membrane-bound acidic compartments, whereas weak acids are excluded from these sites (for a discussion of these general concepts see reference [34], and for an application to subcellular compartments see reference [35]).

β-Lactam antibiotics are thought to cross the cell membrane by passive diffusion to gain access to the cellular medium. The equilibrium concentration of these antibiotics becomes equal on either side of the membrane, resulting in an accumulation factor of approximately 1 [36-38]. Being weak acids, however, β-lactams are largely excluded from lysosomes and related acidic vacuoles. Quinolones likely also enter most cells by simple diffusion, but are more concentrated inside the cells than outside at equilibrium, for reasons which are still unclear [39,40•,41,42]. Macrolides are among the antibiotics with the highest capacity for accumulation in eukaryotic cells [43]. Because of their weak basic character, cell-associated macrolides are largely trapped in their positively charged, less diffusible form in lysosomes, with dicationic molecules (eg, azithromycin, erythromycylamine and telithromycin) reaching higher levels of accumulation than monocationic molecules (eg, erythromycin, roxithromycin, clarithromycin and cethromycin) [44-47,48•].

Endocytosis

Endocytosis is a non-specific mechanism that drives poorly diffusible molecules (ie, molecules that are 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 (for a review, see reference [49]).

Aminoglycosides are the best-characterized example of antibiotics that enter cells (kidney and ear) via a double process of adsorptive and receptor-mediated endocytosis. These highly polar molecules are polyaminated and bind to the negatively charged phospholipids of the membrane and the endocytic receptor megalin. Megalin is a protein that acts as a receptor for polyaminated compounds, and is particularly abundant in renal proximal tubules, as well as in the hair cells of the inner ear (for a review, see reference [50]). Glycopeptides, which are voluminous molecules, also enter cells via this endocytic route, and their level of accumulation in the lysosomes varies considerably depending on the type of glycopeptide. Amphiphilic glycopeptides, such as teicoplanin, dalbavancin, telavancin or oritavancin, reach much higher levels of accumulation in cells than more hydrophilic molecules such as vancomycin [51-53]. This effect is particularly evident in the case of oritavancin, the intracellular concentration of which is several hundred times higher than the extracellular concentration, which is suspected to be the result of a high level of adsorption of the molecule at the cell surface.

Inward transport

Inward transport of drugs is observed for molecules that have sufficient similarity to endogenous substrates of transporters. Active inward transport of antibiotics has been demonstrated at the surface of epithelia. This method of intracellular accumulation contributes to the intestinal absorption or re-absorption by renal tubular cells, and therefore governs the pharmacokinetics profile of antibiotics. The intestinal absorption of β-lactams (peptidomimetic drugs bearing a free acid function) is mediated by transporters of small peptides (eg, PEPT1 [54,55]) or of monocarboxylate compounds (eg, MCT1 [56]), while tubular re-absorption of β-lactams occurs via peptide transporter PEPT2 [54,55] and organic ion transporters such as OCTN2 [57]. It is worth noting that there is a huge variation in the level of recognition of different β-lactams by these transporters [55], which may explain the considerable variation in the oral bioavailability or rate of elimination of these antibiotics. Active transport is also suspected to take place in non-polarized, phagocytic cells. For example, it has been suggested that transporters of purines contribute to the accumulation of quinolones (bicyclic aromatic nuclei) in monocytes [58].

Efflux

Efflux transporters expressed at the surface of eukaryotic cells are involved in the extrusion of either polar, non-

diffusible metabolites produced within the cells, or of diffusible molecules capable of freely invading the cells. These transporters usually exhibit a broad substrate specificity by being able to recognize molecules mainly on the basis of their amphiphilicity and of the presence of ionizable functions [59]. Among these transporters, multidrug transporters of the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily (including the multidrug-resistance protein P-glycoprotein (P-gp) and multiple drug-resistance proteins (MRPs)) are ubiquitous, while organic cation or anion transporters are mainly found at the surface of epithelia [60]. When expressed in non-polarized cells, multidrug transporters reduce the cellular accumulation of drugs and, hence, affect the pharmacological activity of the drugs toward intracellular targets. For antibiotics, this transport has been demonstrated to have deleterious consequences on intracellular activity [61]. When localized at the surface of biological barriers, such as the intestine, the blood-brain barrier, the liver and the kidneys, efflux transporters contribute to reduced absorption of drugs, poor penetration of the central nervous system, or accelerated elimination by hepatic or renal routes, making serum drug concentrations suboptimal [60,62].

P-gp is thought to be involved in the transport of β-lactams, macrolides, quinolones, tetracyclines, streptogramins and trimethoprim, and MRPs are thought to transport β-lactams, macrolides, quinolones and rifamycins, at the level of epithelial cells bordering biological barriers or of phagocytic or transfected cells (see reference [60] and the references cited therein). In addition, different types of organic anion transporters contribute to the renal tubular re-absorption of β-lactams and prevent their access to the central nervous system [63,64].

Accumulation levels and subcellular distribution of the main antibiotic classes in eukaryotic cells

Table 2 summarizes the current knowledge of pharmacokinetics of antibiotics in eukaryotic cells. Macrolides and semisynthetic glycopeptides accumulate at higher levels in cells than other antibiotics, but are mainly concentrated within lysosomal vacuoles [44,45,51]. Quinolones accumulate at moderate levels and are found in the cytosolic fraction, possibly due to their high diffusibility [41]. Lincosamides and rifamycins are also concentrated within eukaryotic cells, but their localization is unknown [65,66]. Cellular concentrations of all of these antibiotic classes will be higher than serum concentrations, suggesting the potential treatment of intracellular infections located in the compartment where the drug is concentrated. Accordingly, macrolides, rifamycins and quinolones are classically considered as drugs of choice for treating intracellular infections [43,67-71]. β-Lactams penetrate, but do not accumulate within the cells, with cellular concentrations being close to extracellular concentrations, and are therefore generally considered to be of no interest for treating intracellular infections [72-74]. However, because serum levels are often quite high for this antibiotic class (peak levels > 50 mg/l), cellular concentrations might be expected to be higher than the MIC of intracellular pathogens under the conditions of their clinical use. Appropriate doses (ie, high concentration) and prolonged time of exposure (as suggested by the time-dependent activity of β-lactams in extracellular models of infection) may therefore compensate for the lack of accumulation, and confer intracellular activity to β-lactams, as was recently demonstrated in *in vitro* models [75•,76••]. Aminoglycosides accumulate slowly within cells, such that they reach active concentrations only upon prolonged exposure. They are therefore used in the management of chronic intracellular infections, such as tuberculosis [77].

Cellular pharmacodynamics of antibiotics

Based on pharmacokinetic considerations alone, it might be tempting to conclude that the intracellular activity of antibiotics can be predicted from their accumulation level. In terms of translating data obtained from cellular models into *in vivo* situations, however, a more accurate view would be obtained by considering cellular concentrations rather than accumulation factors, because this parameter also takes into account the fact that serum concentrations of antibiotics can vary considerably between classes. Some cellular concentration values are provided in Table 2, although some may be overestimated for antibiotics that are highly protein-bound, because it is essentially only the free fraction that can enter cells. It is also worth noting that local concentrations in specific compartments may be higher for antibiotics that are not distributed uniformly throughout cells.

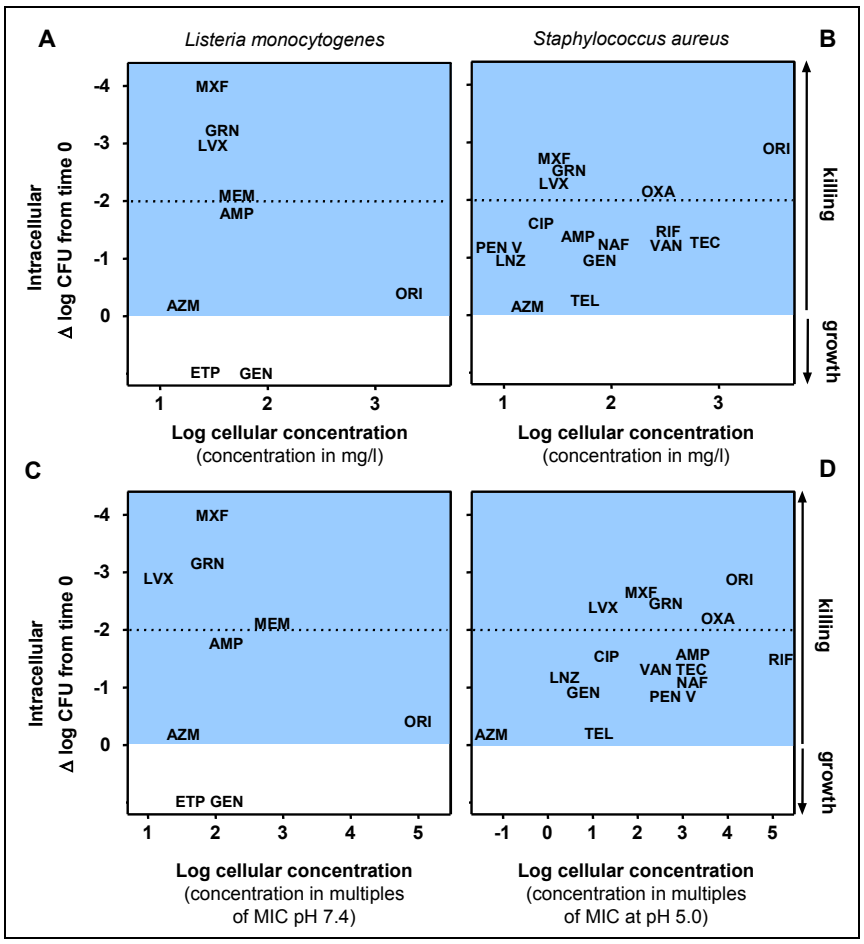
Although the cell concentrations of all antibiotics appear to be well above the MICs of susceptible organisms, studies systematically comparing the extracellular and intracellular activity of antibiotics from different classes have led to two unanticipated observations. First, there is no simple correlation between the cellular concentrations of antibiotics and intracellular activity [10••,76••,78••,79,80]. This concept is illustrated in Figure 2 (panels A and B), in which the intracellular activity of a series of antibiotics against cytosolic (*Listeria monocytogenes*) and phagolysosomal (*S aureus*) bacteria is examined and plotted as a function of log cellular concentration in THP1 macrophages, as determined in cells exposed to an extracellular concentration corresponding to the human C_{max} of each drug for 24 h. Against *L monocytogenes*, most of the antibiotics tested reached a cellular concentration of 10 to 100 mg/l, but displayed effects ranging from inactivity (bacterial growth) to a reduction in bacterial counts (-4 log). Oritavancin, which accumulated to a larger extent, was almost inactive against intracellular *L monocytogenes*, but this can be explained based on its lysosomal localization (this explanation can also be applied for the inactivity of gentamicin). Based on this explanation, it is not surprising that oritavancin is active against intracellular *S aureus*; however, it is not more bactericidal than other drugs having lower cellular concentration, such as quinolones. Second, although cellular concentrations are generally higher than extracellular concentrations, antibiotic activity can be lower intracellularly than extracellularly, at least against a phagolysosomal bacterium such as *S aureus*. The extracellular and intracellular activity of the same antibiotics as in Figure 2, are correlated in Figure 3. Against *L monocytogenes*, however, the

Table 2. Cellular pharmacokinetics of the main antibiotic classes within eukaryotic cells

Pharmacochemical class	Antibiotic	Accumulation level at equilibrium (C_C/C_E)[a]	Cellular concentration at equilibrium (mg/l)[b]	Time to equilibrium	Accelerated efflux due to active transport[c]	Predominant subcellular localization
β-Lactams	All	< 1	~ 20 to 50	Fast	P-gp, MRP, anion/cation transporters	Cytosol
Macrolides	Erythromycin	4 to 10	~ 40 to 150	Moderate (a few hours)	P-gp	2/3 Lysosomes 1/3 Cytosol
	Clarithromycin Roxithromycin Telithromycin	10 to 50	~ 20 to 400			
	Azithromycin	40 to 300	~ 16 to 120			
Fluoroquinolones	Ciprofloxacin Levofloxacin Grepafloxacin	4 to 10	~ 16 to 40	Fast (< 1 h) to very fast (< 5 min)	MRP, P-gp, anion/ cation transporters	Cytosol
	Moxifloxacin Garenoxacin Gemifloxacin	10 to 20	~ 40 to 80		Marginal or unknown	
Aminoglycosides	All	2 to 4 (after several days)	~ 40 to 80	Slow (several days)	Improbable	Lysosomes
Lincosamides	Clindamycin	5 to 20	~ 50 to 200	Fast	Unknown	Unknown
	Lincomycin	1 to 4	~ 15 to 60		None	
Tetracyclines	Probably all	1 to 4	~ 2 to 12	Unknown	P-gp	Unknown
Ansamycins (rifamycins)	Rifampin	2 to 10	~ 36 to 180	Unknown	MRP	Unknown
	Rifapentine	60 to 80	~ 1200 to 1600	Unknown		
Glycopeptides	Vancomycin	8 (after 24 h)	~ 400	Slow (several hours)	Improbable	Lysosomes (in kidney)
	Teicoplanin	60	~ 6000			Unknown
	Oritavancin	150 to 300 (after 24 h)	~ 3750 to 7500			Lysosomes
	Telavancin	50 (after 24 h)	~ 4500			Lysosomes
Oxazolidinones	Linezolid	~ 1	~ 20	Unknown	Unknown	Unknown

[a]C_C/C_E represents the accumulation factor (ie, the ratio between the cellular concentration (C_C) and the extracellular concentration (C_E)) in cultured macrophages. [b]Calculated from the accumulation ratio in cultured macrophages, using the average human C_{max} for the antibiotic under consideration as the C_E value. [c]P-glycoproteins (P-gps) and multiple drug-resistance proteins (MRPs) belong to the ABC superfamily of transporters energized by ATP hydrolysis, which are expressed in epithelial and phagocytic cells. Cation, anion and peptide transporters belong to different transporter families, but are all energized by ion gradients and are expressed essentially in epithelial cells. Table 2 is based on data from references [10••], [32] and [60].

Figure 2. Relationship between the intracellular activity of antibiotics and their cellular concentration.



The graphs show the intracellular activity of a series of antibiotics against *Listeria monocytogenes* (left) and *Staphylococcus aureus* (right) in a model of THP1 human macrophages. Activity is expressed as the change in bacterial counts following 24 h of exposure (or 5 h of exposure for oritavancin in the *L monocytogenes* model) to each of the selected antibiotics at an extracellular concentration corresponding to its human C_{max}. The cellular concentrations were all measured under the same conditions, and are expressed in terms of log of the mg/l values (panels **A** and **B**) or log of multiples of the minimum inhibitory concentration (MIC) as determined in broth at the pH of the corresponding infected compartment (panels **C** and **D**). The blue zones correspond to bacterial killing, while the dotted lines show the limit of bactericidal effect (-2 log according to the recommendations of the Clinical and Laboratory Standards Institute). The limit of detection was -4.2 log, and all values below this limit were set at -5 log. The graphs are based on data from references [75•], [76••] and [107].
AMP Ampicillin, **AZM** azithromycin, **CFU** colony forming units, **CIP** ciprofloxacin, **ETP** ertapenem, **GEN** gentamicin, **GRN** garenoxacin, **LNZ** linezolid, **LVX** levofloxacin, **MEM** meropenem, **MXF** moxifloxacin, **NAF** nafcillin, **ORI** oritavancin, **OXA** oxacillin, **PEN V** penicillin V, **RIF** rifampin, **TEC** teicoplanin, **TEL** telithromycin, **VAN** vancomycin.

intracellular activity of antibiotics is lower, similar (eg, for quinolones), or even higher (eg, for some β-lactams) than extracellular activity. Importantly, macrolides, which are among the antibiotics accumulating in cells at a higher level, are poorly active intracellularly (see reference [69]), probably due to their intrinsic bacteriostatic nature.

The general low intracellular activity of some antibiotics could result from: (i) poor bioavailability of the accumulated antibiotic, making pharmacokinetic predictions incorrect; or (ii) a shift of MICs toward higher values in the intracellular milieu, underlining the importance of pharmacodynamic considerations. Such changes in MICs could be due to either an impaired expression of antibacterial activity within the intracellular environment, or an altered bacterial responsiveness within eukaryotic cells.

Cellular bioavailability of antibiotics

In extracellular models, activity is best predicted from the free serum concentration of antibiotics, which represents the fraction diffusing through tissues and reaching bacterial targets [81]. The absence of a correlation between the total amount of antibiotic associated with cells and the intracellular activity may suggest that part of the accumulated drug is not bioavailable because of binding to cellular constituents. The interaction of antibiotics with cellular proteins has not been documented in the literature to date, but is highly probable. What is known, however, is

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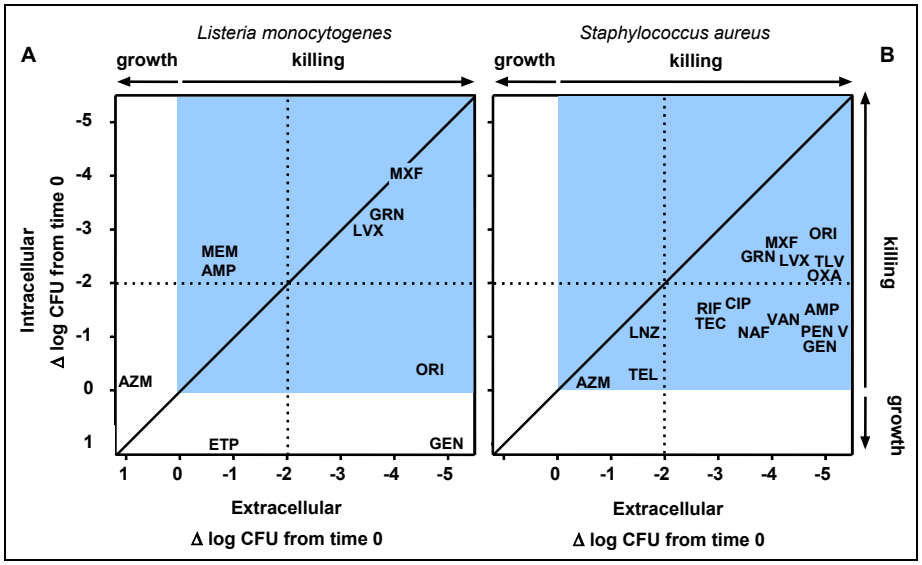
Figure 3. Correlation between the intracellular and the extracellular activity of antibiotics.



The graph shows the correlation between the intracellular and extracellular activity of a series of antibiotics against *Listeria monocytogenes* (panel **A**) and *Staphylococcus aureus* (panel **B**), in a model of THP1 human macrophages. Activity is expressed as the change in bacterial count following 24 h of exposure (or 5 h of exposure for oritavancin in the *L monocytogenes* model) to each of the selected antibiotics at an extracellular concentration corresponding to its human C_{max}, both extracellularly (x-axis) and in infected macrophages (y-axis). The blue zones correspond to bacterial killing, while the dotted lines point to the limit of bactericidal effect (-2 log according to the recommendations of the Clinical and Laboratory Standards Institute). The limit of detection was -4.2 log, and all values below this limit were set at -5 log. The diagonal line delineates the experimental points expected for drugs displaying equal extracellular and intracellular activities, with points above this line corresponding to bactericidal activities that are higher intracellularly than extracellularly, and below the line to activities that are higher extracellularly than intracellularly. The graphs are based on data from references [75•], [76••] and [107].
AMP ampicillin, **AZM** azithromycin, **CFU** colony forming units, **CIP** ciprofloxacin, **ETP** ertapenem, **GEN** gentamicin, **GRN** garenoxacin, **LNZ** linezolid, **LVX** levofloxacin, **MEM** meropenem, **MXF** moxifloxacin, **NAF** nafcillin, **ORI** oritavancin, **OXA** oxacillin, **PEN V** penicillin V, **RIF** rifampin, **TEC** teicoplanin, **TEL** telithromycin, **VAN** vancomycin.

that some antibiotic classes such as aminoglycosides and macrolides, and also oritavancin, tightly bind to the lipid constituents of membranes, causing even lipid deposition within the lysosomes [46,82,83].

Intracellular expression of antibiotic activity

Environmental effects on antibiotic expression of activity can partly be taken into account by plotting activity as a function of the cellular concentration, expressed in multiples of the MIC, as determined at neutral pH for the cytosolic *L monocytogenes*, but at acidic pH for the phagolysosomal *S aureus*. Figures 2C and 2D show that, in acidic milieu, this correction negatively affects the cellular concentration of macrolides, gentamicin and, to a lesser extent, quinolones, but enhances the cellular concentration of rifampin, and marginally that of β-lactams, while not altering the cellular concentration of glycopeptides and linezolid. This correction does not, however, improve the correlation between cellular concentration and intracellular activity, suggesting that the influence of the cellular environment extends beyond pH effects.

Among other factors specific to the intracellular milieu of phagocytes, cell defense mechanisms can either cooperate with or antagonize antibiotic action. For example, inhibiting oxidative burst in macrophages reduces the intracellular activity of quinolones against *L monocytogenes*, suggesting that oxidant species reinforce the efficacy of this class of antibiotic [84]. In contrast, global impairment of cell defense mechanisms does not prevent the unanticipated intracellular bactericidal effect of β-lactams against *L monocytogenes* [85], suggesting that bacteria have increased susceptibility to these antibiotics within the cells.

Intracellular bacterial responsiveness to antibiotics

Bacteria growing inside eukaryotic cells may undergo drastic changes in their metabolism to adapt to the new and sometimes hostile environment of cells compared with the extracellular environment. Such changes have been well characterized for obligate and facultative bacteria, which need to produce additional proteins to escape from phagosomes and move in the cytosol (as observed for *Listeria* or *Shigella* [86,87]), or to prevent the fusion of phagosomes with lysosomes to enable the infection of phagosomes (as observed for *Legionella* or *Chlamydia* [88]). Recent studies examining, in a global fashion, genetic expression or protein profiles of intracellular bacteria or bacteria exposed to a mild acidic environment have demonstrated multiple metabolic modifications [89-91]. It is probable that some of these changes may influence antibiotic action, as suggested above, which might explain the increased sensitivity of intracellular *Listeria* to β-lactams. Also, the growth rate of some bacteria is generally reduced inside the cells [92-94], highlighting their need to adapt to a hostile environment. This delay in growth can contribute to

impaired antibiotic activity, since many classes of antibiotics act upon bacteria in the active stage of multiplication, as demonstrated by the growth-cycle-dependent efficacy of antibiotics against *Chlamydia* [95]. Moreover, local pH can affect not only antibiotic action, but also bacterial response to antibiotics. A surprising example of this is methicillin-resistant *S aureus*, which becomes sensitive to β-lactams intracellularly [96], probably because of a favorable effect of acidity [97]. Finally, mechanisms of resistance can affect bacterial responses to antibiotics, although it is not known how the intracellular environment may influence the expression of inducible mechanisms. Among such mechanisms, efflux pump overexpression is widespread and contributes to antibiotic resistance, both in Gram-positive and Gram-negative bacteria [98], including in pathogens capable of surviving inside eukaryotic cells. The expression of efflux pumps is essentially based on the role they play in bacterial virulence or survival within the host, as demonstrated for Gram-negative bacteria [99,100]; whether or not efflux pumps express *in vivo* is, however, still under debate. In this context, studies aimed at determining whether bacterial efflux pumps are expressed within eukaryotic cells and whether they reduce the concentration, and consequently the activity, of antibiotics in this compartment, would be welcome in the future.

Strategies to optimize intracellular activity of antibiotics

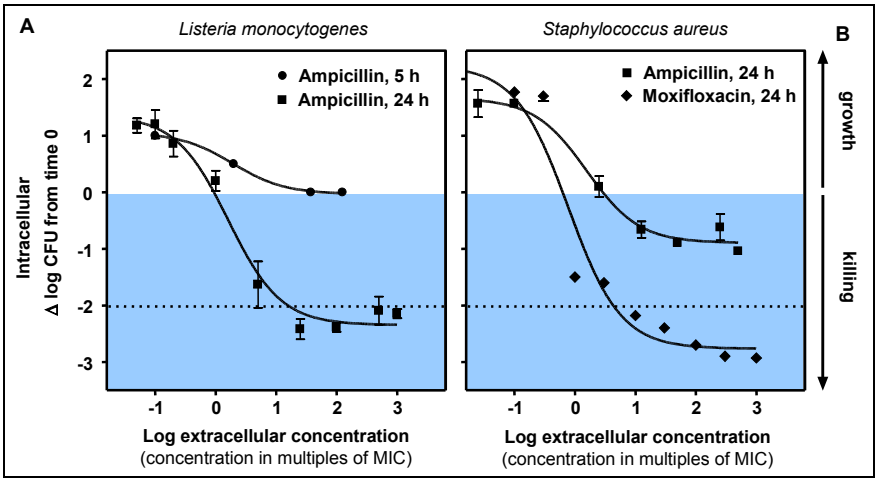
Optimizing cellular pharmacokinetics

Although there is no correlation between accumulation *per se* and activity, the intracellular activity of a given antibiotic can be optimized by maximizing its cellular concentration and the time of exposure. This effect is exemplified in Figure 4 (panel A), in which the influence of the time and concentration on the activity of a β-lactam against *L monocytogenes* is shown. The activity develops in a sigmoidal manner with concentration, and a marked bactericidal effect is obtained only with high concentrations of, and prolonged exposure to, antibiotics. Strategies aimed at optimizing drug content inside the cells over time are therefore liable to improve intracellular activity.

In the case of β-lactams, which do not accumulate to a large extent in eukaryotic cells, appropriate chemical modifications may alter their cellular pharmacokinetic profile and favor their uptake by eukaryotic cells. For example, grafting a weak basic function to and masking the acidic character of penicillin makes the molecule prone to accumulate within the lysosomes [37], whereas masking the acidic character of ampicillin in a cleavable prodrug ester markedly increases the cellular concentration of free ampicillin as well as its activity against intracellular *L monocytogenes* [38]. In the case of aminoglycosides, which slowly accumulate in cells, using an appropriate formulation such as antibiotic-loaded microspheres, improves intracellular activity by increasing the phagocytic rate of the drug [101,102]. This strategy is also efficient for increasing the efficacy of rifampin toward *Mycobacterium tuberculosis*, by allowing the slow release of the drug inside infected cells [103].

On the other hand, for antibiotics extruded out of cells by active efflux mechanisms, inhibition of the corresponding transporters increases the cellular drug content and, as a consequence, the intracellular activity. This is demonstrated by the increase in activity of quinolones against *L monocytogenes* in the presence of gemfibrozil, or the increase in activity of macrolides against both *L monocytogenes* and *S aureus* when cells are exposed to verapamil [104,105].

Figure 4. Influence of time and of concentration on the intracellular activity of antibiotics.



A Shows the influence of time and concentration on the intracellular activity of ampicillin against *Listeria monocytogenes* in infected THP1 macrophages exposed for 5 or 24 h to increasing concentrations of the drug, expressed as the log of multiples of its minimum inhibitory concentration (MIC). **B** Provides a comparison of the dose-effect relationship of the activity of ampicillin and moxifloxacin against *Staphylococcus aureus* in infected THP1 macrophages exposed for over 24 h to increasing multiples of their MIC. The blue zones correspond to bacterial killing, while the dotted lines show the limit of bactericidal effect (-2 log according to the recommendations of the Clinical and Laboratory Standards Institute). The graphs are based on data from references [75•], [76••] and [78••].

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Taking into account cellular pharmacodynamics

Currently, we have only a partial view of factors influencing antibiotic activity or bacterial responsiveness to antibiotics inside cells. Antibiotic selection should be based on MIC data, as determined at the pH of the compartment in which infection develops [76••,80,96]; however, it has been observed that many parameters other than pH affect the intracellular activity of antibiotics, such that the final choice of a drug should also be based on studies of pertinent *in vitro* cellular models, in which pharmacokinetic parameters are optimized. This is exemplified in Figure 4 (panel B), in which the dose-effect relationship of two antibiotics with similar MICs are compared against intracellular *S aureus* over 24 h and at neutral pH. The pharmacological responses appear to differ in maximal effect and EC_{50} values, suggesting the importance of examining the bacterial response to a drug within the physiological environment.

Developing appropriate models

Models need to be developed that closely mimic the clinical conditions of antibiotic use in terms of concentration and antibiotic exposure, and that integrate these pharmacokinetic parameters with pharmacodynamic considerations. Currently, most *in vitro* models use constant static concentrations of antibiotics, but modulate either the time of exposure or the extracellular concentration [26,69,75•,76••,78••,94,106-109]. These current models may be appropriate to study the impact of antibiotic combinations on pharmacokinetics (eg, competition for transport [110]) and pharmacodynamics (eg, synergy or antagonism [111-113]). In addition, modeling of the variation in antibiotic concentrations over time using dynamic *in vitro* models (Figure 1) could help to reproduce more accurately the actual exposure of infected cells to antibiotics. Currently, however, *in vitro* models often use facultative intracellular pathogens, which are grown in broth [114,115••,116]. A more ideal situation would be to develop dynamic models with bacteria growing inside eukaryotic cells [117••]. Recent efforts have also been directed toward developing methodologies that allow for the sensitive and rapid detection of intracellular bacteria [118••,119••] or for the routine evaluation of intracellular efficacy of antibiotics [120,121]. These types of studies should be included in the early development of new antibiotics, especially if their spectrum of activity includes bacteria capable of intracellular survival (eg, see references [51], [69] and [122] to [125]).

In vivo models have been developed for several opportunistic or facultative bacteria [126-129], and these are essential for the appraisal of therapeutic schemes established based on *in vitro* data, to correctly address drug bioavailability issues and assess cooperation with host defenses. In this respect, direct measurement of intracellular concentrations of antibiotics *in vivo* through non-invasive approaches [130•] will allow significant progress to be made in correlating activity with actual drug concentration at the infected site. *In vivo* models can confirm unanticipated intracellular activity observed *in vitro*, for example, in the case of a new derivative of ethambutol that proved to be as active *in vivo* as *in vitro* in infected cells against *M tuberculosis*, and demonstrated a high tissue concentration, despite low oral bioavailability [131], or for quinolones, which proved to be efficient *in vivo* against *L monocytogenes* [132], in accordance with their *in vitro* behavior in infected macrophages [78••]. *In vivo* models can also help to provide a greater understanding of the pharmacokinetic issues responsible for lack of efficacy, such as the inappropriate dosing of the β-lactam mecillinam (which generates concentrations that remain above the MIC for only 6 to 7 h) associated with intracellular survival of *E coli* [29•], or the poor bioavailability of aminoglycoside-loaded microspheres, which originally showed promise *in vitro* against *Brucella abortus*, but which proved extremely disappointing *in vivo* [133].

Conclusion

The cellular accumulation of antibiotics has long been considered to be predictive of activity against intracellular infections. This concept needs to be revisited, based on recent observations that expression of activity of antibiotics and bacterial responsiveness may be considerably modified in the intracellular environment. Activity should therefore be tested in appropriate models of intracellular infections that take into account pharmacokinetic considerations (eg, time of exposure and concentrations achievable *in vivo*), and which can be used to investigate the parameters modulating pharmacodynamic behavior.

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References

•• of outstanding interest
• of special interest

1. Bosso JA: **The antimicrobial armamentarium: Evaluating current and future treatment options.** *Pharmacotherapy* (2005) **25**(10 Pt 2):55S-62S.

2. Amsden GW, Ballow CH, Bertino JSJ: **Pharmacokinetics and pharmacodynamics of anti-infective agents.** In: *Principles and Practice of Infectious Diseases*. Mandell GL, Bennet JE, Dolin R (Eds), Churchill Livingstone, Philadelphia, PA, USA (2000):253-261.

3. Andes D, Anon J, Jacobs MR, Craig WA: **Application of pharmacokinetics and pharmacodynamics to antimicrobial therapy of respiratory tract infections.** *Clin Lab Med* (2004) **24**(2):477-502.

4. Andes D, Craig WA: **Understanding pharmacokinetics and pharmacodynamics: Application to the antimicrobial formulary decision process**. In: *Antibiotic Optimization: Concepts and Strategies in Clinical Practice*. Owens RC Jr, Ambrose PG, Nightingale CH (Eds), Marcel Dekker, New York, NY, USA (2005):65-88.

5. Craig WA: **Pharmacokinetic/pharmacodynamic parameters: Rationale for antibacterial dosing of mice and men.** *Clin Infect Dis* (1998) **26**(1):1-10.

6. Craig WA: **Basic pharmacodynamics of antibacterials with clinical applications to the use of β-lactams, glycopeptides and linezolid.** *Infect Dis Clin North Am* (2003) **17**(3):479-501.

7. Mouton JW, Dudley MN, Cars O, Derendorf H, Drusano GL: **Standardization of pharmacokinetic/pharmacodynamic (PK/PD) terminology for anti-infective drugs: An update.** *J Antimicrob Chemother* (2005) **55**(5):601-607.

•• *Provides a review of the terminology used in the field of PK/PD of antibiotics.*

8. McKinnon PS, Davis SL: **Pharmacokinetic and pharmacodynamic issues in the treatment of bacterial infectious diseases.** *Eur J Clin Microbiol Infect Dis* (2004) **23**(4):271-288.

9. Barger A, Fuhst C, Wiedemann B: **Pharmacological indices in antibiotic therapy.** *J Antimicrob Chemother* (2003) **52**(6):893-898.

10. Carryn S, Chanteux H, Seral C, Mingeot-Leclercq MP, Van Bambeke F, Tulkens PM: **Intracellular pharmacodynamics of antibiotics.** *Infect Dis Clin North Am* (2003) **17**(3):615-634.

•• *Provides a review of the cellular pharmacodynamics of antibiotics.*

11. Hackstadt T: **The diverse habitats of obligate intracellular parasites.** *Curr Opin Microbiol* (1998) **1**(1):82-87.

12. Young D, Hussell T, Dougan G: **Chronic bacterial infections: Living with unwanted guests.** *Nat Immunol* (2002) **3**(11):1026-1032.

13. Valentin-Weigand P: **Intracellular invasion and persistence: Survival strategies of *Streptococcus suis* and *Mycobacterium avium* ssp paratuberculosis.** *Berl Munch Tierarztl Wochenschr* (2004) **117**(11-12):459-463.

14. Cosma CL, Sherman DR, Ramakrishnan L: **The secret lives of the pathogenic mycobacteria.** *Annu Rev Microbiol* (2003) **57**:641-676.

15. Hammerschlag MR: **The intracellular life of chlamydiae.** *Semin Pediatr Infect Dis* (2002) **13**(4):239-248.

16. Clement S, Vaudaux P, Francois P, Schrenzel J, Huggler E, Kampf S, Chaponnier C, Lew D, Lacroix JS: **Evidence of an intracellular reservoir in the nasal mucosa of patients with recurrent *Staphylococcus aureus* rhinosinusitis.** *J Infect Dis* (2005) **192**(6):1023-1028.

17. Ellington JK, Harris M, Webb L, Smith B, Smith T, Tan K, Hudson M: **Intracellular *Staphylococcus aureus*. A mechanism for the indolence of osteomyelitis.** *J Bone Joint Surg Br* (2003) **85**(6):918-921.

18. Brouillette E, Grondin G, Shkreta L, Lacasse P, Talbot BG: ***In vivo* and *in vitro* demonstration that *Staphylococcus aureus* is an intracellular pathogen in the presence or absence of fibronectin-binding proteins.** *Microb Pathog* (2003) **35**(4):159-168.

19. Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP: **Survival of *Staphylococcus aureus* inside neutrophils contributes to infection.** *J Immunol* (2000) **164**(7):3713-3722.

20. Marouni MJ, Barzilai A, Keller N, Rubinstein E, Sela S: **Intracellular survival of persistent group A streptococci in cultured epithelial cells.** *Int J Med Microbiol* (2004) **294**(1):27-33.

21. Mandell GL: **Uptake, transport, delivery and intracellular activity of antimicrobial agents.** *Pharmacotherapy* (2005) **25**(12):130S-133S.

•• *Provides a review of the cellular pharmacokinetics of antibiotics.*

22. Blaser MJ, Atherton JC: ***Helicobacter pylori* persistence: Biology and disease.** *J Clin Invest* (2004) **113**(3):321-333.

23. Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ: **Intracellular bacterial biofilm-like pods in urinary tract infections.** *Science* (2003) **301**(5629):105-107.

24. Anderson GG, Dodson KW, Hooton TM, Hultgren SJ: **Intracellular bacterial communities of uropathogenic *Escherichia coli* in urinary tract pathogenesis.** *Trends Microbiol* (2004) **12**(9):424-430.

25. Butts JD: **Intracellular concentrations of antibacterial agents and related clinical implications.** *Clin Pharmacokinet* (1994) **27**(1):63-84.

26. Bakker-Woudenberg IA, van Vianen W, van Soolingen D, Verbrugh HA, van Agtmael MA: **Antimycobacterial agents differ with respect to their bacteriostatic versus bactericidal activities in relation to time of exposure, mycobacterial growth phase and their use in combination.** *Antimicrob Agents Chemother* (2005) **49**(6):2387-2398.

27. Nuermberger E, Grosset J: **Pharmacokinetic and pharmacodynamic issues in the treatment of mycobacterial infections.** *Eur J Clin Microbiol Infect Dis* (2004) **23**(4):243-255.

28. Brouillette E, Martinez A, Boyll BJ, Allen NE, Malouin F: **Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure *in vivo*.** *FEMS Immunol Med Microbiol* (2004) **41**(1):35-41.

29. Kerrn MB, Struve C, Blom J, Frimodt-Moller N, Krogfelt KA: **Intracellular persistence of *Escherichia coli* in urinary bladders from mecillinam-treated mice.** *J Antimicrob Chemother* (2005) **55**(3):383-386.

• *Persistence of intracellular E coli upon inappropriate dosage of β-lactams in vivo.*

30. Krut O, Sommer H, Kronke M: **Antibiotic-induced persistence of cytotoxic *Staphylococcus aureus* in non-phagocytic cells.** *J Antimicrob Chemother* (2004) **53**(2):167-173.

31. Steinberg TH: **Cellular transport of drugs.** *Clin Infect Dis* (1994) **19**(5):916-921.

32. Tulkens PM: **Intracellular distribution and activity of antibiotics.** *Eur J Clin Microbiol Infect Dis* (1991) **10**(2):100-106.

33. Rang HP, Dale MM, Ritter JM, Moore PK: **Absorption and distribution of drugs.** In: *Pharmacology*. Churchill Livingstone Inc, Edinburgh, Scotland (2003):91-105.

34. Wilkinson GR: **Pharmacokinetics: The dynamics of drug absorption, distribution and elimination.** In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. Hardman JG, Limbird LE (Eds), McGraw-Hill Publishing Co, New York, NY, USA (2001):3-30.

35. de Duve C, de Barsy T, Poole B, Trouet A, Tulkens P, Van Hoof F: **Commentary. Lysosomotropic agents.** *Biochem Pharmacol* (1974) **23**(18):2495-2531.

36. Laufen H, Wildfeuer A, Rader K: **Uptake of antimicrobial agents by human polymorphonuclear leucocytes.** *Arzneimittel-Forsch* (1985) **35**(7):1097-1099.

37. Renard C, Vanderhaeghe HJ, Claes PJ, Zenebergh A, Tulkens PM: **Influence of conversion of penicillin G into a basic derivative on its accumulation and subcellular localization in cultured macrophages.** *Antimicrob Agents Chemother* (1987) **31**(3):410-416.

38. Chanteux H, Mingeot-Leclercq MP, Sonveaux E, Van Bambeke F, Tulkens PM: **Intracellular accumulation and activity of ampicillin used as free drug and as its phthalimidomethyl or pivaloyloxymethyl ester (pivampicillin) against *Listeria monocytogenes* in J774 macrophages.** *J Antimicrob Chemother* (2003) **52**(4):610-615.

39. Dorian M, Grellet J, Saux MC: **Uptake of fluoroquinolones in human monocytes isolated from peripheral blood.** *J Pharm Pharmacol* (1998) **50**(7):783-788.

40. Michot JM, Seral C, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM: **Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin and moxifloxacin) in J774 macrophages.** *Antimicrob Agents Chemother* (2005) **49**(6):2429-2437.

• *Describes a comparative study of the accumulation and efflux of quinolones in macrophages.*

41. Carlier MB, Scorneaux B, Zenebergh A, Desnottes JF, Tulkens PM: **Cellular uptake, localization and activity of fluoroquinolones in uninfected and infected macrophages.** *J Antimicrob Chemother* (1990) **26**(Suppl B):27-39.

42. Rispal P, Grellet J, Celerier C, Breilh D, Dorian M, Pellegrin JL, Saux MC, Leng B: **Comparative uptake of sparfloxacin and ciprofloxacin into human THP 1 monocytic cells.** *Arzneimittel-Forsch* (1996) **46**(3):316-319.

228 Current Opinion in **Drug Discovery & Development** 2006 Vol 9 No 2

43. Amsden GW: **Advanced-generation macrolides: Tissue-directed antibiotics.** *Int J Antimicrob Agents* (2001) **18**(Suppl 1):S11-S15.

44. Carlier MB, Zenebergh A, Tulkens PM: **Cellular uptake and subcellular distribution of roxithromycin and erythromycin in phagocytic cells.** *J Antimicrob Chemother* (1987) **20**(Suppl B):47-56.

45. Carlier MB, Garcia-Luque I, Montenez JP, Tulkens PM, Piret J: **Accumulation, release and subcellular localization of azithromycin in phagocytic and non-phagocytic cells in culture.** *Int J Tissue React* (1994) **16**(5-6):211-220.

46. Montenez JP, Van Bambeke F, Piret J, Brasseur R, Tulkens PM, Mingeot-Leclercq MP: **Interactions of macrolide antibiotics (erythromycin A, roxithromycin, erythromycylamine [dirithromycin] and azithromycin) with phospholipids: Computer-aided conformational analysis and studies on acellular and cell culture models.** *Toxicol Appl Pharmacol* (1999) **156**(2):129-140.

47. Pascual A, Ballesta S, Garcia I, Perea EJ: **Uptake and intracellular activity of ketolide HMR 3647 in human phagocytic and non-phagocytic cells.** *Clin Microbiol Infect* (2001) **7**(2):65-69.

48. Bosnar M, Kelneric Z, Munic V, Erakovic V, Parnham MJ: **Cellular uptake and efflux of azithromycin, erythromycin, clarithromycin, telithromycin and cethromycin.** *Antimicrob Agents Chemother* (2005) **49**(6):2372-2377.
• *Provides a comparison of the cellular accumulation of macrolides.*

49. Pastan I, Willingham MC: **The pathway of endocytosis.** In: *Endocytosis*. Plenum Press, New York, NY, USA (1985):1-44.

50. Nagai J, Takano M: **Molecular aspects of renal handling of aminoglycosides and strategies for preventing the nephrotoxicity.** *Drug Metab Pharmacokinet* (2004) **19**(3):159-170.

51. Van Bambeke F, Carryn S, Seral C, Chanteux H, Tyteca D, Mingeot-Leclercq MP, Tulkens PM: **Cellular pharmacokinetics and pharmacodynamics of the glycopeptide antibiotic oritavancin (LY333328) in a model of J774 mouse macrophages.** *Antimicrob Agents Chemother* (2004) **48**(8):2853-2860.

52. Bulgheroni A, Jabes D, Pollini W, Carrano L, Desperati V, Romagnoli M, Rovida C, Colombo M, Garofalo F: **Dalbavancin (DAL) uptake by murine macrophages.** *44th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Washington, DC, USA (2004):A1490.

53. Barcia-Macay M, Mingeot-Leclercq MP, Tulkens PM, Van Bambeke F: **Telavancin (TEL) accumulates in cultured macrophages and is active against intracellular *S aureus*.** *45th Interscience Conference on Antimicrobial Agents and Chemotherapy,* Washington, DC, USA (2005):A-1831.

54. Ganapathy ME, Brandsch M, Prasad PD, Ganapathy V, Leibach FH: **Differential recognition of β-lactam antibiotics by intestinal and renal peptide transporters, PEPT 1 and PEPT 2.** *J Biol Chem* (1995) **270**(43):25672-25677.

55. Luckner P, Brandsch M: **Interaction of 31 β-lactam antibiotics with the H^+/peptide symporter PEPT2: Analysis of affinity constants and comparison with PEPT1.** *Eur J Pharm Biopharm* (2005) **59**(1):17-24.

56. Li YH, Tanno M, Itoh T, Yamada H: **Role of the monocarboxylic acid transport system in the intestinal absorption of an orally active β-lactam prodrug: Carindacillin as a model.** *Int J Pharm* (1999) **191**(2):151-159.

57. Ganapathy ME, Huang W, Rajan DP, Carter AL, Sugawara M, Iseki K, Leibach FH, Ganapathy V: **β-Lactam antibiotics as substrates for OCTN2, an organic cation/carnitine transporter.** *J Biol Chem* (2000) **275**(3):1699-1707.

58. Bounds SJ, Nakkula R, Walters JD: **Fluoroquinolone transport by human monocytes: Characterization and comparison to other cells of myeloid lineage.** *Antimicrob Agents Chemother* (2000) **44**(10):2609-2614.

59. Van Bambeke F, Balzi E, Tulkens PM: **Antibiotic efflux pumps.** *Biochem Pharmacol* (2000) **60**(4):457-470.

60. Van Bambeke F, Michot JM, Tulkens PM: **Antibiotic efflux pumps in eukaryotic cells: Occurrence and impact on antibiotic cellular pharmacokinetics, pharmacodynamics and toxicodynamics.** *J Antimicrob Chemother* (2003) **51**(5):1067-1077.

61. Nichterlein T, Kretschmar M, Schadt A, Meyer A, Wildfeuer A, Laufen H, Hof H: **Reduced intracellular activity of antibiotics against *Listeria monocytogenes* in multidrug resistant cells.** *Int J Antimicrob Agents* (1998) **10**(2):119-125.

62. Leslie EM, Deeley RG, Cole SP: **Multidrug resistance proteins: Role of P-glycoprotein, MRP1, MRP2 and BCRP (ABCG2) in tissue defense.** *Toxicol Appl Pharmacol* (2005) **204**(3):216-237.

63. Sugiyama Y, Kusuhara H, Suzuki H: **Kinetic and biochemical analysis of carrier-mediated efflux of drugs through the blood-brain and blood-cerebrospinal fluid barriers: Importance in the drug delivery to the brain.** *J Control Release* (1999) **62**(1-2):179-186.

64. Inui KI, Masuda S, Saito H: **Cellular and molecular aspects of drug transport in the kidney.** *Kidney Int* (2000) **58**(3):944-958.

65. Hand WL, King-Thompson NL: **Membrane transport of clindamycin in alveolar macrophages.** *Antimicrob Agents Chemother* (1982) **21**(2):241-247.

66. Hand WL, Corwin RW, Steinberg TH, Grossman GD: **Uptake of antibiotics by human alveolar macrophages.** *Am Rev Respir Dis* (1984) **129**(6):933-937.

67. Darouiche RO, Hamill RJ: **Antibiotic penetration of and bactericidal activity within endothelial cells.** *Antimicrob Agents Chemother* (1994) **38**(5):1059-1064.

68. Ulrich M, Albers C, Moller JG, Dalhoff A, Korfmann G, Kunkele F, Doring G: **Moxifloxacin and azithromycin but not amoxicillin protect human respiratory epithelial cells against *Streptococcus pneumoniae in vitro* when administered up to 6 hours after challenge.** *Antimicrob Agents Chemother* (2005) **49**(12):5119-5122.

69. Baltch AL, Bopp LH, Smith RP, Michelsen PB, Ritz WJ: **Antibacterial activities of gemifloxacin, levofloxacin, gatifloxacin, moxifloxacin and erythromycin against intracellular *Legionella pneumophila* and *Legionella micdadei* in human monocytes.** *J Antimicrob Chemother* (2005) **56**(1):104-109.

70. Smith RP, Baltch AL, Franke MA, Michelsen PB, Bopp LH: **Levofloxacin penetrates human monocytes and enhances intracellular killing of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.** *J Antimicrob Chemother* (2000) **45**(4):483-488.

71. Eick S, Pfister W: **Efficacy of antibiotics against periodonto-pathogenic bacteria within epithelial cells: An *in vitro* study.** *J Periodontol* (2004) **75**(10):1327-1334.

72. Scaglione F, Demartini G, Dugnani S, Fraschini F: **A new model examining intracellular and extracellular activity of amoxicillin, azithromycin and clarithromycin in infected cells.** *Chemotherapy* (1993) **39**(6):416-423.

73. Chiu CH, Lin TY, Ou JT: ***In vitro* evaluation of intracellular activity of antibiotics against non-typhoid *Salmonella*.** *Int J Antimicrob Agents* (1999) **12**(1):47-52.

74. Edelstein PH, Edelstein MA: ***In vitro* extracellular and intracellular activities of clavulanic acid and those of piperacillin and ceftriaxone alone and in combination with tazobactam against clinical isolates of *Legionella* species.** *Antimicrob Agents Chemother* (1994) **38**(2):200-204.

75. Lemaire S, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM: **Activity of three β-lactams (ertapenem, meropenem and ampicillin) against intraphagocytic *Listeria monocytogenes* and *Staphylococcus aureus*.** *J Antimicrob Chemother* (2005) **55**(6):897-904.
• *Provides a demonstration of the unanticipated intracellular activity of β-lactams.*

76. Barcia-Macay M, Seral C, Mingeot-Leclercq MP, Tulkens PM, Van Bambeke F: **Pharmacodynamic evaluation of the intracellular activity of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages.** *Antimicrob Agents Chemother* (2006) **50**(3):841-851.
•• *Provides a comparison of cellular pharmacokinetics and pharmacodynamics of antibiotics against S aureus as a model of phagolysosomal infection.*

77. Fitzgerald D, Haas DW: ***Mycobacterium tuberculosis*.** In: *Principles and Practice of Infectious Diseases*. Mandell GL, Bennet JE, Dolin R (Eds), Elsevier Churchill Livingstone, Philadelphia, PA, USA (2005):2852-2886.

78. Carryn S, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM: **Comparative intracellular (THP-1 macrophage) and extracellular activities of β-lactams, azithromycin, gentamicin and fluoroquinolones against *Listeria monocytogenes* at clinically relevant concentrations.** *Antimicrob Agents Chemother* (2002) **46**(7):2095-2103.
•• *Provides a comparison of the activity of antibiotics against L monocytogenes as a model of cytosolic infection.*

79. Hand WL, King-Thompson NL: **Contrasts between phagocyte antibiotic uptake and subsequent intracellular bactericidal activity.** *Antimicrob Agents Chemother* (1986) **29**(1):135-140.

80. Seral C, Barcia-Macay M, Mingeot-Leclercq MP, Tulkens PM, Van Bambeke F: **Comparative activity of quinolones (ciprofloxacin, levofloxacin, moxifloxacin and garenoxacin) against extracellular and intracellular infection by *Listeria monocytogenes* and *Staphylococcus aureus* in J774 macrophages.** *J Antimicrob Chemother* (2005) **55**(4):511-517.

81. Scaglione F: **Predicting the clinical efficacy of antibiotics: Toward definitive criteria.** *Pediatr Infect Dis J* (1997) **16**(3 Suppl):S56-S59.

82. Aubert-Tulkens G, Van Hoof F, Tulkens P: **Gentamicin-induced lysosomal phospholipidosis in cultured rat fibroblasts. Quantitative ultrastructural and biochemical study.** *Lab Invest* (1979) **40**(4):481-491.

83. Van Bambeke F, Saffran J, Mingeot-Leclercq MP, Tulkens PM: **Mixed-lipid storage disorder induced in macrophages and fibroblasts by oritavancin (LY333328), a new glycopeptide antibiotic with exceptional cellular accumulation.** *Antimicrob Agents Chemother* (2005) **49**(5):1695-1700.

84. Ouadrhiri Y, Scorneaux B, Sibille Y, Tulkens PM: **Mechanism of the intracellular killing and modulation of antibiotic susceptibility of *Listeria monocytogenes* in THP-1 macrophages activated by γ interferon.** *Antimicrob Agents Chemother* (1999) **43**(5):1242-1251.

85. Van de Velde S, Carryn S, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM: **Increased susceptibility of intracellular *Listeria monocytogenes* to ampicillin: Studies with Caco-2 cells and THP-1 macrophages.** *15th International Symposium on Problems of Listeriosis (ISOPOL)*, Uppsala, Sweden (2004):P123.

86. Gouin E, Welch MD, Cossart P: **Actin-based motility of intracellular pathogens.** *Curr Opin Microbiol* (2005) **8**(1):35-45.

87. Portnoy DA, Auerbuch V, Glomski IJ: **The cell biology of *Listeria monocytogenes* infection: The intersection of bacterial pathogenesis and cell-mediated immunity.** *J Cell Biol* (2002) **158**(3):409-414.

88. Duclos S, Desjardins M: **Subversion of a young phagosome: The survival strategies of intracellular pathogens.** *Cell Microbiol* (2000) **2**(5):365-377.

89. Fisher MA, Plikaytis BB, Shinnick TM: **Microarray analysis of the *Mycobacterium tuberculosis* transcriptional response to the acidic conditions found in phagosomes.** *J Bacteriol* (2002) **184**(14):4025-4032.

90. Weinrick B, Dunman PM, McAleese F, Murphy E, Projan SJ, Fang Y, Novick RP: **Effect of mild acid on gene expression in *Staphylococcus aureus*.** *J Bacteriol* (2004) **186**(24):8407-8423.

91. Nicholson TL, Olinger L, Chong K, Schoolnik G, Stephens RS: **Global stage-specific gene regulation during the developmental cycle of *Chlamydia trachomatis*.** *J Bacteriol* (2003) **185**(10):3179-3189.

92. Tierrez A, Garcia-del Portillo F: **New concepts in *Salmonella* virulence: The importance of reducing the intracellular growth rate in the host.** *Cell Microbiol* (2005) **7**(7):901-909.

93. Theus SA, Cave MD, Eisenach KD: **Intracellular macrophage growth rates and cytokine profiles of *Mycobacterium tuberculosis* strains with different transmission dynamics.** *J Infect Dis* (2005) **191**(3):453-460.

94. Seral C, Van Bambeke F, Tulkens PM: **Quantitative analysis of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin and oritavancin (LY333328) activities against intracellular *Staphylococcus aureus* in mouse J774 macrophages.** *Antimicrob Agents Chemother* (2003) **47**(7):2283-2292.

95. Siewert K, Rupp J, Klinger M, Solbach W, Gieffers J: **Growth cycle-dependent pharmacodynamics of antichlamydial drugs.** *Antimicrob Agents Chemother* (2005) **49**(5):1852-1856.

96. Lemaire S, Van Bambeke F, Mingeot-Leclercq MP, Glupzynski Y, Tulkens PM: **Carbapenems (ETP, MEM) are active against intraphagocytic MRSA in a THP-1 macrophage model.** *45th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Washington, DC, USA (2005):A1832.

97. Sabath LD, Wallace SJ, Gerstein DA: **Suppression of intrinsic resistance to methicillin and other penicillins in *Staphylococcus aureus*.** *Antimicrob Agents Chemother* (1972) **2**(5):350-355.

98. Van Bambeke F, Glupzynski Y, Plesiat P, Pechere JC, Tulkens PM: **Antibiotic efflux pumps in prokaryotic cells: Occurrence, impact for resistance and strategies for the future of antimicrobial therapy.** *J Antimicrob Chemother* (2003) **51**(5):1167-1173.

99. Hirakata Y, Srikumar R, Poole K, Gotoh N, Suematsu T, Kohno S, Kamihira S, Hancock RE, Speert DP: **Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*.** *J Exp Med* (2002) **196**(1):109-118.

100. Thanassi DG, Cheng LW, Nikaido H: **Active efflux of bile salts by *Escherichia coli*.** *J Bacteriol* (1997) **179**(8):2512-2518.

101. Prior S, Gander B, Blarer N, Merkle HP, Subira ML, Irache JM, Gamazo C: ***In vitro* phagocytosis and monocyte-macrophage activation with poly(lactide) and poly(lactide-co-glycolide) microspheres.** *Eur J Pharm Sci* (2002) **15**(2):197-207.

102. Prior S, Gander B, Lecaroz C, Irache JM, Gamazo C: **Gentamicin-loaded microspheres for reducing the intracellular *Brucella abortus* load in infected monocytes.** *J Antimicrob Chemother* (2004) **53**(6):981-988.

103. Makino K, Nakajima T, Shikamura M, Ito F, Ando S, Kochi C, Inagawa H, Soma G, Terada H: **Efficient intracellular delivery of rifampicin to alveolar macrophages using rifampicin-loaded PLGA microspheres: Effects of molecular weight and composition of PLGA on release of rifampicin.** *Colloids Surf B Biointerfaces* (2004) **36**(1):35-42.

104. Rudin DE, Gao PX, Cao CX, Neu HC, Silverstein SC: **Gemfibrozil enhances the listeriacidal effects of fluoroquinolone antibiotics in J774 macrophages.** *J Exp Med* (1992) **176**(5):1439-1447.

105. Seral C, Carryn S, Tulkens PM, Van Bambeke F: **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* (2003) **51**(5):1167-1173.

106. Mandell GL, Coleman EJ: **Activities of antimicrobial agents against intracellular pneumococci.** *Antimicrob Agents Chemother* (2000) **44**(9):2561-2563.

107. Carryn S, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM: **Activity of β-lactams (ampicillin, meropenem), gentamicin, azithromycin and moxifloxacin against intracellular *Listeria monocytogenes* in a 24 h THP-1 human macrophage model.** *J Antimicrob Chemother* (2003) **51**(4):1051-1052.

108. Duman N, Cevikbas A, Johansson C: **The effects of rifampicin and fluoroquinolones on tubercle bacilli within human macrophages.** *Int J Antimicrob Agents* (2004) **23**(1):84-87.

109. Takemura H, Yamamoto H, Kunishima H, Ikejima H, Hara T, Kanemitsu K, Terakubo S, Shoji Y, Kaku M, Shimada J: **Evaluation of a human monocytic cell line THP-1 model for assay of the intracellular activities of antimicrobial agents against *Legionella pneumophila*.** *J Antimicrob Chemother* (2000) **46**(4):589-594.

110. Sikri V, Pal D, Jain R, Kalyani D, Mitra AK: **Cotransport of macrolide and fluoroquinolones, a beneficial interaction reversing P-glycoprotein efflux.** *Am J Ther* (2004) **11**(6):433-442.

111. Bamberger DM, Herndon BL, Dew M, Chern RP, Mitchell H, Summers LE, Marcus RF, Kim SC, Suvarna PR: **Efficacies of ofloxacin, rifampin and clindamycin in treatment of *Staphylococcus aureus* abscesses and correlation with results of an *in vitro* assay of intracellular bacterial killing.** *Antimicrob Agents Chemother* (1997) **41**(5):1178-1181.

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112. Nielsen SL, Black FT: **Extracellular and intracellular killing in neutrophil granulocytes of *Staphylococcus aureus* with rifampicin in combination with dicloxacillin or fusidic acid.** *J Antimicrob Chemother* (1999) **43**(3):407-410.

113. Michelet C, Avril JL, Arvieux C, Jacquelinet C, Vu N, Cartier F: **Comparative activities of new fluoroquinolones, alone or in combination with amoxicillin, trimethoprim-sulfamethoxazole or rifampin, against intracellular *Listeria monocytogenes*.** *Antimicrob Agents Chemother* (1997) **41**(1):60-65.

114. Gumbo T, Louie A, Deziel MR, Drusano GL: **Pharmacodynamic evidence that ciprofloxacin failure against tuberculosis is not due to poor microbial kill but to rapid emergence of resistance.** *Antimicrob Agents Chemother* (2005) **49**(8):3178-3181.

115. Gumbo T, Louie A, Deziel MR, Parsons LM, Salfinger M, Drusano GL: **Selection of a moxifloxacin dose that suppresses drug resistance in *Mycobacterium tuberculosis*, by use of an *in vitro* pharmacodynamic infection model and mathematical modeling.** *J Infect Dis* (2004) **190**(9):1642-1651.

•• *Describes the use of mathematical modeling and pharmacodynamic concepts to establish the appropriate quinolone dosage against Mycobacteria*.

116. Alferova IV, Vostrov SN, Portnoy YA, Lubenko IY, Zinner SH, Firsov AA: **Comparative pharmacodynamics of telithromycin and clarithromycin with *Streptococcus pneumoniae* and *Staphylococcus aureus* in an *in vitro* dynamic model: Focus on clinically achievable antibiotic concentrations.** *Int J Antimicrob Agents* (2005) **26**(3):197-204.

117. Tano E, Cars O, Lowdin E: **Pharmacodynamic studies of moxifloxacin and erythromycin against intracellular *Legionella pneumophila* in an *in vitro* kinetic model.** *J Antimicrob Chemother* (2005) **56**(1):240-242.

•• *Describes the first dynamic in vitro model for evaluating the intracellular activity of antibiotics*.

118. Roch N, Maurin M: **Antibiotic susceptibilities of *Legionella pneumophila* strain Paris in THP-1 cells as determined by real-time PCR assay.** *J Antimicrob Chemother* (2005) **55**(6):866-871.

•• *Describes the rapid quantification of intracellular bacteria using real-time PCR*.

119. Qazi SN, Harrison SE, Self T, Williams P, Hill PJ: **Real-time monitoring of intracellular *Staphylococcus aureus* replication.** *J Bacteriol* (2004) **186**(4):1065-1077.

•• *Describes a method for distinguishing viable and non-viable intracellular bacteria using fluorescent and phosphorescent probes*.

120. Malouin F, Brouillette E, Martinez A, Boyll BJ, Toth JL, Gage JL, Allen NE: **Identification of antimicrobial compounds active against intracellular *Staphylococcus aureus*.** *FEMS Immunol Med Microbiol* (2005) **45**(2):245-252.

121. Takii T, Hamasaki S, Hirano K, Abe C, Onozaki K: **Simple fibroblast-based assay to test the pyrazinamide susceptibility of *Mycobacterium tuberculosis*.** *Antimicrob Agents Chemother* (2005) **49**(2):804-807.

122. Cuffini AM, Tullio V, Bonino A, Allocco A, Palarchio AI, Carlone NA: **Entry of sanfetrinem into human polymorphonuclear granulocytes and its cell-associated activity against intracellular, penicillin-resistant *Streptococcus pneumoniae*.** *Antimicrob Agents Chemother* (1998) **42**(7):1745-1750.

123. Edelstein PH, Hu B, Edelstein MA: ***In vitro* and intracellular activities of LBM415 (NVP PDF-713) against *Legionella pneumophila*.** *Antimicrob Agents Chemother* (2005) **49**(6):2533-2535.

124. Gustafsson I, Engstrand L, Cars O: ***In vitro* pharmacodynamic studies of activities of ketolides HMR 3647 (telithromycin) and HMR 3004 against extracellular or intracellular *Helicobacter pylori*.** *Antimicrob Agents Chemother* (2001) **45**(1):353-355.

125. Ordway D, Viveiros M, Leandro C, Arroz MJ, Amaral L: **Intracellular activity of clinical concentrations of phenothiazines including thioridiazine against phagocytosed *Staphylococcus aureus*.** *Int J Antimicrob Agents* (2002) **20**(1):34-43.

126. Edelstein PH, Weiss WJ, Edelstein MA: **Activities of tigecycline (GAR-936) against *Legionella pneumophila in vitro* and in guinea pigs with *L pneumophila* pneumonia.** *Antimicrob Agents Chemother* (2003) **47**(2):533-540.

127. Brouillette E, Grondin G, Lefebvre C, Talbot BG, Malouin F: **Mouse mastitis model of infection for antimicrobial compound efficacy studies against intracellular and extracellular forms of *Staphylococcus aureus*.** *Vet Microbiol* (2004) **101**(4):253-262.

128. Jayaram R, Gaonkar S, Kaur P, Suresh BL, Mahesh BN, Jayashree R, Nandi V, Bharat S, Shandil RK, Kantharaj E, Balasubramanian V: **Pharmacokinetics-pharmacodynamics of rifampin in an aerosol infection model of tuberculosis.** *Antimicrob Agents Chemother* (2003) **47**(7):2118-2124.

129. Chambers HF, Turner J, Schecter GF, Kawamura M, Hopewell PC: **Imipenem for treatment of tuberculosis in mice and humans.** *Antimicrob Agents Chemother* (2005) **49**(7):2816-2821.

130. Langer O, Karch R, Muller U, Dobrozemsky G, Abrahim A, Zeitlinger M, Lackner E, Joukhadar C, Dudczak R, Kletter K, Muller M *et al*: **Combined PET and microdialysis for *in vivo* assessment of intracellular drug pharmacokinetics in humans.** *J Nucl Med* (2005) **46**(11):1835-1841.

• *Describes a non-invasive method for measuring the intracellular concentration of antibiotics in vivo*.

131. Jia L, Tomaszewski JE, Hanrahan C, Coward L, Noker P, Gorman G, Nikonenko B, Protopopova M: **Pharmacodynamics and pharmacokinetics of SQ109, a new diamine-based antitubercular drug.** *Br J Pharmacol* (2005) **144**(1):80-87.

132. Michelet C, Leib SL, Bentue-Ferrer D, Tauber MG: **Comparative efficacies of antibiotics in a rat model of meningoencephalitis due to *Listeria monocytogenes*.** *Antimicrob Agents Chemother* (1999) **43**(7):1651-1656.

133. Prior S, Gander B, Irache JM, Gamazo C: **Gentamicin-loaded microspheres for treatment of experimental *Brucella abortus* infection in mice.** *J Antimicrob Chemother* (2005) **55**(6):1032-1036.

RESULTS

1. Pharmacodynamic evaluation of the intracellular activity of antibiotics against Staphylococcus aureus in a model of THP-1 macrophages

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1.1. Foreword: justification of the model

1.1.1. cell type

In this study, we have developed a model of intracellular infection by *S. aureus,* in THP-1 human macrophages. This cell line, first described in 1980 (254), is derived from the blood of an 1-year-old boy with acute monocytic leukemia.

Monocytes and macrophages are derived from hematopoietic stem cells (Figure 15).

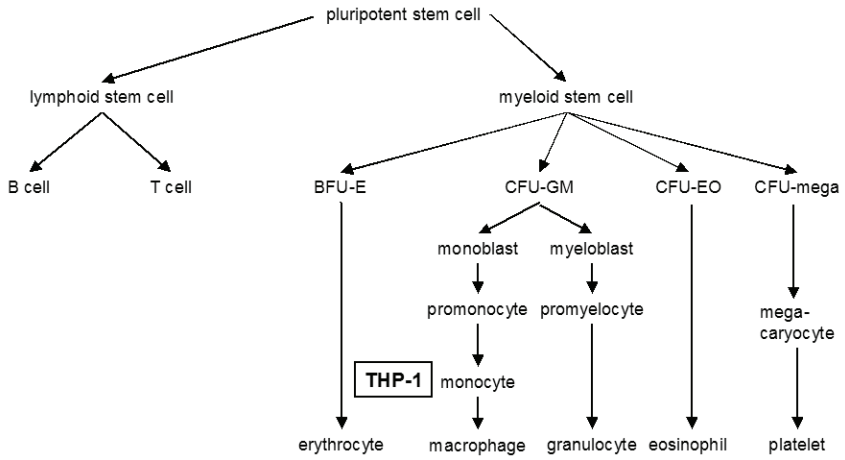

FIGURE 15: hematopoietic cell differentiation; the arrows indicate the differentiation potential. THP-1 cells are boxed (19).

Mature monocytes migrate to different tissues where they differentiate in macrophages, with morphological and functional characteristics typical for the tissue. The multifunctional nature of the monocyte implies that it has to undergo a very complex differentiation process, which involves tissue specific stimuli.

Studies of monocytes/macrophages are often impeded by the limited amount of human cells available. The existence of continuous cell lines like THP-1 may bring an appropriate solution to this problem, and presents the additional advantage of offering an homogeneous population (254). Table 11 lists the main properties of THP-1 cells that make them a suitable model for studying the properties of human monocytes/macrophages.

TABLE 11: main characteristics of the human THP-1 cell line (19,254).

parameter	characteristics
morphological features	morphology resembling that of monocytic leukemia cells: - diameter 12-14 µm - moderate basophile cytoplasm - small azurophiles granules, few vacuoles - nuclei irregular in shape
cytochemical features	- positive for α-naphthyl butyrate esterase - negative reaction with periodic acid-Schiff and Sudan black B - diploid (46,XY) chromosome number
surface antigens and receptors	CD4, CD30, Factor X receptor, Factor Xa receptor, FcRI, FcRII, GM-CSF-receptor, HDL receptor, LDL receptor, TNF receptor, C3b receptor, LFA-1 receptor, Fibronectin receptor, Leu M1, Leu M2, Leu M3, HLA-DR antigens, scavengers receptors
secreted proteins	- hormones, cytokines: TNF-α, IL-1, IL-1b, CSF-1, M-CSF, erythrocyte differentiation factor, PDGF-1 and –2, thymosin B4, killer T cell activating factor, monocyte chemotactic factor - enzymes: lipoprotein lipase, lyzozyme - binding proteins: apoprotein E
functional features	- phagocytosis - production of lyzozyme - capacity to restore the lympocyte T mitogenic responsiveness

1.1.2. bacterial strain

In this study, we have used as standard strain the fully susceptible MSSA strain ATCC25923. This strain was at the origin a clinical isolate (www.ATCC.org). It is now considered as a standard, used as quality control strain or susceptibility testing standard for antibiotics. A Pubmed search on "ATCC 25923" extracted more than 200 papers, demonstrating that this strain is widely used. The use of collection strains is indeed important to compare and standardize intra-laboratories methods.

We selected this strain for our study because of (a) its fully susceptible character, which will allow us to compare antibiotics from various families, and (b) its wide use as standard strain. One can object us however that this strain isolated in 1976 may largely differ from current strains in terms of virulence or pathogenicity determinants. Some collection *staphylococcal* strains have indeed been subcultured from decades since their first isolation and could have lost important pathophysiological characteristics. In fact, struggle for existence leads to the selection of new well-equipped strains to survive adverse conditions, the so-called “survival of the fittest”. Thus, given the plasticity and constant evolution of the *S. aureus* genome, experiments using only collection strains may not be fully adequate to adapt these results to “real world” strains.

We are aware of this limitation, which has pushed us in the second part of the study to extent the model to clinical isolates collected over the last years. As we will see, it is interesting to note, however, that the intracellular fate of these strains does not seem to differ from that of the reference strain used in the first part of our study.

1.1.3. antibiotics

We have examined in the first part of our study a series of antibiotics belonging to different classes. Our choice was essentially based on (a) the potential interest of the molecule in the clinics, (b) the availability of data regarding their capacity to accumulate in eucaryotic cells, and (c) the centers of interest of the laboratory at the time this study started (in this case, we selected several molecules within a single family to evidence potential differences in their behavior).

Thus, the drugs selected were the following:

- beta-lactams, because they are considered as first choice antibiotics in susceptible strains (217), based on their high security of use.
- macrolides, because of their known accumulation in the lysosomal compartment (42).
- quinolones, because previous work of our laboratory showed that they are very active in another model of intracellular infection by *L. monocytogenes* (46,239); we compared drugs for which detailed cellular pharmacokinetic studies were run in parallel (185).
- an aminoglycoside, also known to accumulate in the lysosomes, and characterized by a rapid bactericidal effect extracellularly (18,240).
- glycopeptides, considered at the present time as the alternative of choice for MRSA infections (217,260); we included in our analysis new semi-synthetic derivatives for which cellular pharmacokinetic studies were run in parallel (262 and this work).
- rifampicin, because it is usually recognized as a first choice for intracellular infections (266).
- linezolid, a new drug keeping activity against mutiresistant strains (217).

We did not include tetracyclines and lincosamides for which cellular pharmacokinetic data are scarce, but these drugs would be interesting to test in our model, especially in view of the fact that new derivatives in these families are now in development for the treatment of *S. aureus* infections (tigecycline and VIC-10,5555 [161,171]).

We did neither include sulfamethoxazole-trimethoprim or synercid (quinupristin-dalfopristin) because the combination of two drugs makes more difficult the interpretation of the data.

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Pharmacodynamic Evaluation of the Intracellular Activities of Antibiotics against *Staphylococcus aureus* in a Model of THP-1 Macrophages

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The pharmacodynamic properties governing the activities of antibiotics against intracellular *Staphylococcus aureus* are still largely undetermined. Sixteen antibiotics of seven different pharmacological classes (azithromycin and telithromycin [macrolides]; gentamicin [an aminoglycoside]; linezolid [an oxazolidinone]; penicillin V, nafcillin, ampicillin, and oxacillin [β-lactams]; teicoplanin, vancomycin, and oritavancin [glycopeptides]; rifampin [an ansamycin]; and ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin [quinolones]) have been examined for their activities against *S. aureus* (ATCC 25923) in human THP-1 macrophages (intracellular) versus that in culture medium (extracellular) by using a 0- to 24-h exposure time and a wide range of extracellular concentrations (including the range of the MIC to the maximum concentration in serum [C_{max}; total drug] of humans). All molecules except the macrolides caused a net reduction in bacterial counts that was time and concentration/MIC ratio dependent (four molecules tested in detail [gentamicin, oxacillin, moxifloxacin, and oritavancin] showed typical sigmoidal dose-response curves at 24 h). Maximal intracellular activities remained consistently lower than extracellular activities, irrespective of the level of drug accumulation and of the pharmacological class. Relative potencies (50% effective concentration or at a fixed extracellular concentration/MIC ratio) were also decreased, but to different extents. At an extracellular concentration corresponding to their C_{max}s (total drug) in humans, only oxacillin, levofloxacin, garenoxacin, moxifloxacin, and oritavancin had truly intracellular bactericidal effects (2-log decrease or more, as defined by the Clinical and Laboratory Standards Institute guidelines). The intracellular activities of antibiotics against *S. aureus* (i) are critically dependent upon their extracellular concentrations and the duration of cell exposure (within the 0- to 24-h time frame) to antibiotics and (ii) are always lower than those that can be observed extracellularly. This model may help in rationalizing the choice of antibiotic for the treatment of *S. aureus* intracellular infections.

Staphylococcus aureus, which often causes chronic or relapsing diseases (68), is reported to persist as an opportunistic intracellular organism both in vitro and in vivo (8, 10, 18, 30, 31, 34, 39). Antibiotic treatments should therefore be optimized not only toward the extracellular forms of *S. aureus* but also toward the intracellular forms of *S. aureus* to avoid creating a niche where bacteria may persist, cause cell alterations, and possibly, be selected for resistance if they are exposed to subtherapeutic concentrations (1). A large body of literature on the activities of antibiotics against intracellular *S. aureus* in various cellular models is available (see references 54, 66, 67, and 70 for reviews). Yet, many of these studies yield contradictory results, and we still lack a clear understanding of which parameters are truly critical for the expression of antibiotic activity in the intracellular milieu (11). In a previous study, we measured the activities of selected antibiotics characterized by a fair to high level of cellular accumulation against intracellular *S. aureus* in a model of unstimulated murine J774 macrophages (57). We observed that cellular accumulation was only partially and nonconsistently predictive of activity. In a subsequent pilot study, performed with human THP-1 macrophages, we also noted that β-lactams, which notoriously do not accumulate in cells, actually showed significant activity against intracellular *S. aureus* when their extracellular concentration was brought to a sufficiently high but still clinically meaningful level (36). This triggered us to broaden and systematize our approach. For this purpose, we selected typical representatives of seven classes of antibiotics with known activities against *S. aureus* and included in commonly used guidelines for the handling of staphylococcal infections. We concentrated our effort on THP-1 macrophages because these cells present many of the characteristics of human monocytes while forming a homogeneous and reproducible population (6). THP-1 macrophages have been successfully used in various studies aimed at characterizing the interactions between *S. aureus* and macrophages in a clinical context (19, 28, 49) and to analyze the potential relationship between the accumulation of antibiotics in cells and intracellular activity (48). In contrast to many other models, however, we explored a large array of extracellular concentrations (including the range observed in the serum of patients receiving conventional doses) and used incubation times up to 24 h. Our purpose, indeed, was to analyze the pharmacodynamic parameters governing the activities of these antibiotics against intra-

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cellular forms of *S. aureus* in terms of both the concentration/MIC ratio and the time of exposure. This approach was thought to be necessary to enable us to draw pharmacologically as well as clinically meaningful conclusions.

(Parts of this study were presented at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., 14 to 17 September 2003 [M. Barcia-Macay, C. Seral, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke, Abstr. 43rd Intersci. Conf. Antimicrob Agents Chemother., abstr. A-1174, 2003], and at the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington D.C., 30 October to 2 November 2004 [M. Barcia-Macay, C. Seral, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke, Abstr. 44th Intersci. Conf. Antimicrob Agents Chemother., abstr. A1488, 2004].)

MATERIALS AND METHODS

Bacterial strain, susceptibility testing, and time and dose-kill curve studies in extracellular medium. *S. aureus* (strain ATCC 25923, fully susceptible) was used for all experiments. All conditions for measurement of the MICs (at pH 7.3 and 5.0) and the minimal bactericidal concentrations (MBCs) were exactly the same as those described earlier (57). Dose-kill curve studies were performed as described previously (57), with the following modifications: (i) RPMI 1640 medium supplemented with 10% fetal calf serum rather than broth was systematically used to measure the extracellular activities to better mimic a true extracellular environment, and (ii) enumeration of colonies (for determination of CFU) was performed with an automated detector (14). All samples (diluted as needed) were prepared in a final volume of 1 ml, of which 50 μl was used to seed 8.2-cm-diameter petri dishes containing 12.5 ml of nutrient agar. In the present study, we validated the method by assessing (i) the linearity of the instrument response for counting from 3 to 2,000 colonies per dish ($R^2 = 0.997$; $n = 220$) and (ii) the intraday reproducibilities for samples that yielded 3 to 1,500 colonies/dish (the observed standard deviation [SD] for 10 repeated assays with the same sample was from 0.6 to 1.7 times the theoretical value [which is equal to the square root of n, where n is the value of the actual counts, assuming a Poisson type of distribution], with no trend toward less reproducibility for samples with low counts). Samples with counts more than 1,500 colonies/dish tended to give lower reproducibilities due to the fusion of colonies and larger dilutions were then used. The lowest limit of detection was set at 3 counts/plate (actual SD, 1.1 for 10 repeated assays with the same sample). This lowest value corresponded to 60 CFU in the original samples (if it was undiluted) and to a 4.2-log decrease in the numbers of CFU from a typical initial inoculum of 10^6 bacteria per ml (variation, from 4.1 to 4.4 when an SD value of 1.1 at the level of the determination was considered). All samples yielding less than three colonies were arbitrarily considered to have a 5-log decrease from the typical original inoculum, and this value was used in all illustrations and calculations.

Cells, cell cultures, and intracellular infection. Human THP-1 macrophages were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum exactly as described in a previous publication (55). Infection was performed as described earlier (36), based on a model with murine J774 macrophages (57). Phagocytosis was initiated at a bacterium-macrophage ratio of 4:1, and after removal of unphagocytosed and adherent *S. aureus* cells, the inoculum was typically 1×10^6 to 2×10^6 bacteria per mg of cell protein, as for J774 macrophages (57). At the end of the experiments, the cells were collected by centrifugation, resuspended in phosphate-buffered saline, and centrifuged again to further remove adherent bacteria. The cells were then processed for CFU counting as described above with the same upper and lowest limits of detection. The typical initial intracellular inoculum of approximately 10^6 bacteria per mg of cell protein was diluted in approximately 1 ml after cell collection and processing. The protein concentrations were measured in parallel, as described previously (13). Previous studies showed that the amounts of antibiotic that could be carried over from the cells into the final assay mixture were too low to interfere significantly with CFU determinations, given the high dilution of the cell content during sample preparation (12, 55). Extracellular contamination was assessed in pilot studies by collecting the culture fluid at the end of the observation period, mixing it with all media used to wash the corresponding cell samples, and enumerating the CFU after plating and incubation on Trypticase soy agar.

Determination of cellular antibiotic accumulation. Accumulation studies were performed as described in previous publications (12, 47), but the majority of antibiotics were assayed by a microbiological method (disk diffusion method with *Bacillus subtilis* ATCC 6633 as the test organism and antibiotic medium 11 adjusted to pH 8 for gentamicin; disk diffusion method with *Micrococcus luteus* ATCC 9341 and antibiotic medium 2 adjusted to pH 8 for macrolides; and disk diffusion method with *Micrococcus luteus* ATCC 9341 and antibiotic medium 2 adjusted to pH 7 for β-lactams, rifampin, linezolid, vancomycin, and teicoplanin). For each of these antibiotics, the assay method was checked for linearity (telithromycin, 0.03 to 4 mg/liter; azithromycin, 0.05 to 4 mg/liter; ampicillin, 0.21 to 10 mg/liter, nafcillin, 1.1 to 120 mg/liter; oxacillin, 1.6 to 30 mg/liter; penicillin V, 0.04 to 10 mg/liter; rifampin, 0.2 to 15 mg/liter; linezolid, 12 to 190 mg/liter, vancomycin, 2.7 to 150 mg/liter; teicoplanin, 2.9 to 250 mg/liter; gentamicin, 0.6 to 240 mg/liter) and for reproducibility (coefficient of variation, <10%). Ciprofloxacin, moxifloxacin, and levofloxacin concentrations were measured by fluorimetry (12, 56), and garenoxacin and oritavancin concentrations were measured by radiometry by using ^{14}C-labeled drugs (41, 64). In pilot studies we checked that these assays detected genuine, bioactive drug. The cell concentration of each drug was expressed by reference to the protein content of the corresponding samples, with a conversion factor of 5 μl of cell volume per mg of cell protein used to calculate the apparent cellular drug accumulation (12, 47).

Morphological studies. Infection was carried out at a bacterium-macrophage ratio of approximately 8 to allow visualization of a sufficiently large number of bacteria, with all other conditions similar to those described in the general protocol. The cells were fixed and prepared as described previously (47, 63).

Antibiotics. Whenever possible, antibiotics were obtained as microbiological standards from their corresponding manufacturers: azithromycin (dihydrate salt; potency, 94.4%) from Pfizer Inc., Groton, CT; telithromycin (potency, 99.3%) from Aventis, Romainville, France; ciprofloxacin (potency, 85%) and moxifloxacin (potency, 91%) from Bayer AG, Wuppertal, Germany; oxacillin (potency, 85%) from Bristol-Myers Squibb Co., Syracuse, NY; unlabeled garenoxacin (potency, 79%) and 3-^{14}C-labeled garenoxacin (specific activity, 0.80 MBq/mg) from the Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingsford, CT; and unlabeled oritavancin (as LY333328; potency, 80.6%) and [^{14}C]oritavancin (labeled on the chloro-biphenyl side chain; specific activity, 3.5 μCi/mg) from Eli Lilly & Co., Indianapolis, IN. Ampicillin and penicillin V were purchased from Sigma-Aldrich-Fluka (St. Louis, MO). The other antibiotics were procured as the commercial products registered in Belgium for parenteral use from their respective marketing authorization holders or resellers (gentamicin as Geomycin and vancomycin as Vancocin from Glaxo-SmithKline; rifampin as Rifadine, levofloxacin as Tavanic, and teicoplanin as Targocid from Aventis; and linezolid as Zyvoxid from Pfizer).

Other reagents. Unless stated otherwise, all other reagents were of analytical grade and were purchased from E. Merck AG (Darmstadt, Germany) or Sigma-Aldrich-Fluka. Cell culture or microbiology media were from Invitrogen (Paisley, Scotland) and Difco (Sparks, MD).

Curve fittings and statistical analyses. Curve fittings were done with GraphPad Prism (version 4.02) software for Windows (GraphPad Prism Software, San Diego, CA), and statistical analyses were performed with XLSTAT Pro (version 7.5.2; Addinsoft SARL, Paris, France).

RESULTS

Susceptibility testing. Table 1 shows the MICs (measured at pH 7.3 and 5.0 to mimic the extracellular and phagolysosomal environments, respectively) and the MBCs for the *S. aureus* strain used in this study. Except for azithromycin, all MICs measured at pH 7.3 were considerably lower than the peak concentration in serum (C_{max} [total drug; see Table 1 for estimates of the fraction of free drug at that concentration in human serum]) commonly observed in patients receiving conventional dosages of the corresponding antibiotics. Lowering of the pH to 5.0 had contrasting effects on MICs, with a marked increase (more than eightfold) for gentamicin and the macrolides, a moderate increase (two- to eightfold) for linezolid and the quinolones, no or little change for the glycopeptides, and a modest (1 to 2 dilutions) but reproducible decrease for the β-lactams and rifampin. The MBCs were close to the MICs measured at pH 7.3 (≤2-dilution difference) for gentamicin, rifampin, the β-lactams, oritavancin, and the quin-

TABLE 1. MICs and MBCs of antibiotics under study against *S. aureus* ATCC 25923 compared to the C_{max}[a]

Antibiotic[b]	MIC (mg/liter)		MBC (mg/liter)	Dosage and route[c]	Human C_{max} (mg/liter) (Total drug)	% Free drug	Reference(s)
	pH 7.3	pH 5.0					
Azithromycin	0.5	512	8	500 mg p.o.	0.5	88	22
Telithromycin	0.06	4	2	800 mg p.o.	2	30	44, 71
Gentamicin	0.5	16	2	6 mg/kg i.v.	18	80	26, 43
Linezolid	2	4	32	600 mg i.v.	21	69	5
Penicillin V	0.015	<0.015	0.06	500 mg p.o.	6.3	20	4, 50
Nafcillin	0.25	0.06	1	1,000 mg i.v.	40	3	4, 27
Ampicillin	0.06	0.03	0.25	1,000 mg i.v.	47.6	85	4, 50
Oxacillin	0.125	0.06	0.25	500 mg i.v.	63	10	4, 50
Teicoplanin	0.25	0.5	64	12 mg/kg i.v.	100	10	20
Vancomycin	1	1	16	15 mg/kg i.v.	50	45–90	20
Oritavancin	0.25	0.25	1	3 mg/kg i.v.	25	10	9, 65
Rifampin	0.0075	0.002	0.03	600 mg i.v.	18	10–20	4, 29
Ciprofloxacin	0.125	1	1	750 mg p.o.	4.3	63	32, 33
Levofloxacin	0.125	1	0.125	400 mg p.o.	4	62–76	21
Garenoxacin	<0.03	0.125	0.03	400 mg p.o.	4	25	23[d]
Moxifloxacin	0.06	0.25	0.06	400 mg p.o.	4	52	58, 59

[a] Commonly observed maximal concentration in serum (and estimated percentage of free drug) after intravenous or oral administration of conventional doses to humans, based on the references indicated.
[b] The molecules are ranked by pharmacological classes, with each class appearing by order of its mean level of intracellular activity (as shown in Fig. 5).
[c] p.o., oral; i.v., intravenous.
[d] Reference for protein binding: A. Bello, D. Hollenbaugh, D. A. Gajjar, L. Christopher, and D. M. Grasela, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. A-45, 2001.

olones (except ciprofloxacin). In contrast, the macrolides and linezolid had higher MBCs (equal to or higher than their corresponding C_{max}s). Vancomycin, teicoplanin, and ciprofloxacin showed intermediate behaviors, with their MBCs being considerably higher than their MICs but still lower than their C_{max}s.

Validation of the intracellular model. We first examined whether the accumulation of antibiotics in THP-1 cells was consistent with their known behavior in other cell types (see reference 11 for a review). Whenever possible, the extracellular concentration was set at the C_{max} of the drug (as defined in Table 1), but poor assay sensitivity forced us to use higher concentrations for a number of molecules. The 24-h time point was selected since this corresponded to the maximal duration of our studies with infected cells. Data are presented in Table 2. Linezolid, β-lactams, and gentamicin showed no or only modest accumulation (from 0.5- to 4.4-fold). The quinolones, vancomycin, and teicoplanin reached slightly higher levels (5- to 10-fold). Rifampin, azithromycin, and telithromycin achieved higher levels (17- to 38-fold); and oritavancin accumulated up to almost 150-fold.

We then characterized the course of the infection of THP-1 cells by the strain of *S. aureus* that we used (Fig. 1). In the absence of antibiotic, the number of bacteria collected from cells (after the washing procedure) increased almost at the same rate as that for bacteria incubated in complete culture medium in the absence of cells (extracellular infection). The medium of the infected cells, however, showed visible acidification at 24 h (compared to the medium of the uninfected cells), with the number of viable bacteria in low speed super-

TABLE 2. Cellular accumulation factor of antibiotics in THP-1 cells after 24 h of incubation at a fixed extracellular concentration

Antibiotic[a]	Cellular accumulation[b]	Extracellular concn (mg/liter)
Azithromycin	37.8 ± 1.3	5[c]
Telithromycin	27.9 ± 1.3	2[d]
Gentamicin	4.4 ± 0.1	250[c]
Linezolid	0.5 ± 0.0	250[c]
Penicillin V	1.2 ± 0.1	150[c]
Nafcillin	2.6 ± 0.1	400[c]
Ampicillin	1.0 ± 0.1	150[c]
Oxacillin	4.0 ± 0.1	250[c]
Teicoplanin	7.4 ± 0.2	150[c]
Vancomycin	6.3 ± 0.1	100[c]
Oritavancin	148.0 ± 12.0	25[d]
Rifampin	17.6 ± 0.9	50[c]
Ciprofloxacin	5.1 ± 0.1	4.3[d]
Levofloxacin	7.0 ± 0.6	4[d]
Garenoxacin	9.1 ± 0.3	4[d]
Moxifloxacin	7.6 ± 0.3	4[d]

[a] The molecules are ranked by pharmacological classes, with each class appearing by order of its mean level of intracellular activity (as shown in Fig. 5).
[b] Apparent cellular concentration-to-extracellular concentration ratio, based on a cell volume of 5 μl per mg of cell protein (12,47).
[c] A concentration larger than the C_{max} was used because of a lack of sensitivity of the microbiological assay.
[d] Concentration corresponding to the C_{max} (as defined in Table 1).

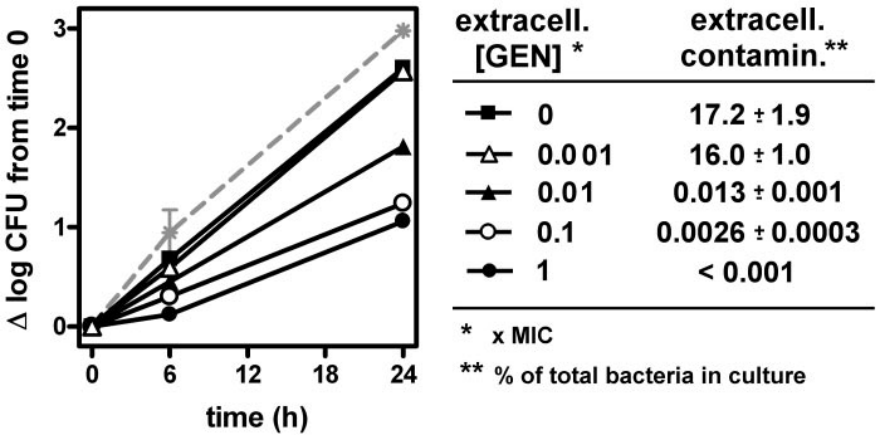


FIG. 1. Kinetics of growth of *S. aureus* (i) in THP-1 macrophages exposed to increasing extracellular concentrations of gentamicin (solid lines) or (ii) in culture medium in the absence of antibiotic (dotted gray line). The graph shows the change in the number of CFU (Δ log CFU; means ± SDs; $n = 3$; most SD bars are smaller than the symbols), starting from an initial inoculum of approximately 10^6 bacteria per mg of protein (intracellular) or per ml of medium (extracellular). The table shows the contamination (contamin.) of the culture medium by *S. aureus* at the end of the experiment. extracell., extracellular; GEN, gentamicin.

natants and washing media amounting to approximately 17% of the total sample content (medium plus cells). Based on previous experience with J774 macrophages (57), gentamicin was added to the culture medium to prevent this contamination. As shown in Fig. 1, a gentamicin concentration as low as 0.01 its MIC reduced the extracellular contamination to an almost negligible level, while it still allowed a marked increase in the number of cell-associated CFU (to about 65% of what was seen without antibiotic). A further increase in the extracellular concentration of gentamicin to its MIC allowed extracellular contamination to go to undetectable levels, but with a further decrease in the cell-associated CFU, demonstrating interference with the intracellular multiplication of the bacteria. Yet, optical and electron microscopy of cells incubated for 24 h with gentamicin at the MIC still revealed the presence of actively multiplying bacteria within membrane-bound structures, consistent with intraphagolysosomal localization (see Fig. 6). Because of all those uncertainties in the true level of intracellular growth of *S. aureus* and the potential impact of even low concentrations of gentamicin, intracellular activities were therefore examined and expressed not as the difference from the controls but in terms of variations of the cell-associated CFU from the original, postphagocytosis inoculum. Finally, careful examination of the bacterial cultures obtained from cell samples exposed to gentamicin or to other antibiotics (see below) failed to identify so-called small-colony variants.

Kinetics of antibacterial effects at a fixed, large concentration (C_{max}). Time-kill curves were obtained for eight molecules selected on the basis of (i) their increasing MBC/MIC ratios (from 1 [moxifloxacin] to 33 [telithromycin]; see Table 1) when they were tested in broth and (ii) their increasing levels of cellular accumulation (apparent cellular-concentration-to-extracellular-concentration ratio from less than 1 [linezolid] to about 150 [oritavancin] in uninfected cells; see Table 2). The results are shown in Fig. 2. By first considering the extracellular activities, it appears that gentamicin, rifampin, and oritavancin acted very fast, with bacterial counts reaching the limit of detection within 6 h or less, whereas linezolid and telithromycin, although they were tested at concentrations equal or close to their MBCs, were only slowly and poorly bactericidal. Oxacillin, vancomycin, and moxifloxacin reached the limit of detection upon prolonged incubation. There was thus only a poor correlation between the rates and extents of killing and the MBC/MIC ratios. By next considering the intracellular bacteria, overall decreases in the rates and extents of killing of

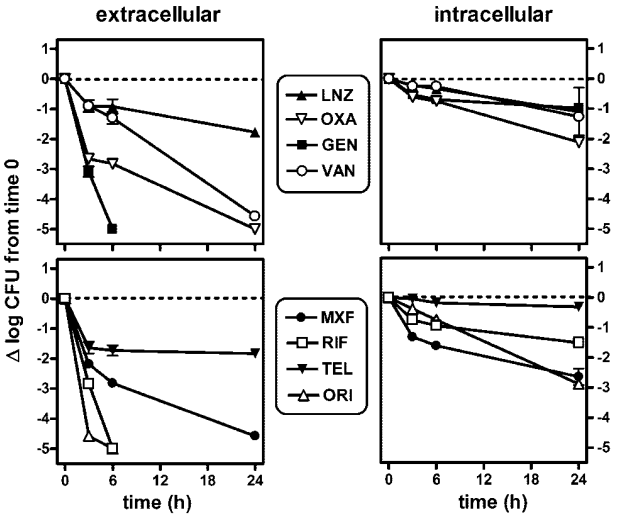


FIG. 2. Influence of time on the rate and the extent of the activities of the antibiotics against extracellular and intracellular *S. aureus* upon incubation at a fixed extracellular concentration. The graphs show the change in the number of CFU (Δ log CFU; means ± SDs; $n = 3$; most SD bars are smaller than the symbols) per ml of culture medium (extracellular) or in THP-1 macrophages (intracellular) per mg of cell protein. The molecules are listed in order from the upper to the lower panel by increasing level of cellular accumulation (as determined in uninfected cells; Table 2). Each antibiotic was added at a concentration corresponding to its C_{max} in humans (total drug, as defined in Table 1). LNZ, linezolid; OXA, oxacillin; GEN, gentamicin; VAN, vancomycin; MXF, moxifloxacin; RIF, rifampin; TEL, telithromycin; ORI, oritavancin.

intracellular bacteria compared to those of extracellular bacteria were observed, but some antibiotics were more affected than others. Only oritavancin, moxifloxacin, and oxacillin achieved bactericidal effects (as defined by a 2-log decrease from the original inoculum) at 24 h. Rifampin and gentamicin, which were highly bactericidal toward extracellular bacteria, did not reach this limit (and their intracellular activities were actually close to those of vancomycin and linezolid). Telithromycin was essentially bacteriostatic. There was no correlation between intracellular activity and cellular accumulation among the eight drugs tested.

Kinetics and influence of concentration on antibacterial effects in the MIC-C_{max} range. Six molecules were then selected from among the bactericidal drugs to examine the influence of concentration on the rate and extent of killing (Fig. 3). By first considering extracellular activities, the extent of killing was significantly concentration dependent for all drugs over the range of concentrations investigated. The rate of killing also increased with concentration for all drugs except rifampin, for which a low concentration (but still above the MIC) caused the antibiotic activity to plateau after 6 h. By next considering the intracellular activities, both the rate and the extent of killing of intracellular bacteria were considerably reduced compared to those of extracellular bacteria; but significant concentration-dependent effects were still observed with respect to both of these parameters for vancomycin, oxacillin, and oritavancin and with respect to the extent of killing for moxifloxacin and rifampin. For gentamicin, an increase in the extracellular concentration from 5 to 18 mg/liter (10- to 36-fold the MIC) was without significant effect at 6 h but caused a modest, albeit statistically significant, increase in activity at 24 h, the extent of which remained, however, very limited.

These experiments were then repeated with all drugs included in this study but were limited to the examination of the 24-h time point and to three critical concentrations (the MIC, 10 times the MIC, and C_{max}, except for rifampin, in view of its very low MIC [see the Fig. 4 legend for the concentrations of rifampin used]). The results are shown in Fig. 4 in a synoptic fashion for ease of direct comparison of the results between molecules and, for each molecule, between its extracellular and intracellular levels of activity. The data show that (i) the macrolides were always bacteriostatic toward both extracellular and intracellular bacteria, whichever concentration was tested; (ii) the largest discrepancy between extracellular and intracellular activities occurred for gentamicin; and (iii) oxacillin (among the four penicillins tested), levofloxacin, garenoxacin, and moxifloxacin (among the four quinolones tested) and oritavancin were bactericidal toward intracellular bacteria (and the level of activity was in that order) but had to be used at concentrations close to or equal to their C_{max}s to achieve such an effect. There was, again, no simple correlation between intracellular bactericidal effects and the MBC/MIC ratios or the levels of cellular accumulation (as measured in uninfected cells).

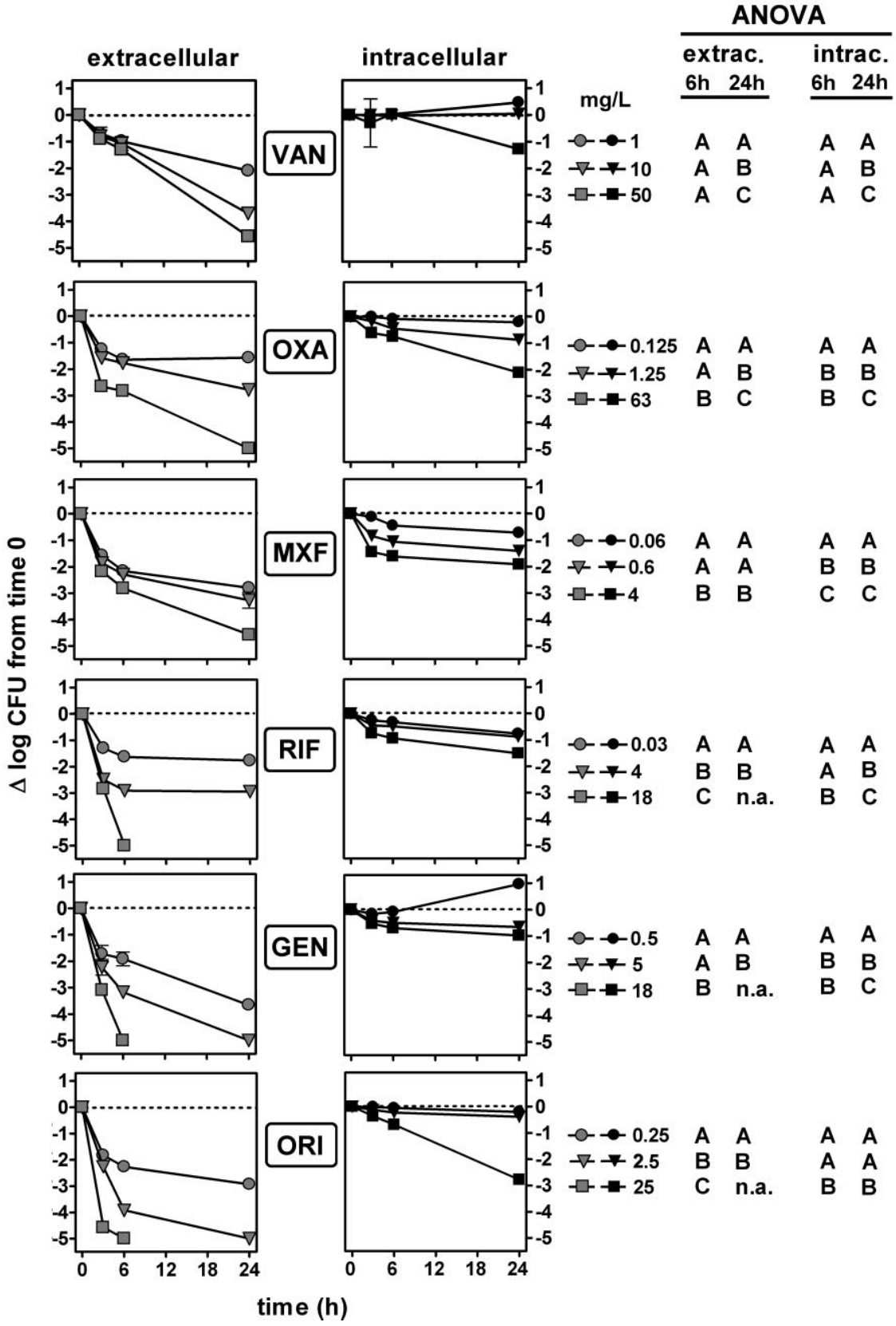
Wide range of concentration-effect relationships (pharmacological comparisons). Four molecules (oxacillin, gentamicin, moxifloxacin, and oritavancin) were selected to obtain full pharmacological dose-response curves based on (i) their demonstrated dose-effect relationships in the MIC-C_{max} range and (ii) their contrasting behaviors with respect to their intracellular activity/extracellular activity ratios. Figure 5 shows the results, with the regression parameters and a detailed statistical analysis presented in Table 3. Against extracellular bacteria, all four drugs displayed similar relative potencies (50% effective concentrations [EC_{50}s]) (53) and static concentrations at about their MICs and 0.3 their MICs, respectively. Their relative efficacies (maximum effects [E_{max}s]), however, were significantly different (oxacillin $<$ moxifloxacin $<$ oritavancin $\approx$ gentamicin). Against intracellular bacteria, all four drugs had significant decreases in their relative efficacies (E_{max}), but these decreases were roughly similar (E_{max} against intracellular bacteria/E_{max} against extracellular bacteria ratios, 0.42 [minimum] to 0.64 [maximum]; because we could not reliably assess inoculum decreases larger than 4.2 log and arbitrarily set all larger values to 5, these ratios may actually be overestimated for highly bactericidal antibiotics such as oritavancin and gentamicin). In contrast, the relative potencies (EC_{50}s) were very differentially affected, with oxacillin and moxifloxacin showing no significant change compared to their corresponding potencies against extracellular bacteria, whereas marked decreases (9- to 14-fold) in potency (indicated by an increase in EC_{50}) were noted for gentamicin and oritavancin. This partially translated into an increase in the static concentrations of about 2-, 4-, 7-, and 17-fold for moxifloxacin, oxacillin, gentamicin, and oritavancin, respectively.

Morphological studies. Electron microscopy (Fig. 6) was used to examine the morphological changes of phagocytosed *S. aureus* after exposure to the antibiotics in order to ascertain that the decreases in the numbers of CFU seen in our experiments were associated with visible changes in the number and/or morphology of the bacteria. Oxacillin and oritavancin were selected for use in this study, since both are reported to act on cell wall biosynthesis and/or the cell wall structure, making their potential action on the bacteria more easily recognizable. In the absence of these antibiotics (but in the presence of gentamicin at the MIC, to fully avoid extracellular contamination), phagocytosed bacteria were darkly stained (Fig. 6A), often actively multiplying, and surrounded by a thick cell wall (Fig.B and C). In cells incubated with oxacillin, a large number of intracellular bacteria appeared as ghosts with a rarefied cytoplasmic material (Fig. 6D and E) or with large, electron-lucent vacuoles (Fig. 6F). Ghosts were also commonly observed in infected cells incubated with oritavancin (Fig. 6G), with profiles often showing granular material sometimes apposed on the periphery of the bacterial body (Fig. 6H).

DISCUSSION

The data presented in this paper underline three main properties of antibiotics in relation to their intracellular activities that may not have been sufficiently detected in previous studies because of an insufficient duration of exposure and the investigation of a limited range of concentrations. The model used here has specifically tried to address these issues and has been validated to exclude the significant contribution of extracellular growth within the limits of the experimental setup.

A first and unanticipated property is that all classes of antibiotics tested, with the exception of the macrolides, showed significant intracellular killing when their extracellular concentration was brought to a sufficiently high level and the time of


extracellular
intracellular
ANOVA
extrac.
intrac.
6h 24h
6h 24h
mg/L
VAN
1 A A A A
10 A B A B
50 A C A C
OXA
0.125 A A A A
1.25 A B B B
63 B C B C
MXF
0.06 A A A A
0.6 A A B B
4 B B C C
RIF
0.03 A A A A
4 B B A B
18 C n.a. B C
GEN
0.5 A A A A
5 A B B B
18 B n.a. B C
ORI
0.25 A A A A
2.5 B B A A
25 C n.a. B B
Δ log CFU from time 0
time (h)
0 6 12 18 24

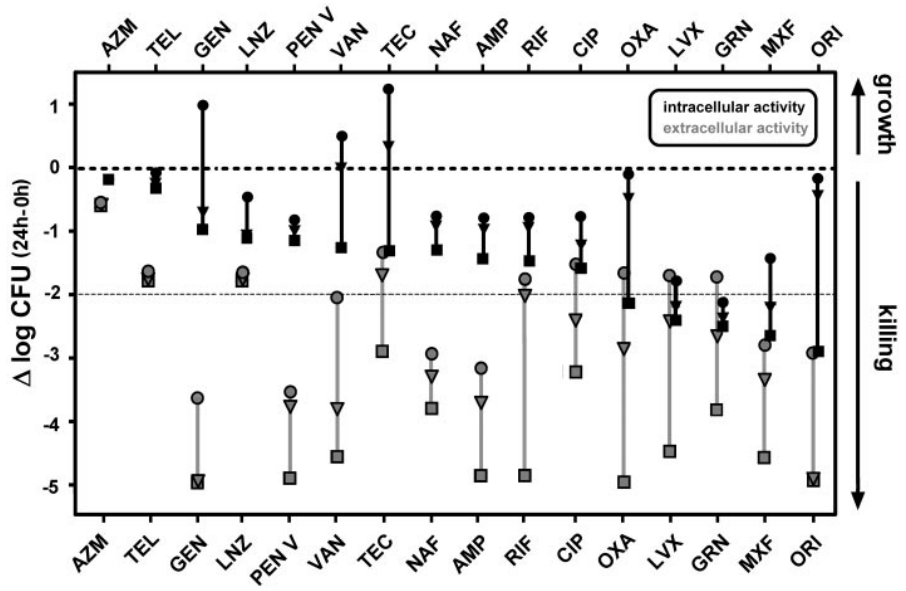


FIG. 4. Influence of concentration on the extent of antibiotic activity against extracellular and intracellular *S. aureus*. The graphs show the change in the number of CFU (Δ log CFU; means ± SDs; $n = 3$; most SD bars are smaller than the symbols) per ml of culture medium (extracellular) or in THP-1 macrophages (intracellular) per mg of cell protein. Each antibiotic was added at concentrations corresponding to its MIC (circles; 4 times the MIC for rifampin), 10 times its MIC (triangles; 530 times the MIC for rifampin), or its C_{max} in humans (squares; total drug [Table 1]). Thick dotted line, static effect; thin dotted line, −2-log change (bactericidal effect, as defined by the Clinical and Laboratory Standards Institute for bacteria growing in broth). AZM, azithromycin; TEL, telithromycin; GEN, gentamicin; LNZ, linezolid; PEN V, penicillin V; VAN, vancomycin; TEC, teicoplanin; NAF, nafcillin; AMP, ampicillin; RIF, rifampin; CIP, ciprofloxacin; OXA, oxacillin; LVX, levofloxacin; GRN, garenoxacin; MXF, moxifloxacin; ORI, oritavancin.

exposure was prolonged to 24 h. For two molecules at least (oxacillin and oritavancin), we could show that the decreases in cell-associated CFU are accompanied by evidence of severe morphological alterations of the intracellular bacteria, consistent with their known modes of action (3, 25), indicating true intracellular expression of drug-related activity. This property is actually the direct consequence of two factors. The first is that all antibiotics studied here, with the exception of the macrolides, show concentration-dependent effects (for the four molecules tested in detail, we even observed typical pharmacological dose-response curves with the classical basic properties of threshold, slope, and maximal effects upon increasing concentration [53], irrespective of their specific modes of action). This definitely helps to provide an understanding of why contradictory results are reported when only narrow ranges of extracellular concentrations are explored. The second factor, which is perhaps as critical as the first one, is that all drugs, with the exception of the macrolides and, surprisingly, rifampin, showed time-dependent effects when they were tested at low multiples of their MICs. Both concentration and time therefore appear to modulate the final response and need to be taken into account when results from different models are compared. We know that this first part of our conclusion may appear to be at variance with what has been drawn from previous studies of the pharmacodynamics of antibiotics, namely, that the activities of some drugs (most notably, the β-lactams) are predominantly time dependent, whereas the activities of others (most notably, the aminoglycosides and the fluoroquinolones) are mainly concentration dependent (15, 16). Our observations being what they are, we suggest that the way that the drugs appear and can be differentiated from one another in most models essentially depends on two factors, namely, (i) the value of the E_{max} parameter of the pharmacological response (maximal activity) and the concentrations at which effects approaching E_{max} are obtained and (ii) the size (how large) of the concentration range examined. (The E_{max} values shown in Table 3 are negative numbers, since they pertain to decreases in bacterial counts. Greater activity is, therefore, strictly speaking, associated with a smaller E_{max}. Since this is rather counterintuitive, we use the term "maximal activity" throughout this

FIG. 3. Influence of concentration on the rate and the extent of the activities of antibiotics against extracellular and intracellular *S. aureus*. The graphs show the change in the number of CFU (Δ log CFU; means ± SDs; $n = 3$; most SD bars are smaller than the symbols) per ml of culture medium (extracellular) or in THP-1 macrophages per mg of cell protein (intracellular). Molecules are ordered by increasing bactericidal potential, as determined by their MIC/MBC ratios in broth (Table 1). Except for rifampin, each antibiotic was tested at three increasing concentrations corresponding to its MIC (circles), 10 times the MIC (triangles), and the C_{max} in humans (squares; total drug); rifampin was used at 4 (circles) and 530 (triangles) times the MIC and at the C_{max} in humans (squares; total drug). Dotted line, static effect. For analysis of variance (ANOVA), the same letter indicates no statistically significant difference between values; different letters indicate a P value <0.05. n.a., not applicable (below the detection level); extrac., extracellular; intrac., intracellular; VAN, vancomycin; OXA, oxacillin; MXF, moxifloxacin; RIF, rifampin; GEN, gentamicin; ORI, oritavancin.

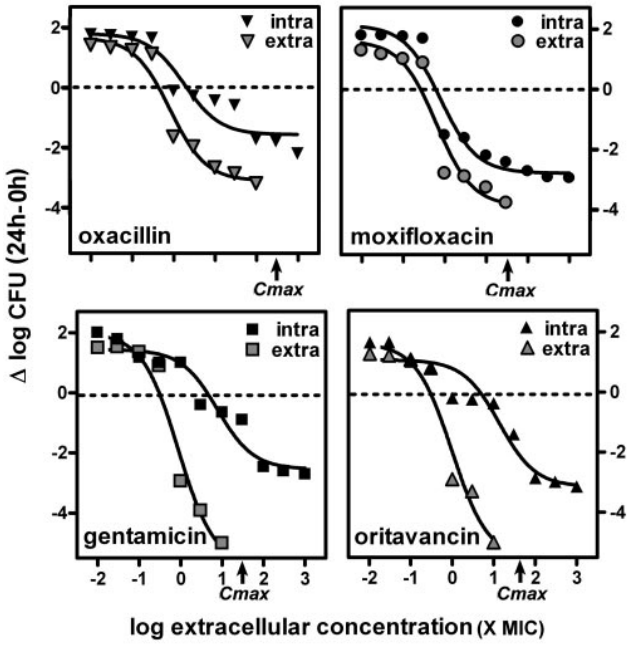


FIG. 5. Dose-response curves of four selected antibiotics against extracellular (extra) and intracellular (intra) *S. aureus*. The graphs show the change in the number of CFU (Δ log CFU; means ± SD; $n = 3$; most SD bars are smaller than the symbols) per ml of culture medium (extracellular; closed gray symbols) or in THP-1 macrophages (intracellular; closed black symbols) per mg of cell protein. Dotted line, static effect. The sigmoidal function was used (Hill coefficient = 1 [42]); goodness of fit and regression parameters are shown in Table 3). Vertical arrow, C_{max} in humans (total drug; Table 1).

discussion.) This explains why the activities of some drugs may have mainly been considered concentration dependent if they were examined within a concentration range that is close to their EC_{50}, whereas the activities of others, tested over a higher concentration range, are essentially reported as time dependent. In Fig. 5 we show where the C_{max} of each drug in humans would fall on the abcissa to help delineate what could be the microbiologically and clinically meaningful range of concentrations to be considered (MIC-C_{max}). In this context, gentamicin and oritavancin, which have higher maximal activities than oxacillin against extracellular bacteria within this MIC-C_{max} range, will be expected to be markedly influenced by the concentration within that range. Conversely, oxacillin, the C_{max} of which (as defined in Table 1) is much larger than its MIC and which has a weaker maximal effect, will be expected to be less influenced by the concentration, which in turn will make time a more predominant parameter. Moxifloxacin has an intermediate behavior, with its activity being largely concentration dependent when it is evaluated with concentrations close to its MIC but with its activity becoming less concentration dependent when it is tested at concentrations close to its reported C_{max} in humans. Since the maximal activities against intracellular *S. aureus* are systematically lower than those against extracellular bacteria, the impact of the concentration on bacterial survival is accordingly less marked, which makes gentamicin and oritavancin behave more like oxacillin within the MIC-C_{max} range of extracellular concentrations. This will increase the impact of the time during which the bacteria are exposed to the antibiotic and suggests that the activities of all these antibiotics actually appear to be mainly time dependent. Extrapolation of our data for the categorization of the activities of the drugs as concentration or time dependent in vivo cannot, however, be done without caution. A first uncertainty relates to the effective availability of the antibiotics in blood and extracellular fluids, which can be severely impaired by binding to proteins or other biological constituents. As a help to the reader, however, we have provided in Table 1 an estimation of the percentage of free drug in human serum for each C_{max} used in our study. If it is assumed that it is only the free drug that drives activity, one could surmise that the clinically meaningful concentration range of antibiotics that are highly protein bound will shift toward lower values, making the activities of most of them more and more concentration dependent as their effective concentrations approach the EC_{50}s. Unfortunately, the model used here does not easily lend itself to a pertinent evaluation of even this simple effect of serum protein binding, because (i) the serum concentration is low, resulting in only weak and limited binding of antibiotics that are usually reported to be highly protein bound (36); (ii) this concentration cannot be markedly changed without causing cell death, thereby preventing most concentration-effect studies; and (iii) the serum is of bovine and not human origin. A second uncertainty is whether the results obtained with a constant concentration over a 24-h period are predictive of what may be observed in vivo with fluctuating concentrations, as will be the case unless drugs are administered by continuous infusion. This will need to be specifically addressed in future studies. However, recent data from a study examining the pharmacodynamics of erythromycin against intracellular *Legionella pneumophila* by the use of both static and kinetic models failed to reveal significant differences in behavior related to the type of exposure (60).

A second property that appears from the comparative analysis of the dose-effect is that intracellular activities consistently remain lower than the extracellular ones, whether one considers what can be obtained at any given extracellular concentration or the maximal achievable effects (the E_{max} parameter; because we could not reliably assess inoculum decreases greater than 4.2 log, the intracellular E_{max}/extracellular E_{max} ratios observed for drugs highly bactericidal toward extracellular bacteria may actually be underestimated). This property was seen for all molecules studied and is probably more related to bacterial or cellular parameters than to drug pharmacodynamic or pharmacokinetic ones. The present study offers no insight into the underlying mechanism. However, we know that *S. aureus* cells phagocytosed by macrophages sojourn and thrive in phagolysosmes (35, 52). We may reasonably suggest that the metabolic changes triggered by the exposure of bacteria to this specific environment and to an acid pH in particular (46) could play a critical role (69). Alternatively, it is possible that those bacteria that apparently remain insensitive to antibiotics are physically protected from direct contact with the drugs. These hypotheses need to be addressed in future work but may face the difficulty of the specific analysis of what may concern only a small, albeit significant, part of the original inoculum. Thus, we could not directly examine the role of the so-called small-colony variants, which have been linked to per-

TABLE 3. Pertinent regression parameters[a] (with confidence intervals) and statistical analysis of the dose-response curves illustrated in Fig. 5

Antibiotic[b]	Extracellular[c]				Intracellular[d]				*P* value[e]
	E_{max}[f] (CI[g])	EC_{50}[h] (CI)	C_{static}[i]	R^2	E_{max} (CI)	EC_{50} (CI)	C_{static}	R^2	
Oxacillin	−3.13 (−3.81 to −2.44)a;A	0.84 (0.34 to 2.04)a;A	0.45	0.966	−1.58 (−2.20 to −0.96)a;B	1.85 (0.51 to 6.53)a;A	2.09	0.909	<0.001
Moxifloxacin	−3.86 (−5.22 to −2.51)b;A	0.64 (0.16 to 2.56)a;A	0.27	0.936	−2.77 (−3.31 to −2.22)b;B	0.81 (0.34 to 1.9)1b;A	0.63	0.956	<0.001
Gentamicin	−5.76 (−7.89 to −3.62)c;B	0.88 (0.28 to 2.78)a;A	0.30	0.969	−2.54 (−3.22 to −1.86)b;B	7.73 (2.86 to 20.9)c;B	2.09	0.943	<0.001
Oritavancin	−5.55 (−8.03 to −3.07)c;B	1.00 (0.25 to 4.0)1a;A	0.29	0.956	−3.15 (−4.04 to −2.57)c;B	13.77 (4.48 to 42.4)c;B	4.79	0.927	<0.001

[a] By use of all data points from antibiotic concentrations of 0.01 to 1,000 times the MIC. Data for samples without antibiotics were not used since there was evidence of an overestimation of the true value of the intracellular counts when the extracellular concentration of antibiotic was lower than 0.01 the MIC (see Fig. 1). Statistical analyses were performed as follows: analysis per column (one-way analysis of variance by the Tukey test for multiple comparisons between each parameter fof all drugs), data with different lowercase letters are significantly different from each other ($P < 0.05$); analysis per row (unpaired, two-tailed *t* test between corresponding parameters of extracellular and intracellular activities), data with different uppercase letters are significantly different from each other ($P < 0.05$); global analysis (analysis of covariance) by the Tukey test for multiple comparisons, for extracellular versus intracellular concentrations, the curves for each antibiotic are compared between these two conditions; for global analysis between drugs, all four curves of extracellular or intracellular activities are compared between drugs.

[b] By analysis of covariance, there was no significant difference between drugs.

[c] In complete cell culture medium where the original inoculum (time zero) was $1.01 \pm 0.20 \times 10^6$ CFU/ml ($n = 3$).

[d] Original (postphagocytosis) inoculum (time zero), $1.91 \pm 0.18 \times 10^6$ CFU/mg protein ($n = 3$).

[e] *P* values were determined by analysis of covariance and are for the extracellular concentration versus the intracellular concentration.

[f] CFU decrease (in $\log_{10}$ units) at 24 h from the corresponding original inoculum, as extrapolated for the antibiotic concentration at infinity; counts of less than 3 colonies/dish were considered below the detection level.

[g] CI, confidence interval.

[h] Concentration (in multiples of the MIC) causing a reduction of the inoculum half-way between the initial (E_0) and the maximal (E_{max}) values, as obtained from the Hill equation (by using a slope factor of 1; SD of log EC_{50} values, 0.163 [minimum] to 0.241 [maximum]).

[i] Concentration (in multiples of the MIC) resulting in no apparent bacterial growth (the number of CFU was identical to that in the original inoculum), as determined by graphical intrapolation.

sistent and relapsing infections (51), since we failed to detect them in significant numbers in our experimental conditions. Likewise, it may prove difficult to determine to what extent a small subpopulation of all intracellular bacteria are sojourning in poorly accessible compartments.

A third property, and probably the most critical one, to be considered in drug selection is the fact that the relative potencies (as measured by the EC_{50} parameter) of some molecules are markedly decreased against intracellular bacteria compared with those against extracellular bacteria. Gentamicin and oritavancin appear to be the most affected, even though both drugs primarily concentrate in lysosomes and related vacuoles (62, 64), where *S. aureus* is thought to localize. These vacuoles are acidic, which will markedly decrease the activities of aminoglycosides (as is well known and which has been confirmed here for the strain of *S. aureus* used). In this context, it is interesting that alkalinization of lysosomes has been associated with improved intracellular activities of aminoglycosides (38). Yet, the activity of oritavancin is unaffected by acidity (as shown in a previous publication [64] and confirmed here), which indicates that effects other than pH, such as binding to intralysosomal constituents, need to be taken into consideration. In a broader context, a lack of true bioavailability and the defeating effect of the local physicochemical conditions on activity probably explain why cell accumulation per se is not necessarily predictive of intracellular efficacy for most antibiotics. This even appears to be the case for drugs with apparent large bioavailabilities, such as the fluoroquinolones. Indeed, fluoroquinolones show considerably less activity than is anticipated from their level of cellular accumulation, as demonstrated here and in other recent studies (2, 48, 56). Macrolides may also suffer from the same effects, but their bacteriostatic character is probably the most critical determinant in their lack of an intracellular killing effect. Conversely, the bactericidal effects of β-lactams against intracellular *S. aureus* when these compounds are used at large extracellular concentrations, as seen here for oxacillin and in previous studies with ampicillin and meropenem (36), not only could be due to the fact that these drugs may reach intracellular concentrations that eventually reach far above their MICs but could also be due to the production of cellular factors that enhance their activities (37, 45, 67).

Our results with linezolid and rifampin require attention,

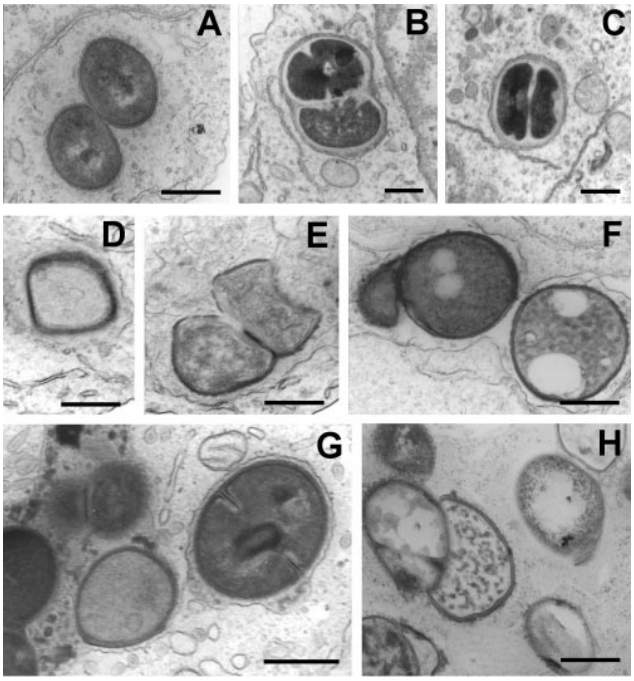


FIG. 6. Electron microscopic appearance of serum-opsonated *S. aureus* in THP-1 macrophages 24 h after phagocytosis. (A to C) Control cells (gentamicin was added at its MIC to prevent extracellular growth); (D to F) cells exposed to oxacillin; (G and H) cells exposed to oritavancin (both at their C_{max}s in humans [total drug; Table 1]). Bars, 0.5 μm.

since both drugs are usually recommended for the treatment of difficult-to-treat staphylococcal infections, but they failed to demonstrate significant intracellular bactericidal effects in our study. This observation is actually not surprising for linezolid, which is essentially bacteriostatic and which does not accumulate in macrophages. Conversely, the weak intracellular activity of rifampin, also seen in murine macrophages (57), was more puzzling since its activity is concentration dependent and its MIC was one of the lowest among those of all drugs tested, especially at acidic pH. A key factor here could be that the activity of rifampin, while it is marked after 3 to 6 h, does not progress over time thereafter, showing the importance of taking this parameter into account when different antibiotics are compared.

The present study used only one strain of fully susceptible *S. aureus*, which may be considered a major limitation for extrapolation of the findings of this study to clinical situations. Actually, the strain studied here has been widely used for the evaluation of the in vitro activities of new antibiotics in broth (61) as well as in phagocytes (24). The choice of a unique, well-characterized strain was actually essential for addressing the question of antibiotic intracellular activity per se and avoiding the blurring of the results because of other factors that can modulate the intracellular response to antibiotics, such as virulence and variations in the expression of resistance mechanisms. Given this caveat and pending further studies with clinical strains, the data presented in this paper may provide unambiguous pharmacological support to the use of new quinolones (7, 17) or oritavancin (40), as an alternative to β-lactams (68), for the treatment of recurrent *S. aureus* infections, provided that sufficient extracellular concentration/MIC ratios are obtained for a sufficient period of time. These conditions may not be obtainable for more toxic drugs such as aminoglycosides or conventional glycopeptides and will not be met with bacteriostatic antibiotics. We also suggest that in vitro models are useful for the appropriate design of animal and clinical studies aimed at evaluating the efficacies of antibiotics against intracellular pathogens, provided that they are made as relevant to the in vivo situation as possible in terms of the drug concentration and the duration of exposure.

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REFERENCES

1. **Alexander, E. H., and M. C. Hudson.** 2001. Factors influencing the internalization of Staphylococcus aureus and impacts on the course of infections in humans. Appl. Microbiol. Biotechnol. **56:**361–366.
2. **Al Nawas, B., and P. M. Shah.** 1998. Intracellular activity of ciprofloxacin and moxifloxacin, a new 8-methoxyquinolone, against methicillin-resistant Staphylococcus aureus. J. Antimicrob. Chemother. **41:**655–658.
3. **Allen, N. E., and T. I. Nicas.** 2003. Mechanism of action of oritavancin and related glycopeptide antibiotics. FEMS Microbiol. Rev. **26:**511–532.
4. **Amsden, G. W., and J. J. Schentag.** 1995. Tables of antimicrobial agent pharmacology, p. 492–529. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, New York, N.Y.
5. **Anonymous.** 2005. Zyvox US full prescribing information. http://www.pfizer.com/pfizer/download/uspi_zyvox.pdf. Pharmacia & Upjohn, Division of Pfizer Inc, New York, N.Y. (last updated, May 2005; last accessed, 18 July 2005).
6. **Auwerx, J.** 1991. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. Experientia **47:**22–31.
7. **Berrington, A. W., R. J. Koerner, J. D. Perry, H. H. Bain, and F. K. Gould.** 2001. Treatment of Staphylococcus aureus endocarditis using moxifloxacin. Int. J. Med. Microbiol. **291:**237–239.
8. **Bishayi, B., and M. Sengupta.** 2003. Intracellular survival of Staphylococcus aureus due to alteration of cellular activity in arsenic and lead intoxicated mature Swiss albino mice. Toxicology **184:**31–39.
9. **Braun, D. K., J. K. Chien, D. S. Farlow, D. L. Phillips, M. W. Wasilewski, and M. L. Zeckel.** 2001. Oritavancin (LY333328): a dose-escalation safety and pharmacokinetics study in patients. Clin. Microbiol. Infect. **7:**P434.
10. **Brouillette, E., G. Grondin, L. Shkreta, P. Lacasse, and B. G. Talbot.** 2003. In vivo and in vitro demonstration that Staphylococcus aureus is an intracellular pathogen in the presence or absence of fibronectin-binding proteins. Microb. Pathog. **35:**159–168.
11. **Carryn, S., H. Chanteux, C. Seral, M. P. Mingeot-Leclercq, F. Van Bambeke, and P. M. Tulkens.** 2003. Intracellular pharmacodynamics of antibiotics. Infect. Dis. Clin. N. Am. **17:**615–634.
12. **Carryn, S., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens.** 2002. Comparative intracellular (THP-1 macrophage) and extracellular activities of beta-lactams, azithromycin, gentamicin, and fluoroquinolones against *Listeria monocytogenes* at clinically relevant concentrations. Antimicrob. Agents Chemother. **46:**2095–2103.
13. **Carryn, S., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens.** 2003. Activity of beta-lactams (ampicillin, meropenem), gentamicin, azithromycin and moxifloxacin against intracellular Listeria monocytogenes in a 24 h THP-1 human macrophage model. J. Antimicrob. Chemother. **51:**1051–1052.
14. **Carryn, S., S. Van de Velde, F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens.** 2004. Impairment of growth of Listeria monocytogenes in THP-1 macrophages by granulocyte macrophage colony-stimulating factor: release of tumor necrosis factor-alpha and nitric oxide. J. Infect. Dis. **189:**2101–2109.
15. **Craig, W. A.** 2003. Basic pharmacodynamics of antibacterials with clinical applications to the use of beta-lactams, glycopeptides, and linezolid. Infect. Dis. Clin. N. Am. **17:**479–501.
16. **Craig, W. A., and S. C. Ebert.** 1990. Killing and regrowth of bacteria in vitro: a review. Scand. J. Infect. Dis. Suppl. **74:**63–70.
17. **Eckart, R. E., D. R. Hospenthal, and J. T. Fishbain.** 2000. Response of complicated methicillin-resistant Staphylococcus aureus endocarditis to the addition of trovafloxacin. Pharmacotherapy **20:**589–592.
18. **Ellington, J. K., M. Harris, L. Webb, B. Smith, T. Smith, K. Tan, and M. Hudson.** 2003. Intracellular Staphylococcus aureus. A mechanism for the indolence of osteomyelitis. J. Bone Joint Surg. Br. **85:**918–921.
19. **Fan, H., G. Teti, S. Ashton, K. Guyton, G. E. Tempel, P. V. Halushka, and J. A. Cook.** 2003. Involvement of G(i) proteins and Src tyrosine kinase in TNFalpha production induced by lipopolysaccharide, group B streptococci and Staphylococcus aureus. Cytokine **22:**126–133.
20. **Feketi, R.** 2000. Vancomycin, teicoplanin, and the streptogramins: quinupristin and dalfopristin, p. 382–392. *In* G. E. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, Philadelphia, Pa.
21. **Fish, D. N., and A. T. Chow.** 1997. The clinical pharmacokinetics of levofloxacin. Clin. Pharmacokinet. **32:**101–119.
22. **Foulds, G., R. M. Shepard, and R. B. Johnson.** 1990. The pharmacokinetics of azithromycin in human serum and tissues. J. Antimicrob. Chemother. **25**(Suppl. A)**:**73–82.
23. **Gajjar, D. A., A. Bello, Z. Ge, L. Christopher, and D. M. Grasela.** 2003. Multiple-dose safety and pharmacokinetics of oral garenoxacin in healthy subjects. Antimicrob. Agents Chemother. **47:**2256–2263.
24. **Garcia, I., A. Pascual, S. Ballesta, C. del Castillo, and E. J. Perea.** 2003. Accumulation and activity of cethromycin (ABT-773) within human polymorphonuclear leucocytes. J. Antimicrob. Chemother. **52:**24–28.
25. **Giesbrecht, P., T. Kersten, H. Maidhof, and J. Wecke.** 1998. Staphylococcal cell wall: morphogenesis and fatal variations in the presence of penicillin. Microbiol. Mol. Biol. Rev. **62:**1371–1414.
26. **Gilbert, D. N.** 2000. Aminoglycosides, p. 307–336. *In* G. E. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, Philadelphia, Pa.
27. **Glew, R. H., and R. C. Moellering, Jr.** 1979. Effect of protein binding on the activity of penicillins in combination with gentamicin against enterococci. Antimicrob. Agents Chemother. **15:**87–92.
28. **Gotoh, M., Y. Takamoto, K. Kurosaka, J. Masuda, M. Ida, A. Satoh, E. Takayama, K. Kojima-Aikawa, Y. Kobayashi, and I. Matsumoto.** 2005. An-

nexins I and IV inhibit Staphylococcus aureus attachment to human macrophages. Immunol. Lett. **98:**297–302.
29. **Grosset, J., L. Chauvelot-Moachon, and J. P. Giroud.** 1988. Antituberculeux, p. 1553–1573. *In* J. P. Giroud, G. Mathé, and G. Meyniel (ed.), Pharmacologie clinique, bases de la thérapeutique. Expansion Scientifique Française, Paris, France.
30. **Hebert, A., K. Sayasith, S. Senechal, P. Dubreuil, and J. Lagace.** 2000. Demonstration of intracellular Staphylococcus aureus in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. FEMS Microbiol. Lett. **193:**57–62.
31. **Hess, D. J., M. J. Henry-Stanley, E. A. Erickson, and C. L. Wells.** 2003. Intracellular survival of Staphylococcus aureus within cultured enterocytes. J. Surg. Res. **114:**42–49.
32. **Hoffken, G., H. Lode, C. Prinzing, K. Borner, and P. Koeppe.** 1985. Pharmacokinetics of ciprofloxacin after oral and parenteral administration. Antimicrob. Agents Chemother. **27:**375–379.
33. **Israel, D., J. G. Gillum, M. Turik, K. Harvey, J. Ford, H. Dalton, M. Towle, R. Echols, A. H. Heller, and R. Polk.** 1993. Pharmacokinetics and serum bactericidal titers of ciprofloxacin and ofloxacin following multiple oral doses in healthy volunteers. Antimicrob. Agents Chemother. **37:**2193–2199.
34. **Jett, B. D., and M. S. Gilmore.** 2002. Host-parasite interactions in Staphylococcus aureus keratitis. DNA Cell Biol. **21:**397–404.
35. **Kapral, F. A., and M. G. Shayegani.** 1959. Intracellular survival of staphylococci. J. Exp. Med. **110:**123–128.
36. **Lemaire, S., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens.** 2005. Activity of three β-lactams (ertapenem, meropenem and ampicillin) against intraphagocytic Listeria monocytogenes and Staphylococcus aureus. J. Antimicrob. Chemother. **55:**897–904.
37. **Matsumoto, Y., A. Ikemoto, Y. Wakai, F. Ikeda, S. Tawara, and K. Matsumoto.** 2001. Mechanism of therapeutic effectiveness of cefixime against typhoid fever. Antimicrob. Agents Chemother. **45:**2450–2454.
38. **Maurin, M., and D. Raoult.** 1994. Phagolysosomal alkalinization and intracellular killing of Staphylococcus aureus by amikacin. J. Infect. Dis. **169:**330–336.
39. **Mempel, M., C. Schnopp, M. Hojka, H. Fesq, S. Weidinger, M. Schaller, H. C. Korting, J. Ring, and D. Abeck.** 2002. Invasion of human keratinocytes by Staphylococcus aureus and intracellular bacterial persistence represent haemolysin-independent virulence mechanisms that are followed by features of necrotic and apoptotic keratinocyte cell death. Br. J. Dermatol. **146:**943–951.
40. **Mercier, R. C., and L. Hrebickova.** 2005. Oritavancin: a new avenue for resistant gram-positive bacteria. Expert Rev. Anti. Infect. Ther. **3:**325–332.
41. **Michot, J. M., C. Seral, F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens.** 2005. Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. Antimicrob. Agents Chemother. **49:**2429–2437.
42. **Motulsky, H. J.** 2005. Analyzing data with GraphPad Prism. http://www.graphpad.com. GraphPad Software Inc., San Diego, Calif. (last updated, 1999; last accessed, 2 August 2005).
43. **Myers, D. R., J. DeFehr, W. M. Bennet, G. A. Porter, and G. D. Olsen.** 1978. Gentamicin binding to serum and plasma proteins. Clin. Pharmacol. Ther. **23:**356–360.
44. **Namour, F., D. H. Wessels, M. H. Pascual, D. Reynolds, E. Sultan, and B. Lenfant.** 2001. Pharmacokinetics of the new ketolide telithromycin (HMR 3647) administered in ascending single and multiple doses. Antimicrob. Agents Chemother. **45:**170–175.
45. **Nielsen, S. L., and F. T. Black.** 1998. The effect of dicloxacillin and fusidic acid on the extracellular and intracellular killing of Staphylococcus aureus. J. Antimicrob. Chemother. **42:**221–226.
46. **Ohkuma, S., and B. Poole.** 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. USA **75:**3327–3331.
47. **Ouadrhiri, Y., B. Scorneaux, Y. Sibille, and P. M. Tulkens.** 1999. Mechanism of the intracellular killing and modulation of antibiotic susceptibility of *Listeria monocytogenes* in THP-1 macrophages activated by gamma interferon. Antimicrob. Agents Chemother. **43:**1242–1251.
48. **Paillard, D., J. Grellet, V. Dubois, M. C. Saux, and C. Quentin.** 2002. Discrepancy between uptake and intracellular activity of moxifloxacin in a *Staphylococcus aureus*-human THP-1 monocytic cell model. Antimicrob. Agents Chemother. **46:**288–293.
49. **Peck, O. M., H. Fan, G. E. Tempel, G. Teti, P. V. Halushka, and J. A. Cook.** 2004. Staphylococcus aureus and lipopolysaccharide induce homologous tolerance but heterologous priming: role of interferon-gamma. Shock **21:**254–260.
50. **Philippon, A., G. Paul, F. Brunet, and J. P. Giroud.** 1988. Pénicillines, p. 1295–1329. *In* J. P. Giroud, G. Mathé, and G. Meyniel (ed.), Pharmacologie clinique, bases de la thérapeutique. Expansion Scientifique Française, Paris, France.
51. **Proctor, R. A., J. M. Balwit, and O. Vesga.** 1994. Variant subpopulations of Staphylococcus aureus as cause of persistent and recurrent infections. Infect. Agents Dis. **3:**302–312.
52. **Rogers, D. E., and R. Tompsett.** 1952. The survival of staphylococci within human leukocytes. J. Exp. Med. **95:**209–230.
53. **Ross, E. M., and T. Kenakin.** 2001. Pharmacodynamics: mechanism of drug action and the relationship between drug concentration and effect, p. 31–43. *In* J. Hardman and L. E. Limbird (ed.), Goodman & Gilman's the pharmacological basis of therapeutics. McGraw-Hill Medical Publishing Division, New York, N.Y.
54. **Schwab, J. C., and G. L. Mandell.** 1989. The importance of penetration of antimicrobial agents into cells. Infect. Dis. Clin. N. Am. **3:**461–467.
55. **Scorneaux, B., Y. Ouadrhiri, G. Anzalone, and P. M. Tulkens.** 1996. Effect of recombinant human gamma interferon on intracellular activities of antibiotics against *Listeria monocytogenes* in the human macrophage cell line THP-1. Antimicrob. Agents Chemother. **40:**1225–1230.
56. **Seral, C., M. Barcia-Macay, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke.** 2005. Comparative activity of quinolones (ciprofloxacin, levofloxacin, moxifloxacin and garenoxacin) against extracellular and intracellular infection by Listeria monocytogenes and Staphylococcus aureus in J774 macrophages. J. Antimicrob. Chemother. **55:**511–517.
57. **Seral, C., F. Van Bambeke, and P. M. Tulkens.** 2003. Quantitative analysis of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, and oritavancin (LY333328) activities against intracellular *Staphylococcus aureus* in mouse J774 macrophages. Antimicrob. Agents Chemother. **47:**2283–2292.
58. **Stass, H., A. Dalhoff, D. Kubitza, and U. Schuhly.** 1998. Pharmacokinetics, safety, and tolerability of ascending single doses of moxifloxacin, a new 8-methoxy quinolone, administered to healthy subjects. Antimicrob. Agents Chemother. **42:**2060–2065.
59. **Sullivan, J. T., M. Woodruff, J. Lettieri, V. Agarwal, G. J. Krol, P. T. Leese, S. Watson, and A. H. Heller.** 1999. Pharmacokinetics of a once-daily oral dose of moxifloxacin (Bay 12–8039), a new enantiomerically pure 8-methoxy quinolone. Antimicrob. Agents Chemother. **43:**2793–2797.
60. **Tano, E., O. Cars, and E. Lowdin.** 2005. Pharmacodynamic studies of moxifloxacin and erythromycin against intracellular Legionella pneumophila in an in vitro kinetic model. J. Antimicrob. Chemother. **56:**240–242.
61. **Tome, J. P., M. G. Neves, A. C. Tome, J. A. Cavaleiro, M. Soncin, M. Magaraggia, S. Ferro, and G. Jori.** 2004. Synthesis and antibacterial activity of new poly-S-lysine-porphyrin conjugates. J. Med. Chem. **47:**6649–6652.
62. **Tulkens, P., and A. Trouet.** 1978. The uptake and intracellular accumulation of aminoglycoside antibiotics in lysosomes of cultured rat fibroblasts. Biochem. Pharmacol. **27:**415–424.
63. **Tyteca, D., P. Van Der Smissen, M. Mettlen, F. Van Bambeke, P. M. Tulkens, M. P. Mingeot-Leclercq, and P. J. Courtoy.** 2002. Azithromycin, a lysosomotropic antibiotic, has distinct effects on fluid-phase and receptor-mediated endocytosis, but does not impair phagocytosis in J774 macrophages. Exp. Cell Res. **281:**86–100.
64. **Van Bambeke, F., S. Carryn, C. Seral, H. Chanteux, D. Tyteca, M. P. Mingeot-Leclercq, and 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.
65. **Van Bambeke, F., Y. Van Laethem, P. Courvalin, and P. M. Tulkens.** 2004. Glycopeptide antibiotics: from conventional molecules to new derivatives. Drugs **64:**913–936.
66. **van den Broek, P. J.** 1991. Activity of antibiotics against microorganisms ingested by mononuclear phagocytes. Eur. J. Clin. Microbiol. Infect. Dis. **10:**114–118.
67. **van den Broek, P. J., L. F. Buys, H. Mattie, and R. van Furth.** 1986. Comparison of the effect of phenoxymethylpenicillin, cloxacillin, and flucloxacillin on Staphylococcus aureus phagocytosed by human monocytes. J. Antimicrob. Chemother. **17:**767–774.
68. **Waldvogel, F. A.** 2000. Staphylococcus aureus, p. 2069–2092. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, Philadelphia, Pa.
69. **Weinrick, B., P. M. Dunman, F. McAleese, E. Murphy, S. J. Projan, Y. Fang, and R. P. Novick.** 2004. Effect of mild acid on gene expression in *Staphylococcus aureus*. J. Bacteriol. **186:**8407–8423.
70. **Yancey, R. J., M. S. Sanchez, and C. W. Ford.** 1991. Activity of antibiotics against Staphylococcus aureus within polymorphonuclear neutrophils. Eur. J. Clin. Microbiol. Infect. Dis. **10:**107–113.
71. **Zhanel, G. G., A. K. Wierzbowski, P. Hisanaga, and D. J. Hoban.** 2004. The use of ketolides in treatment of upper respiratory tract infections. Curr. Infect. Dis. Rep. **6:**191–199.

2. Evaluation of the extracellular and intracellular activities (human THP-1 macrophages) of telavancin vs. vancomycin against methicillin-sensitive, methicillin-resistant, vancomycin-intermediate and vancomycin-resistant Staphylococcus aureus

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Evaluation of the extracellular and intracellular activities (human THP-1 macrophages) of telavancin versus vancomycin against methicillin-susceptible, methicillin-resistant, vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus*

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***Objectives*: To compare extracellular and intracellular activities of telavancin (versus vancomycin) against *Staphylococcus aureus* (MSSA, MRSA, VISA and VRSA).**

***Methods*: Determination of cfu changes (3–24 h) in culture medium and in macrophages at concentrations ranging from 0.01 to 1000× MIC.**

***Results*: Extracellularly, telavancin displayed a fast, concentration-dependent bactericidal activity against all strains. The concentration–effect relationship was bimodal for MSSA and MRSA [two successive sharp drops in bacterial counts (0.3–1× MIC and 100–1000× MIC) separated by a zone of low concentration dependency]. When compared at human total drug C_{max} (vancomycin, 50 mg/L; telavancin, 90 mg/L) towards MSSA, MRSA and VISA, telavancin caused both a faster and more marked decrease of cfu, with the limit of detection (>5 log decrease) reached already at 6 versus 24 h for vancomycin. Intracellularly, the bactericidal activity of telavancin was less intense [–3 log (MSSA) to –1.5 log (VRSA) at C_{max} and at 24 h]. A bimodal relationship with respect to concentration (at 24 h) was observed for both MSSA and MRSA. In contrast, vancomycin exhibited only marginal intracellular activity towards intraphagocytic MSSA, MRSA and VISA (max. –0.5 log decrease at 24 h and at C_{max}).**

***Conclusions*: Telavancin showed time- and concentration-dependent bactericidal activity against both extracellular and intracellular *S. aureus* with various resistance phenotypes. The data support the use of telavancin in infections where intracellular and extracellular *S. aureus* are present. Bimodality of dose responses (MSSA and MRSA) could indicate multiple mechanisms of action for telavancin.**

Keywords: lipoglycopeptide, Gram-positive, bactericidal, phagolysosomes, concentration dependence

Introduction

Treatment for *Staphylococcus aureus* infections faces two major issues: (i) recurrent and relapsing character (convincingly associated with the capacity of this organism to survive and multiply within eucaryotic cells)[1–4] and (ii) narrowing choice of available agents due to increased emergence of resistance.[5] Therefore, new agents remaining active against multi-resistant strains and demonstrating bactericidal activity against both extracellular and intracellular bacteria are needed. This is probably all the more important since pharmacodynamic analyses of vancomycin successes and failures in patients with severe infections suggest that considerably higher drug dosages than anticipated may be needed for successful therapy.[6]

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Telavancin, a hydrophobic derivative of vancomycin,[7] displays a more intense bactericidal activity than vancomycin against *S. aureus* and other Gram-positive organisms and remains active against vancomycin-resistant organisms.[8,9] This has been related to its multiple modes of action, which, beyond inhibition of bacterial cell wall synthesis, also includes disruption of bacterial membrane integrity.[10,11] Telavancin is effective in various animal models of difficult-to-treat staphylococcal infections and in biofilms,[12–16] and is in clinical development.[17,18] Moreover, telavancin accumulates within alveolar macrophages,[19] which could be useful for controlling intracellular infections.

In the present study, we compared the extracellular and intracellular activities of telavancin and vancomycin against *S. aureus*, using strains with different resistance phenotypes towards β-lactams and vancomycin, and cultured murine and human macrophages. The antibacterial responses were analysed over a wide range of extracellular concentrations (pharmacological analysis) and discussed in terms of total and free concentrations as they can be observed clinically in humans.[20,21]

Materials and methods

Cells and cell cultures

Human (THP-1) macrophages (grown in suspension) and murine (J774) macrophages (grown as monolayers) were cultured exactly as described previously.[22–25]

Bacterial strains and MIC determinations

The following strains were used: (i) ATCC 25923 (fully susceptible); (ii) ATCC 29213 (β-lactamase producing MSSA); (iii) ATCC 33591 (MRSA with homogeneous resistance to oxacillin) and ATCC 43300 (MRSA with heterogeneous resistance to oxacillin);[26] (iv) NRS23 (HIP08926) and NRS52 (HIP09737) [MRSA with intermediate level of vancomycin resistance (VISA)]; and (v) VRS1 (HIP11714 or Michigan strain)[27] and VRS2 (HIP11983 or Pennsylvania strain)[28] [MRSA with high level of resistance to vancomycin (VRSA)]. MICs were measured by microdilution in Muller–Hinton broth,[22,25] supplemented by 2% NaCl for MRSA [US Clinical Laboratory Standards Institute (CLSI), Wayne, PA].

Determination of antibiotic activity against extracellular S. aureus

Kill curve experiments were performed in the culture medium of macrophages (containing 10% foetal calf serum)[25] and for control purposes also in Muller–Hinton broth, according to previously published and validated methods.[25,29] In brief, all samples (diluted as needed) were prepared in a final volume of 1 mL, and 50 μL was used for seeding standard Petri dishes. After 24 h incubation at 37°C, colonies were counted using an automated detector[29] with validation for the linearity of the response (3–1500 colonies per dish), intra-day reproducibility and lowest limit of detection (3 counts/plate, corresponding to an actual 4.2 log cfu decrease from a typical initial inoculum of 10^6 bacteria per mL; samples yielding fewer than three colonies were arbitrarily considered as corresponding to a 5 log decrease). Antibiotics were considered bactericidal at a given concentration and a given time if causing a 3 log cfu decrease or greater compared with the original inoculum.[30]

Phagocytosis of S. aureus and determination of antibiotic activity against intracellular S. aureus

We used the same methods as those previously with MSSA ATCC 25923,[22,24,25] except that linezolid rather than gentamicin was used to control extracellular contamination when using VRSA [100× MIC for washing; 1× MIC (2 mg/L for VRS1; 100 mg/L for VRS2) during the incubation]. In brief, bacteria were opsonized with non-decomplemented, freshly thawed human serum diluted 1:10 in serum-free culture medium (RPMI 1640). Phagocytosis was performed at a 4:1 bacteria–macrophage ratio. Elimination of non-phagocytosed bacteria and collection of cells at the end of the experiment were made by centrifugation at room temperature [1300 rpm; 8 min; Eppendorf 5810R Centrifuge equipped with a A-4-62 rotor (Eppendorf Gerätgebau GmbH, Engeldorf, Germany)].

Macrophages were then lysed by resuspension in distilled water and the corresponding samples processed for cfu counting as described above and using the same upper and lowest limits of detection. Proteins were measured in parallel as described previously.[31]

Assessment of macrophage cell membrane integrity

Reliable determination of the intracellular activity of antibiotics requires that direct contact between the extracellular drug and the phagocytosed bacteria is avoided.[32] Since telavancin increases membrane permeability in bacteria,[11] we tested its influence on macrophage membrane by measuring the release of the cytosolic enzyme lactate dehydrogenase using a method described previously for assessing the toxicity of large concentrations of macrolides to fibroblasts,[33] of macrophages exposed to large concentrations of fluoroquinolones and of efflux pump inhibitors,[34] and, more recently, to distinguish between gentamicin-induced apoptosis and necrosis in LLC-PK1 cells.[35] In brief, enzyme activity was measured in the medium and in cells (collected by centrifugation as described above) before (initial levels) and after 24 h incubation (post-incubation levels) in the absence or in the presence of the antibiotics. Results were expressed as the per cent increase in the medium/cell activity ratio; therefore, corresponding to a net release of the enzyme from cells. Control cells (no antibiotic added) and cells exposed to telavancin showed the same increase (6.1 ± 0.3%) up to telavancin concentrations of 150 mg/L, but there was a 25.2 ± 2.5% increase for cells incubated with 500 mg/L telavancin, denoting a significant level of cell toxicity. Vancomycin (250 mg/L) was without significant effect compared to control cells.

Confocal and electron microscopy

This was performed exactly as described previously for adherent and non-adherent cells.[22,25]

Materials

Telavancin hydrochloride for microbiological evaluation (purity > 90%) was supplied in powder form by Theravance Inc, South San Francisco, CA, USA. Because of its low solubility, stock solutions (1–10 mg/L in water) were prepared with extensive shaking (at least 30 min) and carefully checked for absence of undissolved material. Although suggested by the manufacturer, no DMSO and/or acid addition was made since these interfered with macrophage viability. Vancomycin and linezolid were procured as the corresponding branded products registered in Belgium for parenteral use (VANCOCIN® from GlaxoSmithKline; ZYVOXID® from Pfizer). MSSA and MRSA strains were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA; and VISA

and VRSA isolates from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) at Focus Technologies, Inc., Herndon, VA, USA. Cell culture or microbiology media were from Invitrogen Ltd, Paisley, UK, and from BD Diagnostics Systems (formerly DIFCO Inc.), Sparks, MD, USA. Other reagents were of analytic grade and purchased from E. Merck AG (Darmstadt, Germany) or Sigma-Aldrich-Fluka (St Louis, MO, USA).

Results

Susceptibility testing

MICs/MBCs (mg/L) of vancomycin were 1/1 and 1/1 for MSSA ATCC 25923 and ATCC 29213; 2/4 and 2/2 for MRSA ATC33591 and ATCC 43300; 4/4 and 4/4 for VISA NRS23 and NRS52 (MICs for VRSA VRS1 and VRS2 were >128 and 16). MICs/MBCs (mg/L) of telavancin were 0.5/0.5 for MSSA (ATCC 25923 and ATCC 29213), 0.5/1 and 0.5/0.5 for MRSA (ATCC 33591 and ATCC 43300), 0.5/0.5 for VISA (NRS23 and NRS52), and 4/8 and 2/8 for VRSA (VRS1 and VRS2; the MICs observed for those two VRSA are the same as those reported recently by another group of investigators;[36] for VRS2; however, the original publication[37] reported a value of 0.5 mg/L). For all strains, no marked difference was seen when MICs were determined in broth adjusted to pH 5.5 (to mimic the phagolysosomal environment) versus pH 7.3.

Extracellular activity

Figure 1 shows the kinetics of activity of vancomycin and telavancin towards extracellular *S. aureus* exposed to three selected concentrations, namely the MIC, 10× MIC and a concentration mimicking the reported human total drug C_{max}.[20,21] Vancomycin always acted slowly, with a marked influence of the concentration at 24 h only. For all three strains tested, a bactericidal effect (3 log cfu decrease) was obtained only at a concentration of 10× the MIC or higher, and after an incubation time of ~20 h at 10× the MIC and of 15 h at C_{max}. In contrast, telavancin (i) was more concentration dependent; (ii) produced a bactericidal effect for MSSA ATCC 25923 and ATCC 29213, and for MRSA ATCC 33591 within 18 h at 1× the MIC only; (iii) was bactericidal at C_{max} for all strains (including the two VRSA strains) within 2 (MSSA ATCC 25923) to 10 h (MRSA ATCC 43300 and VRS1); (iv) caused apparent complete eradication at C_{max} within 6 h for MSSA ATCC 25923, MRSA ATCC 33591 and NRS52, and at 24 h for MSSA ATCC 29213 and MRSA ATCC 43300. Towards VISA NRS23 and the two VRSA, telavancin was bacteriostatic at its MIC, but caused a 4.5 log decrease at C_{max}.

Figure 2 shows the results observed against MSSA ATCC 25923 using a wide range of drug concentrations (0.01 to 1000× MIC) and after 3 or 24 h of incubation. At 3 h (left panel), telavancin exerted an antibacterial effect that developed in a bimodal fashion, with a first decrease in cfu to reach a plateau at about 2.5 log below the original inoculum for concentrations ranging from 1 to 10× MIC, followed by a second decrease to a value close to the limit of detection at 300× MIC or higher. In contrast, vancomycin caused only a modest decrease in cfu even at the largest concentration tested. At 24 h (right panel), telavancin caused a 4 log cfu decrease at the MIC, and the limit of detection was reached at a concentration of 10× MIC, making the bimodal character of the response difficult to observe. Vancomycin also exhibited a dose-dependent bactericidal activity, but higher multiples of MICs (3- to 10-fold) were needed to achieve similar killing effects.

The concentration dependency of telavancin extracellular activity towards *S. aureus* was further examined for all remaining strains at the same time points (Figure 3; data obtained with strain ATCC 25923 shown in Figure 2 are included for comparison). At 3 h (left panels), (i) an apparent static effect was seen for MRSA, VISA and VRSA strains at a telavancin concentration close to the MIC; (ii) the bimodal response with respect to the concentration was clearly seen for MRSA [with the first plateau (1.5–2.5 log decrease) in the 1–100× MIC range as for MSSA ATCC 29213], but almost not for VISA and not for VRSA (linear decrease in cfu as a function of the drug concentration). For all strains (except MSSA ATCC 25923 which was more susceptible), a bactericidal effect (3 log cfu decrease) at 3 h required concentrations of 300–1000× the MIC. At 24 h (right panels), a bactericidal effect was obtained for concentrations of ~0.85–2× MIC (0.4–1 mg/L) for MSSA and MRSA, and of ~10–44× the MIC (5–22 mg/L) for VISA and VRSA. The limit of detection was obtained at concentrations spanning from 10× MIC (MSSA ATCC 25923) to 250× MIC (NRS23). To check for a potential interference of calf serum in the results shown above, kill curves (3 and 24 h) were repeated for MSSA (ATCC 25923 and ATCC 29213) and MRSA (ATCC 33591 and ATCC 43300) using Muller–Hinton broth. Results not significantly different from those shown in Figure 3 (including the bimodality of the response at 3 h) with an excellent correlation between the two sets of data [linear regression parameters for all data points included in the comparison (n = 77; values below the detection limit were excluded): slope, 0.981 ± 0.02 (95% CI: 0.940–1.024); R^2 = 0.967; $P < 0.0001$].

Intracellular activity (infected macrophages)

We first examined whether our model of *S. aureus* infected J774 and THP-1 macrophages developed with MSSA ATCC 25923[22,25] could be used with the other strains included in this study. In all cases, the intracellular growth could be monitored, and the extracellular growth prevented by the addition of gentamicin (1× MIC for MRSA and VISA), or linezolid (1× MIC for VRSA) when no glycopeptide was added (controls). Intracellular bacteria were unambiguously observed in the macrophages by confocal and/or electron microscopy (data not shown). As discussed previously,[25] cultures maintained in the absence of antibiotic (or with the lowest concentrations [0.01× MIC] of the antibiotics tested) showed a larger bacterial growth [about 2–3 log cfu increase (VRSA strains) over the original inoculum], which was partly due to extracellular bacteria, but without gross deleterious effect on macrophages, as assessed by the measurement of total cell protein [no significant change (J774 macrophages) or modest reduction (23.5% ± 16.8; P = 0.017; n = 24 for THP-1 cells) between infected cultures exposed to telavancin at 0.01 and 1000× MIC, respectively].

The kinetics of intracellular activities of vancomycin (left panel) and telavancin (right panel) was compared towards MSSA ATCC 25923 in THP-1 macrophages exposed to the three selected concentrations (MIC, 10× MIC and the C_{max}) used previously for assessing extracellular activities (Figure 4).

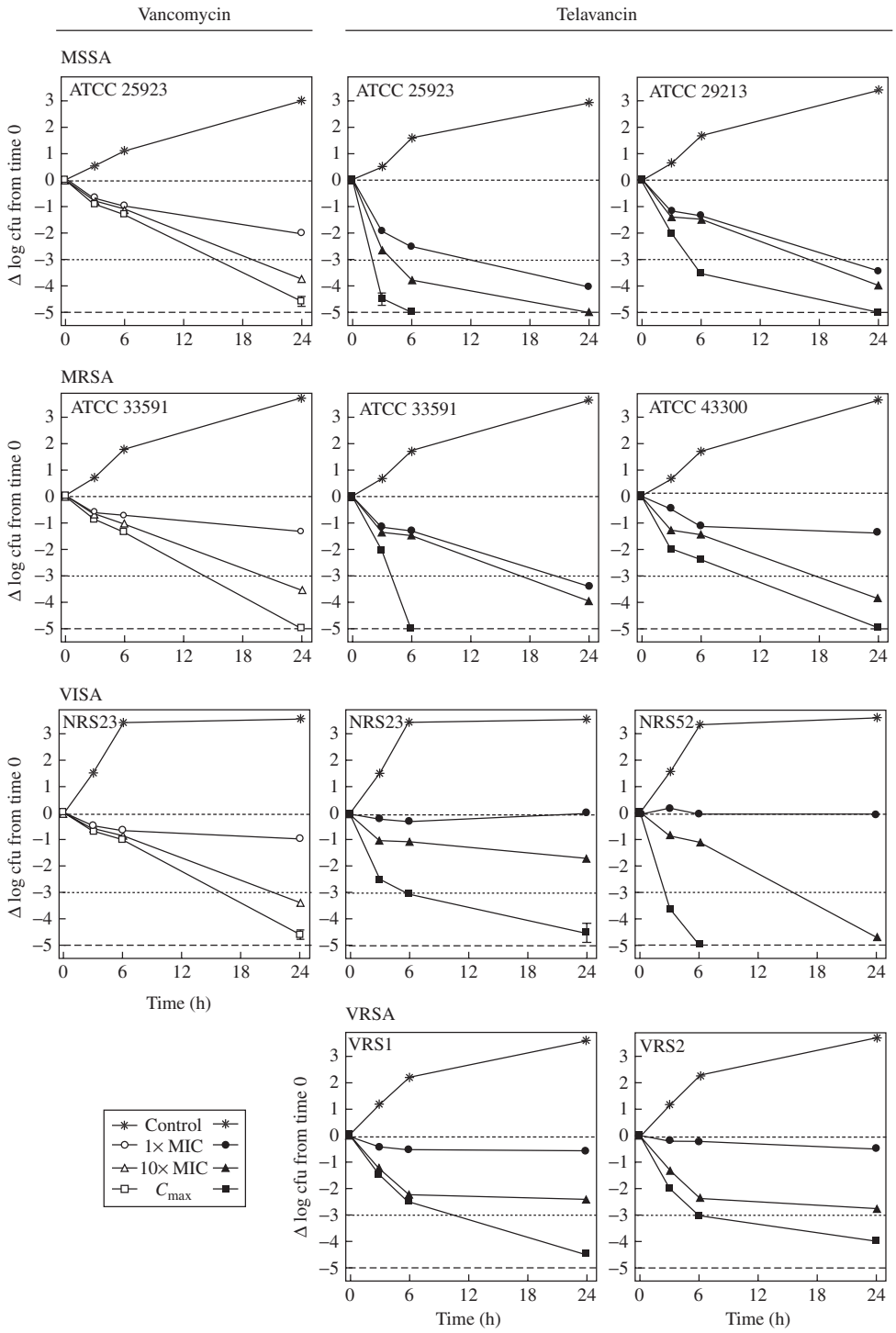

Figure 1. Kinetics of activity of vancomycin and telavancin against the extracellular forms of *S. aureus.* The graphs show the variation in the number of cfu per mL of culture medium upon incubation of *S. aureus* strains [MSSA: ATCC 25923, ATCC 29213; MRSA: ATCC 33591, ATCC 43300; VISA: NRS23, NRS52; VRSA (telavancin only): VRS1, VRS2] for up to 24 h with increasing concentrations of vancomycin and telavancin [corresponding to 1× MIC, 10× MIC, and the human C_{max} (50 mg/L for vancomycin[21]; 90 mg/L for telavancin[20]]. The initial inoculum varied from $10^{5.99}$ to $10^{6.06}$ cfu/mL. Results are given as means ± standard deviation (n = 3; when not visible, SD are smaller than the symbols). The thick dotted line corresponds to a static effect (no change from the initial inoculum); the grey dotted line shows the decrease in cfu (3 log) considered as denoting a bactericidal effect;[30] the dotted line at –5 log corresponds to the lower limit of detection.

Telavancin extracellular and intracellular activities

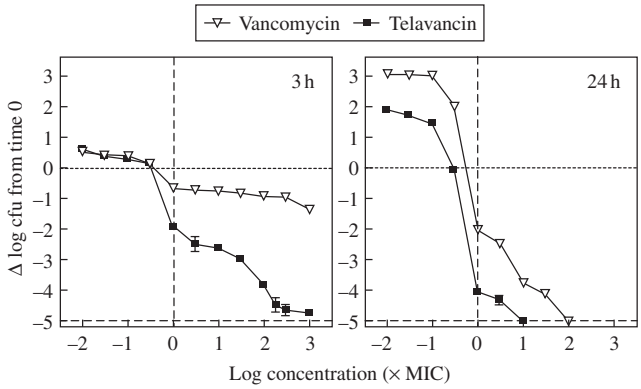

Figure 2. Concentration–effect relationship of the activity of vancomycin and telavancin against the extracellular forms of *S. aureus.* The graphs show the variation in the number of cfu per mL of culture medium upon incubation of *S. aureus* MSSA ATCC 25923 for 3 h (left) or 24 h (right) with increasing concentrations of vancomycin and telavancin (ranging from 0.01 to 1000× MIC). The initial inoculum was 10^6 cfu/mL. Results are given as means ± standard deviation (n = 3; when not visible, SD are smaller than the symbols). The thick dotted line corresponds to a static effect (no change from the initial inoculum); the thin dotted line at –5 log shows the limit of detection.

Vancomycin did not prevent bacterial growth at its MIC, became static at 10× its MIC and achieved intracellular killing (–1.3 log) at its C_{max} only after 24 h (only marginal effects were seen at 3 and 6 h). In contrast, telavancin was rapidly bactericidal at all 3 concentrations tested, achieving a 2 log decrease within 6 h at its C_{max}. No further decrease in bacterial counts, however, was seen upon longer exposure to telavancin.

The concentration dependency of the intracellular activities of vancomycin and telavancin was then examined at 24 h for all strains over a 0.01 to 1000× MIC concentration range. Figure 5 shows the data obtained with THP-1 human macrophages. For both antibiotics, concentration-dependent effects were seen, but with significant differences in the concentrations needed for static and maximal effects. Thus, a bacteriostatic effect was obtained with vancomycin at 3–10× MIC or higher, but already at 1× MIC with telavancin (except for VRSA which required higher concentrations). At higher concentrations of telavancin, a first plateau was then reached at about 1–1.5 log cfu below the original inoculum for extracellular concentrations ranging from 1–5 to 50–100× MIC. This plateau was followed by a second decrease in the number of cfu for MSSA and MRSA at concentrations ranging between 100 and 1000× MIC. For VISA and VRSA, only a first plateau at about 1.5 log decrease from the original inoculum was observed. Similar results were obtained with telavancin in J774 macrophages (not shown).

Discussion

This study shows that telavancin displays a fast bactericidal activity against extracellular as well as intraphagocytic forms of *S. aureus*, including MRSA, VISA and VRSA strains. These properties contrast with the overall behaviour of vancomycin, which displays a slower bactericidal activity towards extracellular bacteria, and a bacteriostatic effect only towards intracellular bacteria.

Telavancin shares with vancomycin the pharmacophore that allows its binding to the bacterial D-Ala-D-Ala motif, causing inhibition of the peptidoglycan biosynthesis.[11] Telavancin, however, also displays a decylaminoethyl side chain[7,8] that confers membrane destabilization properties in bacteria at higher concentrations.[11] This may explain why telavancin (i) acts more quickly and is more bactericidal than vancomycin against vancomycin-susceptible strains; (ii) displays bimodal concentration effects towards MSSA and MRSA, but almost linear concentration effects towards VISA and VRSA, since these are expected to be poorly susceptible (VISA) or resistant (VRSA) to the D-Ala-D-Ala binding-mediated inhibition of peptidoglycan synthesis. For the VRSA strains, the loss of the action mediated by binding to D-Ala-D-Ala may also explain the higher MICs and larger MBC/MIC ratio of telavancin, compared with other strains, since the membrane destabilization-mediated mode of action, which should be the only one to operate in VRSA, appears to require larger concentrations.[11]

The intracellular activity of telavancin was weaker than its extracellular activity (as is the case for all antibiotics examined in our models so far).[22,25] Yet, and in sharp contrast with vancomycin, telavancin nevertheless exhibited a bactericidal activity (defined here as a 3 log decrease from the original inoculum by analogy to what is commonly accepted to categorize an antibiotic as bactericidal and as proposed previously)[25] for all strains tested. This effect is unlikely to result from a direct contact of extracellular telavancin with intraphagocytic *S. aureus*, since we could exclude any gross membrane destabilization of macrophages in our model. Interestingly, bimodal concentration-effect curves were clearly seen for intraphagocytic MSSA and MRSA, and to some extent VISA, suggesting that the multiple modes of action of telavancin observed against the extracellular forms of these strains are also operating in the intracellular environment. We know that telavancin penetrates macrophages *in vitro* and *in vivo*.[19,38] Future studies will therefore need to critically examine key cellular pharmacokinetic/pharmacodynamic parameters of telavancin such as its subcellular disposition, bioavailability and local expression of activity.[39]

The present data obtained *in vitro* may not be extrapolated to the *in vivo* situation without caution. First, we only used two types of immortalized macrophages with poor or no host defences against intracellular infection,[22,29] but this was to obtain a true pharmacological evaluation of telavancin (the activity of which seems less influenced by the immune status of the host than that of vancomycin or linezolid).[14] Second, the persistence of viable intracellular bacteria even after extended exposure to large concentrations of telavancin needs to be critically examined, but this phenomenon is not specific to telavancin.[22,25] Third, we used exposure to constant drug concentrations, which is not in line with the projected clinical use of telavancin.[17,18]

While all these limitations clearly call for the development of more refined, dynamic *in vitro* models, the design of our experiments, nevertheless, allows for potentially useful discussions with respect to dose–effect relationships. Telavancin is bactericidal (using the criterion of 3 log cfu decrease) within 24 h for the extracellular forms of all strains at concentrations ranging from 0.7 (MSSA ATCC 25923) to 22 mg/L (VISA NRS23, the least susceptible strain in our study). *In vivo* pharmacodynamic models suggest that telavancin efficacy is best predicted by the AUC/MIC ratio.[14] Applying this to our conditions, the AUC needed to reach a 3 log cfu decrease within 24 h

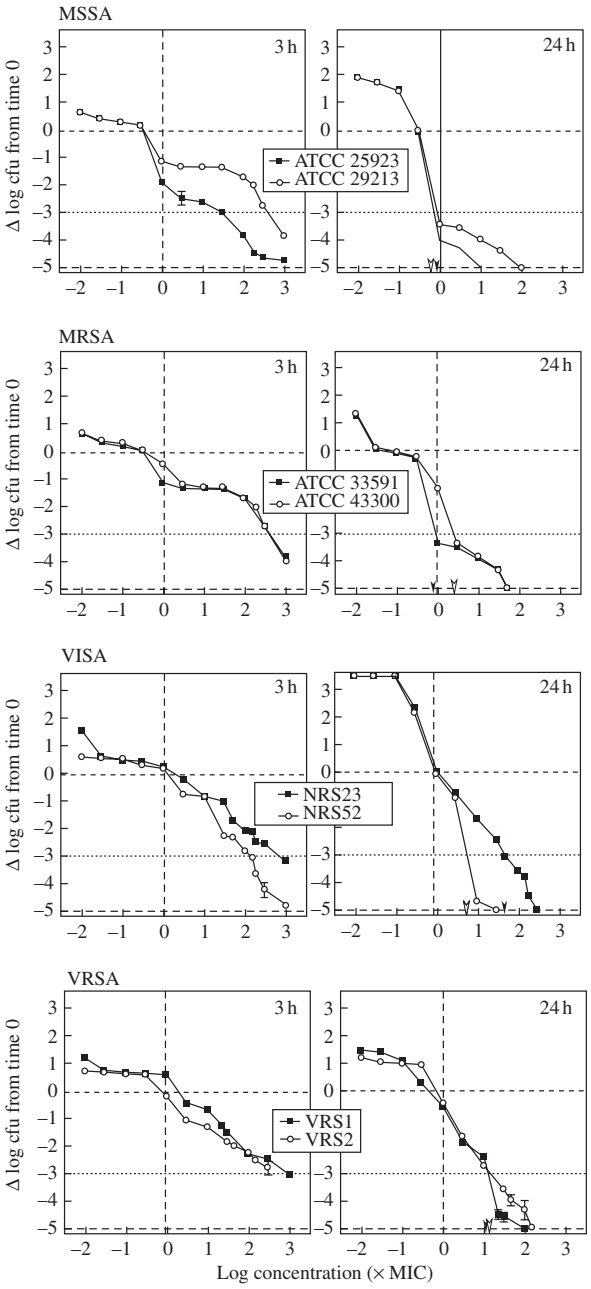

Figure 3. Concentration–effect relationship of the activity of telavancin against the extracellular forms of *S. aureus*. The graphs show the variation in the number of cfu per mL of culture medium upon incubation of *S. aureus* strains (MSSA: ATCC 25923, ATCC 29213; MRSA: ATCC 33591, ATCC 43300; VISA: NRS23, NRS52; VRSA: VRS1, VRS2) for 3 h (left) or 24 h (right) with increasing concentrations of telavancin (ranging from 0.01 to 1000× MIC). The initial inoculum varied between $10^{5.97}$ and $10^{6.13}$ cfu/mL). Results are given as means±standard deviation (n = 3; when not visible, SD are smaller than the symbols). The thick dotted line corresponds to a static effect (no change from the initial inoculum); the grey dotted line shows the decrease in cfu (3 log) considered as denoting a bactericidal effect[30] (with the arrowheads pointing to the corresponding antibiotic concentrations as used for the calculation of the corresponding AUC (open arrowheads, strains with open symbols; closed arrowheads, strains with closed symbols); the thin dotted line at –5 log shows the limit of detection.

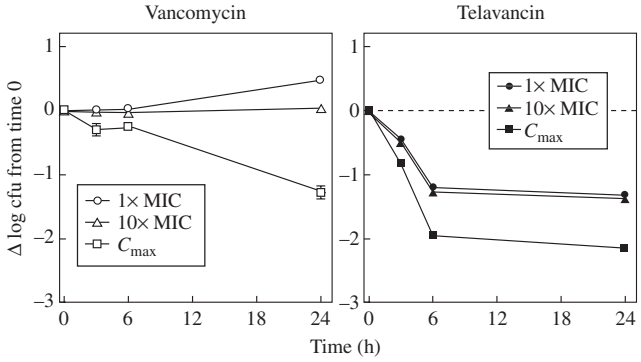

Figure 4. Kinetics of activity of vancomycin and telavancin against the intracellular forms of *S. aureus* in a model of human THP-1 macrophages. The graphs show the variation in the number of cfu per mg cell protein upon incubation of *S. aureus* MSSA ATCC 25923 for up to 24 h with increasing concentrations of vancomycin and telavancin [corresponding to 1× MIC, 10× MIC and the human C_{max} (50 mg/L for vancomycin[21]; 90 µg/mL for telavancin[20])]. The initial inoculum was $10^{6.21}$ cfu/mg of cell protein. Results are given as means ± standard deviation (n = 3; when not visible, SD are smaller than the symbols). The thick dotted line corresponds to a static effect (no change from the initial inoculum).

[AUC = 24 (h) × $C_{3log\ decrease}$ (mg/L), using the data of Figure 3] would be around 10 for MSSA ATCC 25923 and ATCC 29213, around 12 and 25 for MRSA ATCC 39591 and ATCC 43300, around 125 and 500 for VISA NRS52 and NRS23, and around 600 and 1200 for VRSA VRS2 and VRS1). The typical human dose of 10 mg/kg of telavancin (used in the current clinical trials)[17,18] yields a total drug AUC of ∼900 mg · h/L,[20] suggesting that a bactericidal effect will be easily be obtained for MSSA, MRSA and VISA strains and for VRS2, and will be close to being obtained for VRS1. But this does not take into account the high protein binding of telavancin (93%).[20] For most antibiotics, including teicoplanin, another glycopeptide with high protein binding, it is generally agreed that pharmacokinetic/pharmacodynamic indices such as AUC/MIC ratios must use free drug concentrations only.[40,41] If this was also the case for telavancin, we should conclude that bactericidal effects may never be obtained for VISA and VRSA strains *in vivo*, since the minimal AUC needed, based on our data but corrected for protein binding, might be far above what the projected clinical dosage could yield. Recent *in vitro* studies, however, failed to demonstrate a marked influence of serum on the killing capabilities of telavancin,[36] suggesting that using only free drug concentrations to calculate a given target attainment rate would underestimate the real potency of the drug. It is also of interest that kill curves performed in Mueller–Hinton broth or in the cell culture medium (which contains 10% foetal bovine serum) showed no significant differences.

Given these caveats, the present study suggests that telavancin has the potential to display useful activity against *S. aureus* in those infections where not only eradication of extracellular bacteria but also the control of intracellular forms is critical. Reaching both goals may allow decreasing persistence and recurrence, two well-known features of many staphylococcal infections. These may include skin and soft tissues infections, or endocarditis, two diseases in which telavancin efficacy has already been successfully studied.[12,14,17,18]

Telavancin extracellular and intracellular activities

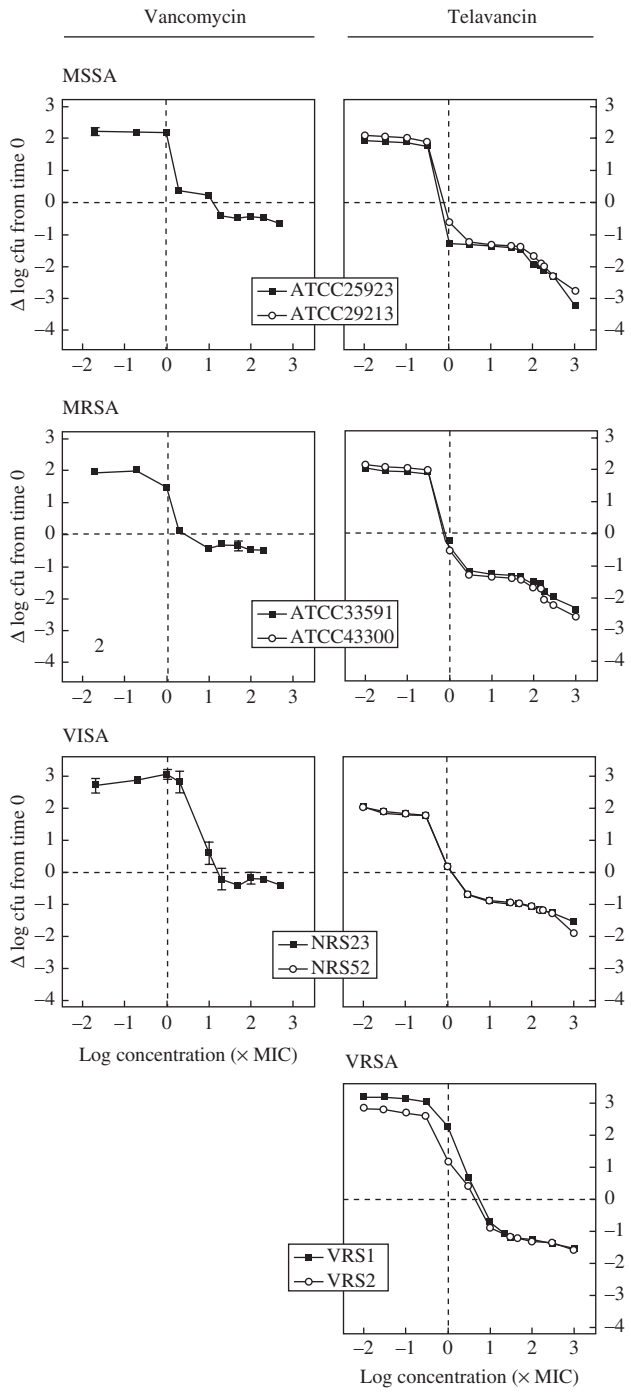

Figure 5. Concentration–effect relationship of the activity of telavancin against the intracellular forms of *S. aureus* in a model using human THP-1 macrophages. The graphs show the variation in the number of cfu per mg cell protein upon incubation of *S. aureus* strains [MSSA: ATCC 25923, ATCC 29213; MRSA: ATCC 33591, ATCC 43300; VISA: NRS23, NRS52; VRSA (telavancin only): VRS1, VRS2] with vancomycin (left) or telavancin (right) at increasing extracellular concentrations (ranging from 0.01 to 1000× MIC) for 24 h. The initial inoculum varied between $10^{6.11}$ and $10^{6.37}$ cfu/mg of cell protein. Results are given as means±standard deviation (n = 3; when not visible, SD are smaller than the symbols). The thick dotted line corresponds to a static effect (no change from the initial inoculum).

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Transparency declaration

None to declare.

References

1. Ellington JK, Harris M, Webb L *et al*. Intracellular *Staphylococcus aureus*. A mechanism for the indolence of osteomyelitis. *J Bone Joint Surg Br* 2003; **85**: 918–21.

2. Hess DJ, Henry-Stanley MJ, Erickson EA *et al*. Intracellular survival of *Staphylococcus aureus* within cultured enterocytes. *J Surg Res* 2003; **114**: 42–9.

3. Lowy FD. Is *Staphylococcus aureus* an intracellular pathogen? *Trends Microbiol* 2000; **8**: 341–3.

4. Clement S, Vaudaux P, Francois P *et al*. Evidence of an intracellular reservoir in the nasal mucosa of patients with recurrent *Staphylococcus aureus* rhinosinusitis. *J Infect Dis* 2005; **192**: 1023–8.

5. Bal AM, Gould IM. Antibiotic resistance in *Staphylococcus aureus* and its relevance in therapy. *Expert Opin Pharmacother* 2005; **6**: 2257–69.

6. Moise-Broder PA, Forrest A, Birmingham MC *et al*. Pharmacodynamics of vancomycin and other antimicrobials in patients with *Staphylococcus aureus* lower respiratory tract infections. *Clin Pharmacokinet* 2004; **43**: 925–42.

7. Leadbetter MR, Adams SM, Bazzini B *et al*. Hydrophobic vancomycin derivatives with improved ADME properties: discovery of telavancin (TD-6424). *J Antibiot (Tokyo)* 2004; **57**: 326–36.

8. Van Bambeke F. Glycopeptides in clinical development: pharmacological profile and clinical perspectives. *Curr Opin Pharmacol* 2004; **4**: 471–8.

9. Pace JL, Judice JK. Telavancin (Theravance). *Curr Opin Investig Drugs* 2005; **6**: 216–25.

10. King A, Phillips I, Kaniga K. Comparative *in vitro* activity of telavancin (TD-6424), a rapidly bactericidal, concentration-dependent anti-infective with multiple mechanisms of action against Gram-positive bacteria. *J Antimicrob Chemother* 2004; **53**: 797–803.

11. Higgins DL, Chang R, Debabov DV *et al*. Telavancin, a multifunctional lipoglycopeptide, disrupts both cell wall synthesis and cell membrane integrity in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2005; **49**: 1127–34.

12. Madrigal AG, Basuino L, Chambers HF. Efficacy of Telavancin in a rabbit model of aortic valve endocarditis due to methicillin-resistant *Staphylococcus aureus* or vancomycin-intermediate *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2005; **49**: 3163–5.

13. Reyes N, Skinner R, Kaniga K *et al*. Efficacy of telavancin (TD-6424), a rapidly bactericidal lipoglycopeptide with multiple

mechanisms of action, in a murine model of pneumonia induced by methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2005; **49**: 4344–6.

14. Hegde SS, Reyes N, Wiens T *et al*. Pharmacodynamics of telavancin (TD-6424), a novel bactericidal agent, against gram-positive bacteria. *Antimicrob Agents Chemother* 2004; **48**: 3043–50.

15. Gander S, Kinnaird A, Finch R. Telavancin: *in vitro* activity against staphylococci in a biofilm model. *J Antimicrob Chemother* 2005; **56**: 337–43.

16. Reyes N, Skinner R, Benton BM *et al*. Efficacy of telavancin in a murine model of bacteraemia induced by methicillin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother* 2006; **58**: 462–5.

17. Stryjewski ME, O'Riordan WD, Lau WK *et al*. Telavancin versus standard therapy for treatment of complicated skin and soft-tissue infections due to gram-positive bacteria. *Clin Infect Dis* 2005; **40**: 1601–7.

18. Stryjewski ME, Chu VH, O'Riordan WD *et al*. Telavancin versus standard therapy for treatment of complicated skin and skin structure infections caused by gram-positive bacteria: FAST 2 Study. *Antimicrob Agents Chemother* 2006; **50**: 862–7.

19. Gotfried M, Duchin K, Shaw JP *et al*. Telavancin penetrates well into human pulmonary epithelial lining fluid and alveolar macrophages and is unaffected by pulmonary surfactant. In: *Programs and Abstracts of the Forty-fifth Interscience Conference on Antimicrobial Agents and Chemotherapy*, *Washington, DC, 2005.* Abstract A-14, p. 3. American Society for Microbiology, Washington, DC, USA.

20. Shaw JP, Seroogy J, Kaniga K *et al*. Pharmacokinetics, serum inhibitory and bactericidal activity, and safety of telavancin in healthy subjects. *Antimicrob Agents Chemother* 2005; **49**: 195–201.

21. Feketi R. Vancomycin, teicoplanin, and the streptogramins: quinupristin and dalfopristin. In: Mandell GE, Bennett JE, Dolin R, eds. *Principles and Practice of Infectious Diseases*, 5th edn. Philadelphia, PA: Churchill Livingstone, 2000; 382–92.

22. Seral C, Van Bambeke F, Tulkens PM. Quantitative analysis of the activity of antibiotics [gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, oritavancin (LY333328)] against intracellular *Staphylococcus aureus* in mouse J774 macrophages. *Antimicrob Agents Chemother* 2003; **47**: 2283–92.

23. Van Bambeke F, Carryn S, Seral C *et al*. Cellular pharmacokinetics and pharmacodynamics of the glycopeptide antibiotic oritavancin (LY333328) in a model of J774 mouse macrophages. *Antimicrob Agents Chemother* 2004; **48**: 2853–60.

24. Lemaire S, Van Bambeke F, Mingeot-Leclercq MP *et al*. Activity of three {beta}-lactams (ertapenem, meropenem and ampicillin) against intraphagocytic *Listeria monocytogenes* and *Staphylococcus aureus*. *J Antimicrob Chemother* 2005; **55**: 897–904.

25. Barcia-Macay M, Seral C, Mingeot-Leclercq MP *et al*. Pharmacodynamic evaluation of the intracellular activity of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages. *Antimicrob Agents Chemother* 2006; **50**: 841–51.

26. Swenson JM, Spargo J, Tenover FC *et al*. Optimal inoculation methods and quality control for the NCCLS oxacillin agar screen test for detection of oxacillin resistance in *Staphylococcus aureus*. *J Clin Microbiol* 2001; **39**: 3781–4.

27. Centers for Disease Control and Prevention. Staphylococcus aureus resistant to vancomycin—United States, 2002. *MMWR Morb Mortal Wkly Rep* 2002; **51**: 565–7.

28. Centers for Disease Control and Prevention. Vancomycin-resistant *Staphylococcus aureus*—Pennsylvania, 2002. *MMWR Morb Mortal Wkly Rep* 2002; **51**: 902.

29. Carryn S, Van de Velde S, Van Bambeke F *et al*. Impairment of growth of *Listeria monocytogenes* in THP-1 macrophages by granulocyte macrophage colony-stimulating factor: release of tumor necrosis factor-alpha and nitric oxide. *J Infect Dis* 2004; **189**: 2101–9.

30. National Committee for Clinical Laboratory Standards. *Methods for Determining Bactericidal Activity of Antimicrobial Agents: Approved Standard M26-A*. NCCLS, Wayne, PA, USA, 1998.

31. Carryn S, Van Bambeke F, Mingeot-Leclercq MP *et al*. Activity of beta-lactams (ampicillin, meropenem), gentamicin, azithromycin and moxifloxacin against intracellular *Listeria monocytogenes* in a 24 h THP-1 human macrophage model. *J Antimicrob Chemother* 2003; **51**: 1051–2.

32. Sanchez MS, Ford CW, Yancey RJ,Jr. Evaluation of antibacterial agents in a high-volume bovine polymorphonuclear neutrophil *Staphylococcus aureus* intracellular killing assay. *Antimicrob Agents Chemother* 1986; **29**: 634–8.

33. Montenez JP, Van Bambeke F, Piret J *et al*. Interactions of macrolide antibiotics (Erythromycin A, roxithromycin, erythromycylamine [Dirithromycin], and azithromycin) with phospholipids: computer-aided conformational analysis and studies on acellular and cell culture models. *Toxicol Appl Pharmacol* 1999; **156**: 129–40.

34. Michot JM, Van Bambeke F, Mingeot-Leclercq MP *et al*. Active efflux of ciprofloxacin from J774 macrophages through an MRP-like transporter. *Antimicrob Agents Chemother* 2004; **48**: 2673–82.

35. Servais H, Van Der SP, Thirion G *et al*. Gentamicin-induced apoptosis in LLC-PK1 cells: involvement of lysosomes and mitochondria. *Toxicol Appl Pharmacol* 2005; **206**: 321–33.

36. Leuthner KD, Cheung CM, Rybak MJ. Comparative activity of the new lipoglycopeptide telavancin in the presence and absence of serum against 50 glycopeptide non-susceptible staphylococci and three vancomycin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother* 2006; **58**: 338–4.

37. Tenover FC, Weigel LM, Appelbaum PC *et al*. Vancomycin-resistant *Staphylococcus aureus* isolate from a patient in Pennsylvania. *Antimicrob Agents Chemother* 2004; **48**: 275–80.

38. Barcia-Macay M, Mingeot-Leclercq MP, Tulkens PM *et al*. Telavancin accumulates in cultured macrophages and is active against intracellular *S. aureus*. In: *Programs and Abstracts of the Forty-fifth Interscience Conference on Antimicrobial Agents and Chemotherapy*, DC, *Washinghton, 2005.* Abstract A-1831, p. 31. American Society for Microbiology, Washington, DC, USA.

39. Van Bambeke F, Barcia-Macay M, Lemaire S *et al*. Cellular pharmacodynamics and pharmacokinetics of antibiotics: current views and perspectives. *Curr Opin Drug Discov Devel* 2006; **9**: 218–30.

40. Knudsen JD, Fuursted K, Raber S *et al*. Pharmacodynamics of glycopeptides in the mouse peritonitis model of *Streptococcus pneumoniae* or *Staphylococcus aureus* infection. *Antimicrob Agents Chemother* 2000; **44**: 1247–54.

41. Chambers HF, Kennedy S. Effects of dosage, peak and trough concentrations in serum, protein binding, and bactericidal rate on efficacy of teicoplanin in a rabbit model of endocarditis. *Antimicrob Agents Chemother* 1990; **34**: 510–4.

3. Telavancin accumulates in eucaryotic cells (J774 mouse macrophages; rat embryo fibroblasts) without affecting their viability or lipid content

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Paper in preparation

ABSTRACT

Background: telavancin is a lipoglycopeptide with multiple mechanisms of action towards bacterial cells. We examined here its interaction with eucaryotic cells with respect to accumulation, distribution, and effects on membrane integrity and lipid content.

Methods: J774 macrophages and rat embryo fibroblasts were exposed for up to 24 h and 72 h to telavancin (5-90 mg/L [human C_{max}]). We determine the drug cellular concentration (radioactive drug), subcellular distribution (cell fractionation), as well as its influence on cellular viability (release of lactate dehydrogenase) and phospholipid or cholesterol content.

Results: telavancin accumulated linearly as a function of the time and of the concentration in both cell types (rate of uptake: ~ 0.01 µg/mg prot per h and per µg/ml of extracellular drug in culture medium added by 10 % serum; twice this value in the absence of serum). Efflux (measured in macrophages) was ~ 5.7-fold slower. Telavancin distribution was similar to that of a lysosomal enzyme (N-acetyl-β-hexosaminidase) in a sucrose gradient. Telavancin did not alter the pericellular membrane integrity (no significant release of lactate dehydrogenase). It did neither significantly modify the cell content in total phospholipids or cholesterol, in contrast to oritavancin, which induced a marked accumulation of both types of lipids.

Conclusion : telavancin accumulates on a slowly reversible manner in the lysosomes of phagocytic and non-phagocytic cells by a non specific mechanism. This accumulation is not associated with any alteration of cell viability or lipid metabolism and may therefore represent an advantage for the eradication of intracellular bacteria.

INTRODUCTION

Telavancin is a lipoglycopeptide derivative of vancomycin,[1] in phase III of clinical development for the treatment of complicated skin and soft tissues infections and of pneumonia caused by multiresistant *Staphylococcus aureus.*[2] As compared to its parent compound, telavancin is characterized by an additional hydrophobic side chain on the vancosamine sugar (decylaminoethyl) and by a (phosphonomethyl)aminomethyl substituant on the cyclic peptidic core. These modifications confer to telavancin new pharmacokinetic and pharmacodynamic properties.

First, telavancin shows a high protein binding (~ 90-93 %), and a reasonably prolonged half-life (~ 7.5 h),[3-5], which make it suitable for once-a-day administration.[6,7] Recent data also suggests a large distribution for this drug, with a high penetration in tissues[8] and a slow accumulation in human alveolar macrophages.[9] The latter property is however difficult to characterize *in vivo*. The first goal of this study was therefore to examine the cellular pharmacokinetics of telavancin, using both phagocytic (J774 mouse macrophages) and non-phagocytic (rat embryo fibroblasts) cultured cells. These models have indeed been successfully used in the past to determine the cellular pharmacokinetic properties of many antibiotics.[10-12]

Second, telavancin is highly and rapidly bactericidal towards multi-resistant gram-positive organisms, including glycopeptide-susceptible organisms as well as glycopeptide-intermediate and vancomycin-resistant *Staphylococcus aureus.*[13,14] This results from its complex mechanism of action, which involves a perturbation of bacterial lipid synthesis and the disruption of the bacterial membrane.[15] The second aim of this study was therefore to examine the specificity of action of telavancin towards bacterial cells, by evaluating the membrane integrity and the content in polar lipids of macrophages and fibroblasts exposed to telavancin. These studies were run in parallel with vancomycin and oritavancin (the latter was indeed shown to cause a marked accumulation of phospholipids and cholesterol in cultured cells[16]).

MATERIALS AND METHODS

Cells and cell cultures. J774 mouse macrophages and rat embryo fibroblasts were cultivated respectively in RPMI 1640 medium or DMEM medium supplemented with 10 % foetal calf serum (unless stated otherwise), as described.[16]

Determination of cellular antibiotic concentration. Cells were incubated with ^{14}C-labeled telavancin, washed three times in ice-cold NaCl 0.9 %, and collected by scraping in distilled water. Telavancin was assayed by radiometry, and its cell concentration was expressed by reference to the protein content (see ref[12] for a more detailed description of the methodology). To check that the concentrations measured corresponded to those of a bioactive product, we used in parallel a microbiological assay (with *Micrococcus luteus* ATCC 9341 as test organism and antibiotic medium 11), in which telavancin concentrations in samples were calculated using a calibration curve constructed from cell lysates spiked with known amounts of the drug. A correlation coefficient of 0.93 was found between cellular contents measured by radiometric and microbiological assays. Validation by the LC-MS/MS method used to measure serum level *in vivo*[5,9] was not feasible here, since preliminary experiments showed that this procedure extracted only 2 to 10 % of telavancin from cell lysates spiked with known amounts of the drug.

Cell fractionation studies. Separation of the main subcellular organelles was made by means of combined differential and isopycnic centrifugation exactly as described in details earlier.[12] Protein and ^{14}C-telavancin contents were determined in the fractions in parallel with the activity of marker enzymes of the main organelles, namely inosine 5'-diphosphatase (E.C. 3.6.1.6.), cytochrome *c* oxidase (E.C. 1.9.3.1.), and N-acetyl-β-glucosaminidase (E.C. 3.2.1.30.). Results are expressed as the percentage of enzyme activity, protein or drug recovered in each fraction in function of the density. Histograms were then standardized for densities of equal increments ranging from 1.08 to 1.21, as described earlier.[17]

Biochemical studies. At the end of the incubation with glycopeptides, culture medium were collected and cell sheets were washed three times in ice-cold NaCl 0.9 %, collected by scraping in distilled water and lysed by sonication. Cell viability assessed by measuring the percentage of lactate dehydrogenase released in the culture medium (using the technique described by Vassault[18]). Total phospholipids and cholesterol were extracted and assayed as in previous publications.[16] Proteins were assayed by the Folin-Ciocalteu/biuret method.[19]

Materials. Telavancin hydrochloride as powder for microbiological evaluation (purity > 90 %) and ^{14}C-telavancin trifluoroacetate (33.8 mCi/mmol; radiochemical purity > 91 %) were supplied by Theravance, Inc., San Francisco, CA. The labelled drug was mixed with unlabelled telavancin to obtain a specific activity of 5 mCi/mmol. Stock solutions were prepared at a final concentration of 1-2 mg/L, by vigorous vortexing in distilled water, so as to avoid any effect of other solvent (acidified DMSO, as suggested by the manufacturer) on cellular viability. Oritavancin (supplied as diphosphate salt fully hydrated) was obtained from Intermune (Brisbane, CA). Vancomycin was procured as VANCOCIN ® (the commercial product registered for clinical use in Belgium and supplied by GlaxoSmithKline Belgium). Cell culture or microbiology media were from Invitrogen (Paisley, Scotland, UK) and Difco (Sparks,MD). Other reagents were of analytic grade and purchased from E. Merck AG (Darmstadt, Germany) or Sigma-Aldrich-Fluka (St Louis, MO).

RESULTS

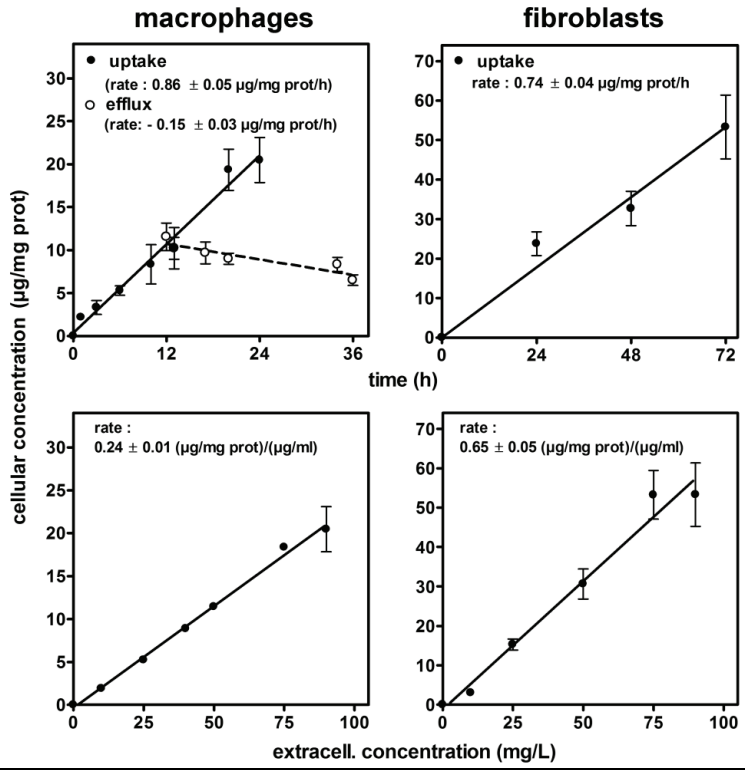
Cellular pharmacokinetics of telavancin

Figure 1 (upper panels) shows the kinetics of accumulation or of efflux of telavancin in cells incubated with an extracellular concentration of 90 mg/L (human Cmax[5]). In J774 mouse macrophages (left), the accumulation of the drug proceeded on a linear fashion over 24 hours. Telavancin efflux was also followed in macrophages exposed to 90 mg/L of telavancin during 12 h, washed, and reincubated in a telavancin free-medium. Kinetics of efflux was linear as well, but slower than influx (rate ~ 5.7-fold inferior to the rate of influx). In rat embryo fibroblasts (right), telavancin uptake remained linear over 3 days, and proceeded at a rate very similar to that observed in macrophages (~ 0.8 µg/mg prot/h).

Figure 1 (lower panels) examines the effect of increasing telavancin extracellular concentration on its cellular accumulation in macrophages at 24 h (left) or in fibroblasts at 72 h (right). In both cell types, the cellular concentration was directly proportional to the extracellular concentration over the whole the range investigated. Thus, since telavancin accumulation proceeded linearly as a function of both the time and the drug extracellular concentration, we calculated that the drug was taken up by macrophages and fibroblasts at a very similar rate of ~ 0.01 µg/mg prot per hour and per µg/ml of extracellular drug.

Since televancin is known as highly bound to serum proteins, we evaluated, in a next series of experiments, the influence of calf serum present in the culture fluid on drug uptake. To this effect, telavancin accumulation was measured in J774 macrophages, using in parallel culture media added or not by calf serum, but limiting the incubation time to 5 h. In these conditions, telavancin uptake remained linear over the 10-90 mg/L range of extracellular concentrations, but proceeded 1.7-fold quicker than in the presence of 10 % calf serum, corresponding to a rate of ~ 0.02 µg/mg prot per hour and per µg/ml of extracellular drug.

Figure 1



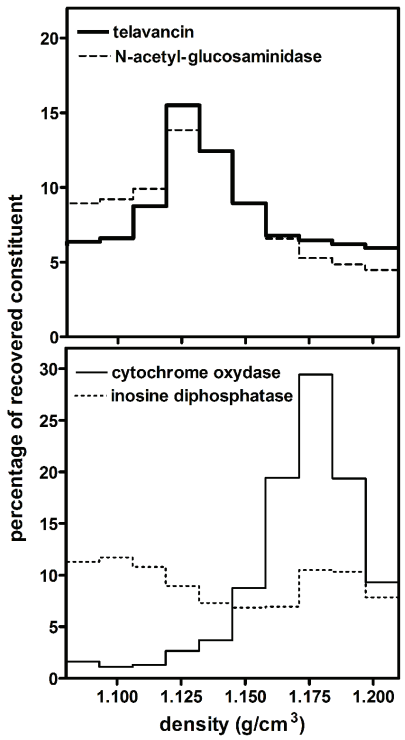
Upper panel: Kinetics of uptake of telavancin (closed symbols and plain line) in J774 macrophages (left) or embryo fibroblasts (right) incubated for the indicated times with an extracellular concentration of 90 mg/L at 37°C in medium supplemented with 10 % foetal calf serum. The left panel also shows the kinetics of efflux of the drug from cells loaded during 12 h in the same conditions and reincubated in a drug-free medium for 24 h (open symbols and dotted line). Lower panel: cellular concentration of telavancin in J774 macrophages (left) or embryo fibroblasts (right) incubated for 24 h at 37°C with the indicated extracellular concentrations of telavancin in medium supplemented with 10 % foetal calf serum. Results are given as arithmetic means ± standard deviation (n=3). R^2 for linear regressions: upper panel: 0.9832 for uptake and 0.8386 for efflux in macrophages; 0.9742 for uptake in fibroblasts; lower panel: 0.9851 for macrophages; 0.9804 for fibroblasts.

Subcellular distribution of telavancin

The subcellular distribution of the cell-associated drug was then determined in homogenates obtained from J774 macrophages incubated for 24 h with 90 mg/L telavancin and separated by differential centrifugation into a "nuclei/unbroken cells" fraction (low speed centrifugation), a "granule fraction" (high speed centrifugation), and a final supernatant. The distribution of cell-associated telavancin in these fractions fitted well with that of the lysosomal enzyme N-acetyl-β-glucosaminidase (respective recovery of telavancin and of this enzyme were 10 % and 6 % in the supernatant, 55 % and 67 % in the "granules fraction", and 35 % and 27 % "nuclei/unbroken cells" fraction). The "granule fraction" was therefore analyzed by isopycnic centrifugation to determine the distribution of telavancin among the organelles present in that fraction (mainly mitochondria, lysosomes, and the vesicles formed by the disruption and resealing of the endoplasmic reticulum and pericellular membranes).

Figure 2 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, demonstrating an effective analytical separation of these organelles, as observed in our previous publications.[12,17] The distribution pattern of telavancin was largely similar to that of the N-acetyl-β-glucosaminidase, strongly suggesting a co-localization in the same organelles (lysosomes).

Figure 2



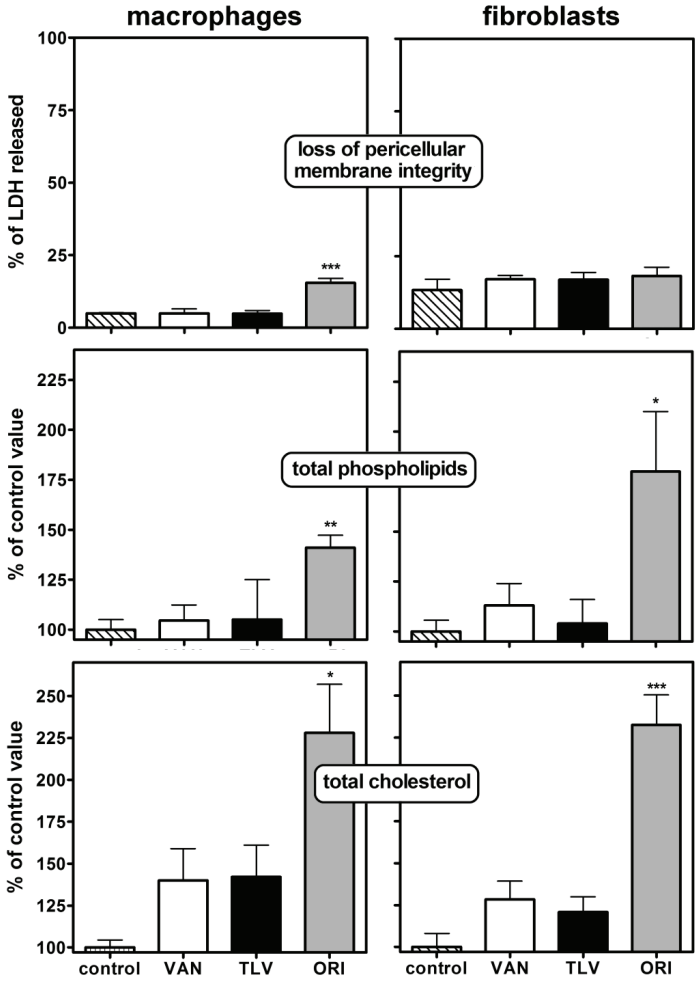
Density distribution of marker enzymes (N-acetyl-β-glucosaminidase [lysosomes], cytochrome *c*-oxidase [mitochondria]), and inosine 5'-diphosphatase [plasma and endoplasmic reticulum membranes]), and of telavancin after isopycnic centrifugation in linear sucrose gradient of a granule fraction prepared from homogenates of J774 cells which were incubated for 24 h with 90 mg/L telavancin at 37°C in medium supplemented with 10 % foetal calf serum. The ordinate shows the percentage of each constituent recovered in each fraction.

Assessment of telavancin influence on cellular viability and lipid metabolism

In the last series of experiments, we examined whether telavancin has the capacity to affect cell viability by altering pericellular membrane integrity, as assessed by the release in the culture medium of lactate dehydrogenase, a cytosolic enzyme. We also searched for an effect on lipid metabolism, based on our previous observations that oritavancin, but not vancomycin, induced a marked increase in the phospholipid and cholesterol content of cultured cells.[16] These glycopeptides were therefore used here in parallel and all were compared at their human Cmax (90 mg/L for telavancin[5]; 50 mg/L for vancomycin[20] and 25 mg/L for oritavancin[21]). Figure 3 illustrated the data obtained after 24 h and 72 h of incubation in macrophages and fibroblasts, respectively. Thus, telavancin did not cause any significant change in cellular viability, neither in the cellular phospholipid or cholesterol

content, just as vancomycin. In sharp contrast, oritavancin induced a slight but significant release of lactate dehydrogenase in macrophages (15 % of the total enzymatic activity, versus 5 % for controls, vancomycin or telavancin) as well as a marked accumulation of lipidic material in both cell types.

Figure 3



Cellular viability (as assessed by the release of lactate dehydogenase in the culture medium; upper panels), total phospholipids content (middle panels) or total cholesterol content (lower panels) of J774 mouse macrophages (left) or rat embryo fibroblasts (right) exposed for 24 h and 72 h, respectively, to glycopeptides at their human C_{max} (VAN, vancomycin 50 mg/L; TLV, telavancin 90 mg/L; ORI, oritavancin 25 mg/L). Results are expressed as percentages of lactate dehydrogenase released in the culture medium at the end of the incubation period, or as percentages of control values for total phospholipids and cholesterol. Values are arithmetic means ± SD, with n=3 for LDH release and n= 6-8 for phospholipids and cholesterol. Values for control macrophages and fibroblasts were respectively 204 ± 10 and 295 ± 8 nmol/mg prot for total phospholipids, and 72 ± 3 and 130 ± 16 nmol/mg prot for total cholesterol. Statistical analysis (ANOVA): ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ as compared to the matching control. Other differences are not significant.

DISCUSSION

The present study documents that telavancin is internalised within eucaryotic cells, but does not exert towards these cells the toxic effects associated to its cidal action against prokaryotic cells (membrane permeabilization[15]), or observed with oritavancin.[16]

Examining cellular pharmacokinetics first, we demonstrate here that telavancin accumulates to high levels and on a slowly reversible fashion in the lysosomes of cultured cells. Together with our previous report on oritavancin,[12] this study therefore presents these lipophilic glycopeptides as a new class of cell-associated antibiotics, which may be a clear advantage for the eradication of intracellular infections. This has indeed been exemplified with bacteria sojourning in the vacuolar apparatus, like *S. aureus.*[14,22].

Despite the gross similarity in the cellular pharmacokinetic profile of these two drugs, some differences can nevertheless be evidenced. In particular, the accumulation of telavancin is slower than that of oritavancin, with a calculated clearance of ~ 10 µL x mg $prot^{-1}$ x h^{-1}, against an initial value of ~ 150 µL x mg $prot^{-1}$ x h^{-1} for oritavancin.[12] This rate, however, is still higher than that of markers of fluid phase pinocytosis (~0.7 µL x mg $prot^{-1}$ x h^{-1}) [23], and therefore compatible with a mechanism of adsorptive endocytosis (as proposed for oritavancin), driving the drug to the lysosomes from where its efflux is slow. The facts that telavancin accumulation remains linear as a function of the time and of the concentration in the ranges investigated, and reaches similar levels in both phagocytic and non-phagocytic cells, nevertheless suggest that telavancin binding sites at the cell surface are far from being saturated, in contrast to what was observed with oritavancin.[12]

Beside their biological interest, these *in vitro* data also need to be put in a pharmacological perspective, and to be examined in the light of preliminary *in vivo* studies, documenting the capacity of telavancin to accumulate in human alveolar macrophages, with cellular concentrations reaching 42 (3.4-83) µg/ml at 24 h.[9] This value seems lower than what was found here *in vitro*, but this can be explained by the following differences. First, our model does not take into account the fluctuations in concentrations over time observed *in vivo,* neither the capacity of the drug to gain access to alveolar macrophages from the blood stream. Second, the protein binding of telavancin is not the same in human serum *in vivo,* or in the culture fluid added by 10 % foetal calf serum we used in the present *in vitro* study. The free fraction of the drug is indeed probably the one which is taken up by cells. Taking therefore into account a protein binding of ~ 90-93 % and a half-life of ~ 7.5 h,[5] we can estimate that *in vivo* free serum concentrations would oscillate between ~ 10 and 0.5 µg/ml. Considering in our models a rate of uptake for the free fraction of ~ 0.02 µg/mg prot per hour and per µg/ml of extracellular drug, cellular concentrations would reach at 24 h a minimum of

~ 0.24 µg/mg prot (i.e. ~ 48 µg/ml, if approximating the mean cellular volume to 5 µl/mg prot [[12] and references cited therein]). Third, *in vivo* cellular contents may be underestimated, based on our observation that the LC-MS/MS method used to measure telavancin concentration in human alveolar macrophages did not fully extract cell-associated telavancin in our models (see methods).

Considering then cellular toxicity issues, we show here that telavancin does not affect the pericellular membrane integrity, even upon prolonged exposure to concentrations much higher than those needed to disrupt the permeability of bacterial cells.[15] We neither detect any significant change in the cellular content of lipids, in sharp contrast to what we described previously for oritavancin.[16] This divergence is most probably due to the fact that the cellular concentrations in telavancin are much lower than those of oritavancin, with the maximal values observed in our experimental conditions being close the threshold cellular contents in oritavancin needed to induce lipid accumulation.[16] We can however not exclude a difference in the intrinsic capacity of both drugs to interfere with lipid metabolism.

As a whole, our *in vitro* data, therefore, bring a mechanistic support to the capacity of telavancin to slowly accumulate within eucaryotic cells *in vivo,* and tend to suggest that high cellular levels could also be achieved upon repeated administration of the drug over the treatment duration, because of the slow reversibility of this accumulation. This accumulation, however, is not associated with cellular alterations of the same nature as those induced by telavancin in bacterial cells or by other lipoglycopeptides in eucaryotic cells. Combined with the bactericidal potency of this drug, a high cellular accumulation can therefore represent an advantage for treating persistent staphylococcal infections, for which intracellular foci are probably present.[24,25]

Acknowledgments

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References

1. Leadbetter MR, Adams SM, Bazzini B *et al.* Hydrophobic vancomycin derivatives with improved ADME properties: discovery of telavancin (TD-6424). *Journal Antibiotics (Tokyo)* 2004; **57**, 326-36.

2. Pace JL, Judice JK. Telavancin (Theravance). *Current Opinion Investigational Drugs* 2005; **6**, 216-25.

3. Pace JL, Yang G. Glycopeptides: Update on an old successful antibiotic class. *Biochem Pharmacol* 2006; **71**, 968-80.

4. Van Bambeke F. Glycopeptides in clinical development: pharmacological profile and clinical perspectives. *Current Opinion Pharmacology* 2004; **4**, 471-8.

5. Shaw JP, Seroogy J, Kaniga K *et al.* Pharmacokinetics, serum inhibitory and bactericidal activity, and safety of telavancin in healthy subjects. *Antimicrob Agents Chemother* 2005; **49**, 195-201.

6. Stryjewski ME, Chu VH, O'Riordan WD *et al.* Telavancin versus Standard Therapy for Treatment of Complicated Skin and Skin Structure Infections Caused by Gram-Positive Bacteria: FAST 2 Study. *Antimicrob Agents Chemother* 2006; **50**, 862-7.

7. Stryjewski ME, O'Riordan WD, Lau WK *et al.* Telavancin versus standard therapy for treatment of complicated skin and soft-tissue infections due to gram-positive bacteria. *Clin Infect Dis* 2005; **40**, 1601-7.

8. Sun HK, Duchin K, Nightingale CH *et al.* Tissue penetration of telavancin after intravenous administration in healthy subjects. *Antimicrob Agents Chemother* 2006; **50**, 788-90.

9. Gotfried M, Duchin K, Shaw JP, Seroogy J, Benton B, Krause K, Kitt M, Barriere S. Telavancin penetrates well into human pulmonary epithelial lining fluid and alveolar macrophages and is unaffected by pulmonary surfactant. In abstracts of the *45th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 2005.* A14.

10. Carlier MB, Garcia-Luque I, Montenez JP *et al.* Accumulation, release and subcellular localization of azithromycin in phagocytic and non-phagocytic cells in culture. *Int J Tissue React* 1994; **16**, 211-20.

11. Tulkens P, Trouet A. The uptake and intracellular accumulation of aminoglycoside antibiotics in lysosomes of cultured rat fibroblasts. *Biochem Pharmacol* 1978; **27**, 415-24.

12. Van Bambeke F, Carryn S, Seral C *et al.* Cellular pharmacokinetics and pharmacodynamics of the glycopeptide antibiotic oritavancin (LY333328) in a model of J774 mouse macrophages. *Antimicrob Agents Chemother* 2004; **48**, 2853-60.

13. Pace JL, Krause K, Johnston D *et al.* In vitro activity of TD-6424 against Staphylococcus aureus. *Antimicrob Agents Chemother* 2003; **47**, 3602-4.

14. Barcia-Macay M, Lemaire S, Mingeot-Leclercq MP *et al.* Evaluation of the extracellular and intracellular activities (human THP-1 macrophages) of telavancin vs. vancomycin against methicillin-sensitive, methicillin-resistant, vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother* 2006; Epub; Oct 24.

15. Higgins DL, Chang R, Debabov DV *et al.* Telavancin, a multifunctional lipoglycopeptide, disrupts both cell wall synthesis and cell membrane integrity in methicillin-resistant Staphylococcus aureus. *Antimicrob Agents Chemother* 2005; **49**, 1127-34.

16. Van Bambeke F, Saffran J, Mingeot-Leclercq MP *et al.* Mixed-lipid storage disorder induced in macrophages and fibroblasts by oritavancin (LY333328), a new glycopeptide antibiotic with exceptional cellular accumulation. *Antimicrob Agents Chemother* 2005; **49**, 1695-700.

17. Renard C, Vanderhaeghe HJ, Claes PJ *et al.* Influence of conversion of penicillin G into a basic derivative on its accumulation and subcellular localization in cultured macrophages. *Antimicrob Agents Chemother* 1987; **31**, 410-6.

18. Vassault A. (1987). Lactate dehydrogenase. In *Methods in Enzymatic Analysis,* Bergemeyer HU, Eds), pp. 118-26. VHC Publishers, Veinheim, Federal Republic of Germany,

19. Lowry OH, N.J.Rosebrough, A.L.Farr *et al.* Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**, 265-75.

20. Feketi R. (2000). Vancomycin, teicoplanin, and the streptogramins: quinupristin and dalfopristin. In *Principles and practice of infectious diseases,* 5th (Mandell GE, Bennett JE, Dolin R, Eds), pp. 382-92. Churchill Livingstone, Philadelphia, Pen,

21. Braun DK, Chien JK, Farlow DS *et al.* Oritavancin (LY333328): a dose-escalation safety and pharmacokinetics study in patients. *Clin Microbiol Infect* 2001; **7**, P434.

22. Barcia-Macay M, Seral C, Mingeot-Leclercq MP *et al.* Pharmacodynamic evaluation of the intracellular activities of antibiotics against Staphylococcus aureus in a model of THP-1 macrophages. *Antimicrob Agents Chemother* 2006; **50**, 841-51.

23. Tyteca D, Van Der Smissen P, Mettlen M *et al.* Azithromycin, a lysosomotropic antibiotic, has distinct effects on fluid-phase and receptor-mediated endocytosis, but does not impair phagocytosis in J774 macrophages. *Experimental Cell Research* 2002; **281**, 86-100.

24. Ellington JK, Harris M, Webb L *et al.* Intracellular Staphylococcus aureus. A mechanism for the indolence of osteomyelitis. *J Bone Joint Surg Br* 2003; **85**, 918-21.

25. Lowy FD. Is Staphylococcus aureus an intracellular pathogen? *Trends Microbiol* 2000; **8**, 341-3.

DISCUSSION

1. Models of intracellular infection

Although the intracellular-facultative bacterium *S. aureus* has been primarily considered an extracellular pathogen, it is now clear that it can be internalized by both phagocytes and non-phagocytic cells (102,167). The intracellular residence of *S. aureus* is considered to play an important role on their persistence (host defenses evasion) and recurrence (antibiotic failure) of infection (38,54,85).

Therefore, the selection of antibiotics for such infections should consider their efficacy not only toward extracellular, but also towards intracellular bacteria (45,261).

Our work has precisely consisted in comparing the activity of a series of antibiotics against the extracellular and intracellular forms of *S. aureus* in similar conditions of exposure (in terms of time of incubation and antibiotic concentrations) so as to delineate how their activity could be modified when bacteria are intracellular. To this effect we have developed and validated an intracellular model in which human monocytes-like macrophages cells are successfully infected by *S. aureus* strains for a period of up to 24-h.

1.1. Interest of the model

Although microbiological standard parameters to test antimicrobial activity are useful to predict antibiotic outcome on extracellular bacteria (64), they do not provide information on intracellular killing. By standardizing some of these parameters it is possible to study “in vitro” adequate antibiotic concentrations and more precisely describe the course of this antimicrobial activity. Many intracellular models have been developed over time, in many cases, infecting cells of shorter life-span or performing shorter experiments (81,82,191,204).

The purpose of developing a model of bacterial infection is to standardize the optimal conditions in which both eukaryotic and prokaryotic cells can live together in relevant numbers, the first type of cells remaining alive while being infected, and the other type being used in an appropriate number to allow its survival and multiplication within the host cell for a set time in a reproducible manner. Extracellular bacteria need to be carefully eliminated, to avoid acidification of the extracellular medium and subsequent macrophage death. Once this optimal ratio has been found, the variations in the bacterial intracellular growth are

minimal. Having done so, we have obtained a highly reproducible method to assess bacteria intracellular survival; this is what we call an intracellular model.

Much has been said about the need for the antimicrobial to join its target at the site of infection in order to exert activity (44,117-119,134,213,256). As such, intracellular models of infection are an important tool for the "in vitro" study, not only of antibiotic cellular pharmacokinetics (accumulation, distribution, degradation, bioavailability, binding to cell constituents, cellular half-life, …), but also of their cellular pharmacodynamics (activity, cooperation with cell defense mechanisms, …(45,261). It could also serve to assess the intracellular fate of the bacteria, including mutations and metabolic changes used for survival. In the present case, we have used *in vitro* models to compare in great detail antibiotic activity on both the intracellular and the extracellular *S. aureus*, and to correlate activity with cellular accumulation. Hence, our models are useful tools for the understanding of general bacteria-to-cell "in vitro" interactions, especially when developing new antibiotic molecules.

The data obtained with our models correlates well with the results provided by other intracellular infection models and in some cases underlines some of the reasons of clinical failures (39,83,84,141,274). Additional studies are however needed to further validate our results, including animal models.

1.1.1. Pharmacodynamics

Many studies of antibiotic activity are based only on parameters measured on the extracellular form of the bacteria, utilizing parameters such as minimum inhibitory concentration (MIC), minimal bactericidal concentration (MBC), maximum antibiotic levels on the serum of treated patients (Cmax), to predict antibiotic efficacy (13,62-64). In contrast, we have used intracellular models to fill the gap between antibiotic extracellular concentrations and intracellular antimicrobial effects, but using clinically relevant ranges of concentrations, (261).

Having been argued for long time that antibiotics have to accumulate in the eukaryotic cells to be active against intracellular microorganisms (256), several experiments were aimed at finding a correlation between antibiotic accumulation in the macrophages and the final outcome of the infection. Our results however, show that accumulation *per se* is not predictive of intracellular bacterial eradication. This suggests that intracellular activity

critically depends on the ability of the bacteria to modulate their susceptibility profile when intracellular and on the influence the intracellular medium can exert on antibiotic activity and/or bacteria metabolism (27,167,280). We have shown for example that an acidic pH markedly reduces the activity of macrolides and aminoglycosides, and to a lesser extent, of quinolones, which may contribute to impair the activity of these antibiotics when tested against a phagolysosomal organism like *S. aureus.* Further studies are however needed to better apprehend the cellular or bacterial parameters that affect antibiotic activity intracellularly.

A main interest of our model is clearly that it allows comparing antibiotics on a pharmacodynamic basis, meaning over a wide range of equipotent concentrations (expressed in multiples of their respective MIC) and over prolonged periods of time (up to 24 h). By doing so, we have been able to demonstrate that all antibiotics display concentration-dependent effects and obey to the classical model of sigmoidal pharmacological dose-responses. This contrasts with the conclusions from *in vivo* studies, which suggest that some antibiotics like beta-lactams are time-dependent but not markedly affected by concentration (64). This discrepancy can however be easily explained by the fact that the beta-lactam concentrations found in serum are in a range where maximal effect is already obtained.
We have also shown that these concentration-relationships are maintained intracellularly, but are systematically characterized by a reduced Emax (maximal effect), associated with a persistence of a low intracellular inoculum. Further studies are needed to determine whether these intracellular persisters are a metabolic variant of the parent population with altered susceptibility to antibiotics, or a subpopulation located in a non-accessible compartment.

1.1.2. Cell type

Unlike some other intracellular models which use cell lines of animal origin (38,72,218,240,255), we have used human THP-1 cells (254). These cells present many of the characteristics of human monocytes while forming an homogeneous and reproducible population (19). They have been successfully used in various studies aimed at characterizing the interactions between *S. aureus* and phagocytes in a clinical context (89,110,209), and to analyze the potential relationship between cell accumulation of antibiotics and intracellular activity, or how the modulation of the intracellular environment may affect antibiotic intracellular activity (191,202). They constitute therefore an appropriate model for the type of studies we wanted to perform.

Moreover, an in sharp contrast with most phagocyte-infected models developed to date, which use short incubation times (105,174,191,192,245,268,282) and most often polymorphonuclear cells, our model presents the advantage of a successful 24-h maintaining of the intracellular infection. This allows us (a) to determine the influence of time on antibiotic activity, and (b) to evaluate the antibiotic activity towards bacteria in active stage of multiplication, which is not the case after 5 hours where the bacteria are still in lag period of intracellular growth (24,240).

1.1.3. Bacterial strains

Our intracellular model can been easily adapted to test bacterial strains with different mechanisms of resistance and/or production of virulence factors (see also [152,197] for applications to other strains in our laboratory). This is of importance in a clinical perspective since multi-resistant *S. aureus* represents one of the major threats both in the hospital and in the community settings.

The rare models of infections by resistant strains described in the literature usually examine a single strain with a defined resistance mechanism in a given model (6,7,198). In contrast, we have been able to use a same model (with same infection rate and same protocol of infection of a single call line), which allows for a direct comparison of antibiotic activity against different strains (23). In other words, the use of a common model allows attributing unequivocally differences in antibiotic activity to differences in the intracellular susceptibility of the investigated bacterial strains.

1.2. Validation of the data

Of importance for their global significance, the conclusions drawn from our *in vitro* data are coherent with *in vivo* data examining the activity of the same antibiotics towards persistent or recurrent staphylococcal infections and/or towards intracellular infections by other bacteria. This is illustrated hereunder by typical examples for each of the main antibiotic classes examined here.

We have shown that aminoglycoside antibiotics are poorly active towards intracellular infections by *S. aureus* due to their slow accumulation inside the cells and to their inactivation at acidic pH.
Accordingly, their use is limited to chronical intracellular infections, like tuberculosis (93), for which a long term antibiotic treatment allows for a sufficient penetration of these drugs inside the infected cells. In the field of chronical staphylococcal infections like osteomyelitis, galenic forms are under development to deliver the antibiotic directly within the infected bone and limit by this way the low biodisponibility of the drug (151).

We have found that beta-lactam antibiotics are active towards intracellular *S. aureus*, despite the fact they do not accumulate inside the cells. This activity, however, only develops over time and high concentrations.
This may contribute to explain the clinical success of beta-lactams and their positioning as first line therapy for MSSA infections, including infections with persistent or recurrent character like skin and soft tissues infections, endocarditis, or osteomyelitis (70,96,221).

We have shown that new hemi-synthetic glycopeptides, in contrast to vancomycin, accumulate to high levels in the lysosomes of cells and display marked intracellular bactericidal effects.
The clinical experience is still limited with these molecules, but animal models demonstrate that their efficacy in staphylococcal endocarditis (136,175). However, they do not prove more efficient than vancomycin against susceptible strains.

We have shown that macrolides, despite their high cellular accumulation, are the less active drugs in our intracellular models, due to the marked increase of their MIC at acidic pH and to their intrinsic bacteriostatic character.
Based on their favorable cellular pharmacokinetics, macrolides have long been considered as drugs of choice for intracellular infections (208). Our data would tend to suggest that they might be useless, at least for bacteria sojourning in acidic compartments (where these drugs precisely accumulate in large amounts). Accordingly, azithromycin proved ineffective for treating *S. aureus* osteomyelitis in rats (196).

Among quinolones, moxifloxacin, levofloxacin and garenoxacin develop an important intra- and extracellular bactericidal activity in our model.
The high diffusibility and bactericidal effect of quinolones probably contribute to the efficacy of these drugs against *S. aureus* in animal models of endocarditis (88) and in the treatment of chronical osteomyelitis (71).

In our model, rifampicin shows a high cellular accumulation and a fast extra- and intracellular effect, which does not progress with time.
Rifampicin is considered as one of the antibiotics of choice for the treatment of intracellular infections and widely used for *Mycobacterium tuberculosis* (93). Its usefulness is acknowledged in many other indications (271). The main limitations to a large use are its poor safety profile and the easy selection of resistance by target mutation, which forces to use it in combination only. In these combinations, the rapid bactericidal effect –evidenced also in our model- of rifampin appears as a main determinant in global efficacy (14,22).

Linezolid, the first oxazolidinone antimicrobial on the market, shows only bacteriostatic activity against both extracellular and intracellular *S. aureus* wild-type.
This static effect may explain why linezolid is poorly effective in short-term therapy in animal models (58), but efficient upon prolonged administration (177), the latter being however limited by its toxicity.

1.3. Limitations of the model

1.3.1. Constant concentrations

One of the setbacks of our intracellular model is that antibiotic concentrations are kept constant over time and do therefore not mimic peak/trough variations as observed *in vivo*. Our model allows however to calculate drug exposure in terms of Area Under the Curve (AUC), as exemplified in the discussion of our study of the pharmacodynamics of telavancin (23). Importantly in this respect, this parameter is considered as predictive of the efficacy of most antibiotics (232).

1.3.2. Protein binding

Another major limitation of our model is that it does not allow reproducing the protein binding of drugs as it is observed in human serum. Many antibiotics, including some of those we have studied here (such as telavancin, oritavancin, teicoplanin, penicillin V, nafcillin, or oxacillin) are indeed characterized by a high level of protein binding (48,260). It is usually accepted that only the free fraction of antibiotics is important for activity because is the one which is able to exert its pharmacological activity (66,160), and probably also in the context of intracellular infection, to penetrate inside the cells. Our cell-culture medium contains only

10% of calf fetal serum and this proportion cannot be markedly modified without causing alteration of cell viability. Therefore, when we use in our models drug concentrations that mimic the range of serum concentration observed in humans, we probably overestimate drug exposure, in the sense that our free concentrations are higher from what is obtained *in vivo*. This point was explicitly discussed in our papers, where we also try to take into consideration the activity observed for concentrations mimicking the free serum concentrations in humans. We can also however argue that for some drugs, like telavancin, *in vitro* data suggest that bactericidal activity is maintained in the presence of serum and that MIC is only marginally increased in human serum (155).

1.3.3. Phagocytes

As explained in the introduction, intracellular replication plays a key role in the persistence of invasive *S. aureus* infections, providing protection against host defenses and antibiotics. Our understanding of the mechanisms and consequences of the specific interactions between the bacteria and the host cells are at present rather limited. In the present work, we have focused our interest on monocytic-like cells, privileging by this way a model where cells can be maintained viable even when infected for prolonged periods of time. These cells also present a low level of intrinsic bactericidal mechanisms, which can be considered as an advantage when willing to examine antibiotic efficacy (they can be considered as a living test tube). The meaning of our results is thus limited by the fact that we cannot extrapolate our conclusions to other cell types of interest, like PMN's, which constitute also a first line of defense to infections but are better equipped with bactericidal mechanisms, or non-phagocytic cells like osteoblasts, keratinocytes, or endothelial cells which are the target of intracellular *S. aureus* in recurrent infections. In these cells, which are lacking of specific defense mechanisms, the fate of staphylococci is indeed different, since the bacteria are found in the cytosol and are able to induce cell death by apoptosis (29,183,255).

2. Ideal properties of antibiotics for S. aureus infections

Based on our experimental data, the ideal properties of an antibiotic for the treatment of *S. aureus* infections could be summarized as follows:

- microbiological properties :
    - activity not affected by the current mechanisms of resistance
    - low MIC, including in acidic media
- pharmacokinetic properties :
    - capacity of reaching cellular (and lysosomal) concentration higher than the MIC of the strain, as determined in acidic broth (a high accumulation is not necessarily predictive of increased efficacy)
    - slow efflux from the cell (for obtaining a maintenance of the active concentration for prolonged period of time)

- pharmacodynamic properties:
    - intrinsic activity not affected, or even better, increased in acidic environments
    - rapid bactericidal character
    - capacity to act both on bacteria in active phase of multiplication and in lag phase of growth
    - absence of cellular toxic effects

Our work has suggested that these properties are best fulfilled for three classes of antibiotics, namely:

- New glycopeptides: they are not affected by acidic pH and rapidly bactericidal towards both the extracellular and intracellular forms of *S. aureus* and marginally affected by their resistance phenotype. The cellular alterations observed with oritavancin (263) call however for caution, even though their biological significance is not established.
- Fluoroquinolones, and moxifloxacin in particular (due to its low MIC): they also display a rapid bactericidal effect towards both extra- and intracellular bacteria, even though there are negatively affected by the acidic pH. An additional advantage of this class of drugs is that they are distributed both in the lysosomes and in the cytosol (43,238), and therefore prove efficient towards bacteria localized also in this compartment (46,238). This may be an important benefit when remembering that *S. aureus* can also develop in the cytosol of non – phagocytic cells (29,183,218). A severe limitation of quinolones, however, consists

in the fact that most hospital-acquired MRSA strains are now resistant to this antibiotic class (184,193).

- Beta-lactams, and oxacillin in particular, proved unexpectedly efficacious towards intracellular MSSA. In contrast to both glycopeptides and fluoroquinolones, however, their bactericidal effect takes time to develop. Another intriguing observation made in parallel studies from our laboratory is that intracellular MRSA recover their susceptibility to beta-lactams ([153]; this effect has been attributed to the acidic pH of the phagolysosomes, which is able to increase the binding of beta-lactams to still undetermined proteins at the bacterial surface [Lemaire *et al*, submitted for publication]). We therefore come to a situation where beta-lactams could be more useful for intracellular than for extracellular infections, which is in complete opposition with what was thought during years (44,117-119,134,213,256) !

PERSPECTIVES

1. Exploring the intracellular fate of S. aureus

As suggested in the introduction, the intracellular fate of *S. aureus* is not yet fully understood (98,167). In relation with this question, an intriguing observation made in this work and also reproduced by other teams (191) is that a complete eradication of intracellular bacteria cannot be achieved in 24 h, whatever the drug used. Hypotheses to explore may include the presence of an intracellular pool poorly accessible to antibiotic or the presence of intracellular bacteria having become insensitive to antibiotics. To examine these possibilities,

- We could examine, in confocal microscopy, infected cells after 24 h of exposure to antibiotics. Bacteria could be labeled by FITC prior infection (240); subcellular compartments could be labeled by appropriate markers (for example, antibodies directed towards LAMP proteins for lysosomes or towards rab-5 for endosomes [8,220]). Difficulties will reside in the fact that high inoculum will be needed to observed a sufficient number of bacteria at the end of the 24-h incubation.
- We could carefully search for *S. aureus* variants like SCV, which have been shown to be induced in the intracellular medium (272), and are known to present a reduced susceptibility to most antibiotics, due to alterations in electron transport system and trans-membrane potential, slow growth, and atypical biochemical characteristics (28,163,180,237). We have not observed them in routine examination of our plates, but we cannot exclude their presence, since they could be detectable only upon more prolonged incubation (2-3 days) in enriched culture media (21,144).
- We could compare the global metabolism of *S. aureus* when extracellular and intracellular, to try evidencing differences explaining its reduced susceptibility to antibiotics. Recent studies have for example shown major changes in the genetic expression of *S. aureus* when exposed to acidic pH (280). Likewise, the intracellular expression of toxins may contribute to the induction of apoptosis in non-phagocytic cells (122). Typical approaches for this type of study could include microarrays (comparisons of the mRNA levels from extracellular and intracellular bacteria) or proteomic approaches (comparison of the protein expression pattern from extracellular and intracellular bacteria). In the experience from our laboratory, however, the latter approach is extremely difficult because it required the careful separation of the proteins from the bacterial and from the host cells (265).

2. "In vitro" dynamic models and "in vivo" models

We have insisted in the general discussion on two main limitations of our model, namely the facts that (a) it does not allow to vary drug concentrations over time, and (b) it does not correctly apprehend to issue of protein binding to serum proteins. These problems could be solved by developing "in vitro dynamic models" (165), in which a system of pumps allow to vary the drug concentration over time in a way that can mimic the human pharmacokinetic profile of the antibiotic under investigation (65). These models have been largely described for antibiotic testing in "extracellular" infections (9,78,91,157), but have only rarely applied to intracellular pathogens (133).

A second step in the validation of our *in vitro* data, which would take into account both variations in drug concentrations over time and the serum protein binding, would consist in examining antibiotic activity in *in vivo* models of intracellular infections (65). Many animal models do exist to examine the activity of antibiotics towards extracellular infections, and these models have allowed establishing the current bases of antibiotic pharmacodynamics (12,13,64,233). Interestingly, a animal model of mixed extracellular-intracellular peritoneal infection has been recently developed (229), in which mice are infected by peritoneal injection of *S. aureus*, treated by antibiotics, and sacrificed. Antibiotic activity is then determined in the peritoneal fluid (total activity) and after centrifugation (extracellular activity in the supernatant and intracellular activity in the pellet of peritoneal macrophages).

3. Antibiotic combinations

Therapeutic strategies towards multi-resistant strains have pointed out the interest of using antibiotic combinations for severe *S. aureus* infections (2). In most cases antimicrobial combinations are employed to broaden and enhance the spectrum of coverage, avoid the selection of resistant strains and increase the bacterial killing (90).

Classical combinations include :

- rifampin associated to another drug like an aminoglycoside a beta-lactam, a quinolone, a glycopeptide, quinupristin-dalfopristin or linezolid (36,68,194,227,230,236,252). Though the main goal of these combinations is to avoid the easy selection of mutants resistant to rifampin, they also often prove most effective that monotherapies.
- an aminoglycoside associated with an antibiotic acting on the cell wall (beta-lactam); this combination is well known to be synergistic (40), because the alteration of the cell wall

integrity by beta-lactams favors the penetration of the aminoglycoside inside the bacteria (69).

Most of the synergistic effects of antibiotic combinations have been studied *in vitro* and they are used in humans based on synergism in test tubes. It would therefore be worthwhile to examine whether favorable interactions between antibiotics do also take place in our intracellular models. It would be of particular interest in this respect to examine whether the concomitant use of two antibiotics would allow reducing to a higher level the intracellular inoculum (we never reach a reduction more important than 2-3 log when using a single drug). We could also investigate in our model whether the combination of a beta-lactam and an aminoglycoside shows a complementarity of effects for the simultaneous control of the intracellular bacteria (by the beta-lactam) and of the extracellular bacteria (by the aminoglycoside). This would be still more interesting for MRSA, which were demonstrated recently in our laboratory to recover their susceptibility to beta-lactams intracellularly (153). In this case indeed, a drug acting on extracellular bacteria remaining resistant to beta-lactams is absolutely required.

REFERENCES

1. **Abele-Horn, M., B. Schupfner, P. Emmerling, H. Waldner, and H. Goring**. 2000. Persistent wound infection after herniotomy associated with small-colony variants of Staphylococcus aureus. Infection **28**:53-54.

2. **Acar, J. F.** 2000. Antibiotic synergy and antagonism. Med. Clin. North Am. **84**:1391-1406.

3. **Acar, J. F., F. W. Goldstein, and P. Lagrange**. 1978. Human infections caused by thiamine- or menadione-requiring Staphylococcus aureus. J. Clin. Microbiol. **8**:142-147.

4. **Agerer, F., S. Lux, A. Michel, M. Rohde, K. Ohlsen, and C. R. Hauck**. 2005. Cellular invasion by Staphylococcus aureus reveals a functional link between focal adhesion kinase and cortactin in integrin-mediated internalisation. J. Cell Sci. **118**:2189-2200.

5. **Ahmad, S., A. Selvapandiyan, and R. K. Bhatnagar**. 2000. Phylogenetic analysis of gram-positive bacteria based on grpE, encoded by the dnaK operon. Int. J. Syst. Evol. Microbiol. **50 Pt 5**:1761-1766.

6. **Al Nawas, B. and P. M. Shah**. 1998. Intracellular activity of ciprofloxacin and moxifloxacin, a new 8-methoxyquinolone, against methicillin-resistant Staphylococcus aureus. J. Antimicrob. Chemother. **41**:655-658.

7. **Al Nawas, B. and P. M. Shah**. 1998. Intracellular activity of vancomycin and Ly333328, a new semisynthetic glycopeptide, against methicillin-resistant Staphylococcus aureus. Infection **26**:165-167.

8. **Al Younes, H. M., T. Rudel, and T. F. Meyer**. 1999. Characterization and intracellular trafficking pattern of vacuoles containing Chlamydia pneumoniae in human epithelial cells. Cell Microbiol. **1**:237-247.

9. **Alferova, I. V., S. N. Vostrov, Y. A. Portnoy, I. Y. Lubenko, S. H. Zinner, and A. A. Firsov**. 2005. Comparative pharmacodynamics of telithromycin and clarithromycin with Streptococcus pneumoniae and Staphylococcus aureus in an in vitro dynamic model: focus on clinically achievable antibiotic concentrations. Int. J. Antimicrob. Agents **26**:197-204.

10. **Allen, N. E. and T. I. Nicas**. 2003. Mechanism of action of oritavancin and related glycopeptide antibiotics. FEMS Microbiol. Rev. **26**:511-532.

11. **Almeida, R. A., K. R. Matthews, E. Cifrian, A. J. Guidry, and S. P. Oliver**. 1996. Staphylococcus aureus invasion of bovine mammary epithelial cells. J. Dairy Sci. **79**:1021-1026.

12. **Andes, D. and W. A. Craig**. 2006. Pharmacodynamics of a new streptogramin, XRP 2868, in murine thigh and lung infection models. Antimicrob. Agents Chemother. **50**:243-249.

13. **Andes, D., M. L. van Ogtrop, J. Peng, and W. A. Craig**. 2002. In vivo pharmacodynamics of a new oxazolidinone (linezolid). Antimicrob. Agents Chemother. **46**:3484-3489.

14. **Antony, S. J.** 2006. Combination therapy with daptomycin, vancomycin, and rifampin for recurrent, severe bone and prosthetic joint infections involving methicillin-resistant Staphylococcus aureus. Scand. J. Infect. Dis. **38**:293-295.

15. **Appelbaum, P. C.** 2006. MRSA-the tip of the iceberg. Clin. Microbiol. Infect. **12 Suppl 2**:3-10.

16. **Appelbaum, P. C.** 2006. The emergence of vancomycin-intermediate and vancomycin-resistant Staphylococcus aureus. Clin. Microbiol. Infect. **12 Suppl 1**:16-23.

17. **Arthur, M., P. Reynolds, and P. Courvalin**. 1996. Glycopeptide resistance in enterococci. Trends Microbiol. **4**:401-407.

18. **Aubert-Tulkens, G., F. Van Hoof, and P. Tulkens**. 1979. Gentamicin-induced lysosomal phospholipidosis in cultured rat fibroblasts. Quantitative ultrastructural and biochemical study. Lab Invest **40**:481-491.

19. **Auwerx, J.** 1991. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. Experientia **47**:22-31.

20. **Baird-Parker, A. C.** 1971. The grouping of staphylococci and micrococci. J. Clin. Pathol. **24**:769-770.

21. **Balwit, J. M., P. van Langevelde, J. M. Vann, and R. A. Proctor**. 1994. Gentamicin-resistant menadione and hemin auxotrophic Staphylococcus aureus persist within cultured endothelial cells. J. Infect. Dis. **170**:1033-1037.

22. **Barberan, J.** 2006. Management of infections of osteoarticular prosthesis. Clin. Microbiol. Infect. **12 Suppl 3**:93-101.

23. **Barcia-Macay, M., S. Lemaire, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke**. 2006. Evaluation of the extracellular and intracellular activities (human THP-1 macrophages) of telavancin versus vancomycin against methicillin-susceptible, methicillin-resistant, vancomycin-intermediate and vancomycin-resistant Staphylococcus aureus. J. Antimicrob. Chemother. **58**:1177-1184.

24. **Barcia-Macay, M., C. Seral, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke**. 2006. Pharmacodynamic evaluation of the intracellular activities of antibiotics against Staphylococcus aureus in a model of THP-1 macrophages. Antimicrob. Agents Chemother. **50**:841-851.

25. **Barrett, J. F.** 2005. MRSA -- what is it, and how do we deal with the problem? Expert. Opin. Ther. Targets. **9**:253-265.

26. **Barrett, J. F.** 2005. Recent developments in glycopeptide antibacterials. Curr. Opin. Investig. Drugs **6**:781-790.

27. **Baudoux, P., N. Bles, S. Lemaire, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke**. J.Antimicrob.Chemother., in press.

28. **Baumert, N., C. von Eiff, F. Schaaff, G. Peters, R. A. Proctor, and H. G. Sahl**. 2002. Physiology and antibiotic susceptibility of Staphylococcus aureus small colony variants. Microb. Drug Resist. **8**:253-260.

29. **Bayles, K. W., C. A. Wesson, L. E. Liou, L. K. Fox, G. A. Bohach, and W. R. Trumble**. 1998. Intracellular Staphylococcus aureus escapes the endosome and induces apoptosis in epithelial cells. Infect. Immun. **66**:336-342.

30. **Beauregard, D. A., D. H. Williams, M. N. Gwynn, and D. J. Knowles**. 1995. Dimerization and membrane anchors in extracellular targeting of vancomycin group antibiotics. Antimicrob. Agents Chemother. **39**:781-785.

31. **Bera, A., R. Biswas, S. Herbert, and F. Gotz**. 2006. The presence of peptidoglycan O-acetyltransferase in various staphylococcal species correlates with lysozyme resistance and pathogenicity. Infect. Immun. **74**:4598-4604.

32. **Bhavnani, S. M., J. S. Owen, J. S. Loutit, S. B. Porter, and P. G. Ambrose**. 2004. Pharmacokinetics, safety, and tolerability of ascending single intravenous doses of oritavancin administered to healthy human subjects. Diagn. Microbiol. Infect. Dis. **50**:95-102.

33. **Biavasco, F., C. Vignaroli, R. Lupidi, E. Manso, B. Facinelli, and P. E. Varaldo**. 1997. In vitro antibacterial activity of LY333328, a new semisynthetic glycopeptide. Antimicrob. Agents Chemother. **41**:2165-2172.

34. **Blumberg, E. A., V. B. Hatcher, and F. D. Lowy**. 1988. Acidic fibroblast growth factor modulates Staphylococcus aureus adherence to human endothelial cells. Infect. Immun. **56**:1470-1474.

35. **Bosso, J. A.** 2005. The antimicrobial armamentarium: evaluating current and future treatment options. Pharmacotherapy **25**:55S-62S.

36. **Brandt, C. M., M. S. Rouse, B. M. Tallan, N. W. Laue, W. R. Wilson, and J. M. Steckelberg**. 1995. Effective treatment of cephalosporin-rifampin combinations against cryptic methicillin-resistant beta-lactamase-producing coagulase-negative staphylococcal experimental endocarditis. Antimicrob. Agents Chemother. **39**:1815-1819.

37. **Brouillette, E., G. Grondin, C. Lefebvre, B. G. Talbot, and F. Malouin**. 2004. Mouse mastitis model of infection for antimicrobial compound efficacy studies against intracellular and extracellular forms of Staphylococcus aureus. Vet. Microbiol. **101**:253-262.

38. **Brouillette, E., G. Grondin, L. Shkreta, P. Lacasse, and B. G. Talbot**. 2003. In vivo and in vitro demonstration that Staphylococcus aureus is an intracellular pathogen in the presence or absence of fibronectin-binding proteins. Microb. Pathog. **35**:159-168.

39. **Brouillette, E., A. Martinez, B. J. Boyll, N. E. Allen, and F. Malouin**. 2004. Persistence of a Staphylococcus aureus small-colony variant under antibiotic pressure in vivo. FEMS Immunol. Med. Microbiol. **41**:35-41.

40. **Calandra, T. and M. P. Glauser**. 1986. Immunocompromised animal models for the study of antibiotic combinations. Am. J. Med. **80**:45-52.

41. **Candiani, G. P., M. Abbondi, M. Borgonovi, G. Romano, and F. Parenti**. 1999. In-vitro and in-vivo antibacterial activity of BI 397, a new semi- synthetic glycopeptide antibiotic. J. Antimicrob. Chemother. **44**:179-192.

42. **Carlier, M. B., I. Garcia-Luque, J. P. Montenez, P. M. Tulkens, and J. Piret**. 1994. Accumulation, release and subcellular localization of azithromycin in phagocytic and non-phagocytic cells in culture. Int. J. Tissue React. **16**:211-220.

43. **Carlier, M. B., B. Scorneaux, A. Zenebergh, J. F. Desnottes, and P. M. Tulkens**. 1990. Cellular uptake, localization and activity of fluoroquinolones in uninfected and infected macrophages. J. Antimicrob. Chemother. **26 Suppl B**:27-39.

44. **Carlone, N. A., A. M. Cuffini, V. Tullio, and G. Cavallo**. 1991. Interactions of antibiotics with phagocytes in vitro. J. Chemother. **3 Suppl 1**:98-104.

45. **Carryn, S., H. Chanteux, C. Seral, M. P. Mingeot-Leclercq, F. Van Bambeke, and P. M. Tulkens**. 2003. Intracellular pharmacodynamics of antibiotics. Infect. Dis. Clin. North Am. **17**:615-634.

46. **Carryn, S., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens**. 2002. Comparative intracellular (THP-1 macrophage) and extracellular activities of beta-lactams, azithromycin, gentamicin, and fluoroquinolones against Listeria monocytogenes at clinically relevant concentrations. Antimicrob. Agents Chemother. **46**:2095-2103.

47. **Cespedes, C., B. Said-Salim, M. Miller, S. H. Lo, B. N. Kreiswirth, R. J. Gordon, P. Vavagiakis, R. S. Klein, and F. D. Lowy**. 2005. The clonality of Staphylococcus aureus nasal carriage. J. Infect. Dis. **191**:444-452.

48. **Chambers, H. F.** 2000. Penicillins, p. 261-274. *In*: G. E. Mandell, J. E. Bennett, and R. Dolin (eds.), Principles and practice of infectious diseases. Churchill Livingstone, Philadelphia, Pen.

49. **Chambers, H. F.** 2005. Community-associated MRSA--resistance and virulence converge. N. Engl. J. Med. **352**:1485-1487.

50. **Chapman, A. L., J. M. Greig, and J. A. Innes**. 2005. MRSA in the community. N. Engl. J. Med. **353**:530-532.

51. **Cho, A., T. W. Glinka, M. Ludwikow, A. T. Fan, M. Wang, and S. J. Hecker**. 2001. New anti-MRSA cephalosporins with a basic aminopyridine at the C-7 position. Bioorg. Med. Chem. Lett. **11**:137-140.

52. **Chuard, C., P. E. Vaudaux, R. A. Proctor, and D. P. Lew**. 1997. Decreased susceptibility to antibiotic killing of a stable small colony variant of Staphylococcus aureus in fluid phase and on fibronectin-coated surfaces. J. Antimicrob. Chemother. **39**:603-608.

53. **Clauditz, A., A. Resch, K. P. Wieland, A. Peschel, and F. Gotz**. 2006. Staphyloxanthin plays a role in the fitness of Staphylococcus aureus and its ability to cope with oxidative stress. Infect. Immun. **74**:4950-4953.

54. **Clement, S., P. Vaudaux, P. Francois, J. Schrenzel, E. Huggler, S. Kampf, C. Chaponnier, D. Lew, and J. S. Lacroix**. 2005. Evidence of an intracellular reservoir in the nasal mucosa of patients with recurrent Staphylococcus aureus rhinosinusitis. J. Infect. Dis. **192**:1023-1028.

55. **Clements, M. O. and S. J. Foster**. 1999. Stress resistance in Staphylococcus aureus. Trends Microbiol. **7**:458-462.

56. **Clements, M. O., S. P. Watson, and S. J. Foster**. 1999. Characterization of the major superoxide dismutase of Staphylococcus aureus and its role in starvation survival, stress resistance, and pathogenicity. J. Bacteriol. **181**:3898-3903.

57. **Cocchiaro, J. L., M. I. Gomez, A. Risley, R. Solinga, D. O. Sordelli, and J. C. Lee**. 2006. Molecular characterization of the capsule locus from non-typeable Staphylococcus aureus. Mol. Microbiol. **59**:948-960.

58. **Conaughty, J. M., J. Chen, O. V. Martinez, G. Chiappetta, K. F. Brookfield, and F. J. Eismont**. 2006. Efficacy of linezolid versus vancomycin in the treatment of methicillin-resistant staphylococcus aureus discitis: a controlled animal model. Spine **31**:E830-E832.

59. **Cooper, R. D., N. J. Snyder, M. J. Zweifel, M. A. Staszak, S. C. Wilkie, T. I. Nicas, D. L. Mullen, T. F. Butler, M. J. Rodriguez, B. E. Huff, and R. C. Thompson**. 1996. Reductive alkylation of glycopeptide antibiotics: synthesis and antibacterial activity. J. Antibiot. (Tokyo) **49**:575-581.

60. **Cotter, P. D. and C. Hill**. 2003. Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiol. Mol. Biol. Rev. **67**:429-53, table.

61. **Courvalin, P.** 2006. Vancomycin resistance in gram-positive cocci. Clin. Infect. Dis. **42 Suppl 1**:S25-S34.

62. **Craig, W. A.** 1998. Choosing an antibiotic on the basis of pharmacodynamics. Ear Nose Throat J. **77**:7-11.

63. **Craig, W. A.** 2001. Does the dose matter? Clin. Infect. Dis. **33 Suppl 3**:S233-S237.

64. **Craig, W. A.** 2003. Basic pharmacodynamics of antibacterials with clinical applications to the use of beta-lactams, glycopeptides, and linezolid. Infect. Dis. Clin. North Am. **17**:479-501.

65. **Craig, W. A.** 2004. Proof of concept: performance testing in models. Clin. Microbiol. Infect. **10 Suppl 2**:12-17.

66. **Craig, W. A. and S. C. Ebert**. 1989. Protein binding and its significance in antibacterial therapy. Infect. Dis. Clin. North Am. **3**:407-414.

67. **da Silva, M. C., J. M. Zahm, D. Gras, O. Bajolet, M. Abely, J. Hinnrasky, M. Milliot, M. C. de Assis, C. Hologne, N. Bonnet, M. Merten, M. C. Plotkowski, and E. Puchelle**. 2004. Dynamic interaction between airway epithelial cells and Staphylococcus aureus. Am. J. Physiol Lung Cell Mol. Physiol **287**:L543-L551.

68. **Dailey, C. F., P. J. Pagano, L. V. Buchanan, J. A. Paquette, J. V. Haas, and J. K. Gibson**. 2003. Efficacy of linezolid plus rifampin in an experimental model of methicillin-susceptible Staphylococcus aureus endocarditis. Antimicrob. Agents Chemother. **47**:2655-2658.

69. **Davis, B. D.** 1982. Bactericidal synergism between beta-lactams and aminoglycosides: mechanism and possible therapeutic implications. Rev. Infect. Dis. **4**:237-245.

70. **Davis, J. S.** 2005. Management of bone and joint infections due to Staphylococcus aureus. Intern. Med. J. **35 Suppl 2**:S79-S96.

71. **Dellamonica, P., E. Bernard, H. Etesse, R. Garraffo, and H. B. Drugeon**. 1989. Evaluation of pefloxacin, ofloxacin and ciprofloxacin in the treatment of thirty-nine cases of chronic osteomyelitis. Eur. J. Clin. Microbiol. Infect. Dis. **8**:1024-1030.

72. **Desnottes, J. F. and N. Diallo**. 1992. Cellular uptake and intracellular bactericidal activity of RP 59500 in murine macrophages. J. Antimicrob. Chemother. **30 Suppl A**:107-115.

73. **Diekema, D. J., M. A. Pfaller, F. J. Schmitz, J. Smayevsky, J. Bell, R. N. Jones, and M. Beach**. 2001. Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. Clin. Infect. Dis. **32 Suppl 2**:S114-S132.

74. **Diekema, D. J., M. A. Pfaller, J. Turnidge, J. Verhoef, J. Bell, A. C. Fluit, G. V. Doern, and R. N. Jones**. 2000. Genetic relatedness of multidrug-resistant, methicillin (oxacillin)-resistant Staphylococcus aureus bloodstream isolates from SENTRY Antimicrobial Resistance Surveillance Centers worldwide, 1998. Microb. Drug Resist. **6**:213-221.

75. **Dinges, M. M., P. M. Orwin, and P. M. Schlievert**. 2000. Exotoxins of Staphylococcus aureus. Clin. Microbiol. Rev. **13**:16-34, table.

76. **Dorr, M. B., D. Jabes, M. Cavaleri, J. Dowell, G. Mosconi, A. Malabarba, R. J. White, and T. J. Henkel**. 2005. Human pharmacokinetics and rationale for once-weekly dosing of dalbavancin, a semi-synthetic glycopeptide. J. Antimicrob. Chemother. **55 Suppl 2**:ii25-ii30.

77. **Drews, T. D., J. L. Temte, and B. C. Fox**. 2006. Community-associated methicillin-resistant Staphylococcus aureus: review of an emerging public health concern. WMJ. **105**:52-57.

78. **Duffull, S. B., E. J. Begg, S. T. Chambers, and M. L. Barclay**. 1994. Efficacies of different vancomycin dosing regimens against Staphylococcus aureus determined with a dynamic in vitro model. Antimicrob. Agents Chemother. **38**:2480-2482.

79. **Dziewanowska, K., A. R. Carson, J. M. Patti, C. F. Deobald, K. W. Bayles, and G. A. Bohach**. 2000. Staphylococcal fibronectin binding protein interacts with heat shock protein 60 and integrins: role in internalization by epithelial cells. Infect. Immun. **68**:6321-6328.

80. **Dziewanowska, K., J. M. Patti, C. F. Deobald, K. W. Bayles, W. R. Trumble, and G. A. Bohach**. 1999. Fibronectin binding protein and host cell tyrosine kinase are required for internalization of Staphylococcus aureus by epithelial cells. Infect. Immun. **67**:4673-4678.

81. **Easmon, C. S.** 1979. The effect of antibiotics on the intracellular survival of Staphylococcus aureus in vitro. Br. J. Exp. Pathol. **60**:24-28.

82. **Easmon, C. S. and J. P. Crane**. 1984. Cellular uptake of clindamycin and lincomycin. Br. J. Exp. Pathol. **65**:725-730.

83. **Ellington JK, H. M., Hudson M, Vishin S, Webb L, and Sherertz R**. 2005. Intracellular S. aureus and antibiotic resistance: implications for treatment of Staphylococcal osteomyelitis. Orthopaedic Research Society : Published by Wiley Periodicals, Inc.87-93.

84. **Ellington, J. K., M. Harris, M. C. Hudson, S. Vishin, L. X. Webb, and R. Sherertz**. 2006. Intracellular Staphylococcus aureus and antibiotic resistance: Implications for treatment of staphylococcal osteomyelitis. J. Orthop. Res. **24**:87-93.

85. **Ellington, J. K., M. Harris, L. Webb, B. Smith, T. Smith, K. Tan, and M. Hudson**. 2003. Intracellular Staphylococcus aureus. A mechanism for the indolence of osteomyelitis. J. Bone Joint Surg. Br. **85**:918-921.

86. **Ellington, J. K., S. S. Reilly, W. K. Ramp, M. S. Smeltzer, J. F. Kellam, and M. C. Hudson**. 1999. Mechanisms of Staphylococcus aureus invasion of cultured osteoblasts. Microb. Pathog. **26**:317-323.

87. **Entenza, J. M., P. Hohl, I. Heinze-Krauss, M. P. Glauser, and P. Moreillon**. 2002. BAL9141, a novel extended-spectrum cephalosporin active against methicillin-resistant Staphylococcus aureus in treatment of experimental endocarditis. Antimicrob. Agents Chemother. **46**:171-177.

88. **Entenza, J. M., J. Vouillamoz, M. P. Glauser, and P. Moreillon**. 2004. Efficacy of garenoxacin in treatment of experimental endocarditis due to Staphylococcus aureus or viridans group streptococci. Antimicrob. Agents Chemother. **48**:86-92.

89. **Fan, H., G. Teti, S. Ashton, K. Guyton, G. E. Tempel, P. V. Halushka, and J. A. Cook**. 2003. Involvement of G(i) proteins and Src tyrosine kinase in TNFalpha production induced by lipopolysaccharide, group B Streptococci and Staphylococcus aureus. Cytokine **22**:126-133.

90. **Fantin, B. and C. Carbon**. 1992. In vivo antibiotic synergism: contribution of animal models. Antimicrob. Agents Chemother. **36**:907-912.

91. **Firsov, A. A., I. Y. Lubenko, S. N. Vostrov, Y. A. Portnoy, and S. H. Zinner**. 2005. Antistaphylococcal effect related to the area under the curve/MIC ratio in an in vitro dynamic model: predicted breakpoints versus clinically achievable values for seven fluoroquinolones. Antimicrob. Agents Chemother. **49**:2642-2647.

92. **Fisher, J. F., S. O. Meroueh, and S. Mobashery**. 2005. Bacterial resistance to beta-lactam antibiotics: compelling opportunism, compelling opportunity. Chem. Rev. **105**:395-424.

93. **Fitzgerald, D. and D. W. Haas**. 2005. Mycobacterium tuberculosis, p. 2852-2886. *In*: G. L. Mandell, J. E. Bennet, and R. Dolin (eds.), Principles and practice of infectious diseases. Elsevier Churchill Livingstone, Philadelphia, PA.

94. **Fitzgerald, J. R., D. E. Sturdevant, S. M. Mackie, S. R. Gill, and J. M. Musser**. 2001. Evolutionary genomics of Staphylococcus aureus: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. Proc. Natl. Acad. Sci. U. S. A **98**:8821-8826.

95. **Fluit, A. C., C. L. Wielders, J. Verhoef, and F. J. Schmitz**. 2001. Epidemiology and susceptibility of 3,051 Staphylococcus aureus isolates from 25 university hospitals participating in the European SENTRY study. J. Clin. Microbiol. **39**:3727-3732.

96. **Fortun, J., E. Navas, J. Martinez-Beltran, J. Perez-Molina, P. Martin-Davila, A. Guerrero, and S. Moreno**. 2001. Short-course therapy for right-side endocarditis due to Staphylococcus aureus in drug abusers: cloxacillin versus glycopeptides in combination with gentamicin. Clin. Infect. Dis. **33**:120-125.

97. **Foster, T. J.** 2004. The Staphylococcus aureus "superbug". J. Clin. Invest **114**:1693-1696.

98. **Foster, T. J.** 2005. Immune evasion by staphylococci. Nat. Rev. Microbiol. **3**:948-958.

99. **Fournier, B. and A. Klier**. 2004. Protein A gene expression is regulated by DNA supercoiling which is modified by the ArlS-ArlR two-component system of Staphylococcus aureus. Microbiology **150**:3807-3819.

100. **Fournier, B. and D. J. Philpott**. 2005. Recognition of Staphylococcus aureus by the innate immune system. Clin. Microbiol. Rev. **18**:521-540.

101. **Fowler, T., S. Johansson, K. K. Wary, and M. Hook**. 2003. Src kinase has a central role in in vitro cellular internalization of Staphylococcus aureus. Cell Microbiol. **5**:417-426.

102. **Fowler, T., E. R. Wann, D. Joh, S. Johansson, T. J. Foster, and M. Hook**. 2000. Cellular invasion by Staphylococcus aureus involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. Eur. J. Cell Biol. **79**:672-679.

103. **Fuda, C. C., J. F. Fisher, and S. Mobashery**. 2005. beta-Lactam resistance in Staphylococcus aureus: the adaptive resistance of a plastic genome. Cell Mol. Life Sci. **62**:2617-2633.

104. **Furuya, E. Y. and F. D. Lowy**. 2006. Antimicrobial-resistant bacteria in the community setting. Nat. Rev. Microbiol. **4**:36-45.

105. **Garcia, I., A. Pascual, S. Ballesta, C. del Castillo, and E. J. Perea**. 2003. Accumulation and activity of cethromycin (ABT-773) within human polymorphonuclear leucocytes. J. Antimicrob. Chemother. **52**:24-28.

106. **Gerding, D. N.** 1997. Is there a relationship between vancomycin-resistant enterococcal infection and Clostridium difficile infection? Clin. Infect. Dis. **25 Suppl 2**:S206-S210.

107. **Gillet, Y., B. Issartel, P. Vanhems, J. C. Fournet, G. Lina, M. Bes, F. Vandenesch, Y. Piemont, N. Brousse, D. Floret, and J. Etienne**. 2002. Association between Staphylococcus aureus strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. Lancet **359**:753-759.

108. **Goerke, C. and C. Wolz**. 2004. Regulatory and genomic plasticity of Staphylococcus aureus during persistent colonization and infection. Int. J. Med. Microbiol. **294**:195-202.

109. **Gonzalez-Zorn, B. and P. Courvalin**. 2003. VanA-mediated high level glycopeptide resistance in MRSA. Lancet Infect. Dis. **3**:67-68.

110. **Gotoh, M., Y. Takamoto, K. Kurosaka, J. Masuda, M. Ida, A. Satoh, E. Takayama, K. Kojima-Aikawa, Y. Kobayashi, and I. Matsumoto**. 2005. Annexins I and IV inhibit Staphylococcus aureus attachment to human macrophages. Immunol. Lett. **98**:297-302.

111. **Gould, I. M.** 2005. The clinical significance of methicillin-resistant Staphylococcus aureus. J. Hosp. Infect. **61**:277-282.

112. **Govan, J. R. and J. W. Nelson**. 1992. Microbiology of lung infection in cystic fibrosis. Br. Med. Bull. **48**:912-930.

113. **Gresham, H. D., J. H. Lowrance, T. E. Caver, B. S. Wilson, A. L. Cheung, and F. P. Lindberg**. 2000. Survival of Staphylococcus aureus inside neutrophils contributes to infection. J. Immunol. **164**:3713-3722.

114. **Guignard, B., J. M. Entenza, and P. Moreillon**. 2005. Beta-lactams against methicillin-resistant Staphylococcus aureus. Curr. Opin. Pharmacol. **5**:479-489.

115. **Hammond, R. K. and D. C. White**. 1970. Carotenoid formation by Staphylococcus aureus. J. Bacteriol. **103**:191-198.

116. **Hanaki, H.** 1997. [Molecular design of anti-MRSA drugs]. Nippon Rinsho **55**:1281-1286.

117. **Hand, W. L. and N. L. King-Thompson**. 1986. Contrasts between phagocyte antibiotic uptake and subsequent intracellular bactericidal activity. Antimicrob. Agents Chemother. **29**:135-140.

118. **Hand, W. L. and N. L. King-Thompson**. 1989. The entry of antibiotics into human monocytes. J. Antimicrob. Chemother. **23**:681-689.

119. **Hand, W. L., N. L. King-Thompson, and J. D. Johnson**. 1984. Influence of bacterial-antibiotic interactions on subsequent antimicrobial activity of alveolar macrophages. J. Infect. Dis. **149**:271-276.

120. **Hanssen, A. M. and J. U. Ericson Sollid**. 2006. SCCmec in staphylococci: genes on the move. FEMS Immunol. Med. Microbiol. **46**:8-20.

121. **Harraghy, N., M. Hussain, A. Haggar, T. Chavakis, B. Sinha, M. Herrmann, and J. I. Flock**. 2003. The adhesive and immunomodulating properties of the multifunctional Staphylococcus aureus protein Eap. Microbiology **149**:2701-2707.

122. **Haslinger, B., K. Strangfeld, G. Peters, K. Schulze-Osthoff, and B. Sinha**. 2003. Staphylococcus aureus alpha-toxin induces apoptosis in peripheral blood mononuclear cells: role of endogenous tumour necrosis factor-alpha and the mitochondrial death pathway. Cell Microbiol. **5**:729-741.

123. **Hauck, C. R.** 2002. Cell adhesion receptors - signaling capacity and exploitation by bacterial pathogens. Med. Microbiol. Immunol. (Berl) **191**:55-62.

124. **Hauck, C. R., F. Agerer, P. Muenzner, and T. Schmitter**. 2006. Cellular adhesion molecules as targets for bacterial infection. Eur. J. Cell Biol. **85**:235-242.

125. **Hauck, C. R. and K. Ohlsen**. 2006. Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by Staphylococcus aureus. Curr. Opin. Microbiol.

126. **Hegde, S. S., N. Reyes, T. Wiens, N. Vanasse, R. Skinner, J. McCullough, K. Kaniga, J. Pace, R. Thomas, J. P. Shaw, G. Obedencio, and J. K. Judice**. 2004. Pharmacodynamics of telavancin (TD-6424), a novel bactericidal agent, against gram-positive bacteria. Antimicrob. Agents Chemother. **48**:3043-3050.

127. **Hess, D. J., M. J. Henry-Stanley, E. A. Erickson, and C. L. Wells**. 2003. Intracellular survival of Staphylococcus aureus within cultured enterocytes. J. Surg. Res. **114**:42-49.

128. **Higgins, D. L., R. Chang, D. V. Debabov, J. Leung, T. Wu, K. M. Krause, E. Sandvik, J. M. Hubbard, K. Kaniga, D. E. Schmidt, Jr., Q. Gao, R. T. Cass, D. E. Karr, B. M. Benton, and P. P. Humphrey**. 2005. Telavancin, a multifunctional lipoglycopeptide, disrupts both cell wall synthesis and cell membrane integrity in methicillin-resistant Staphylococcus aureus. Antimicrob. Agents Chemother. **49**:1127-1134.

129. **Ho, P. L., C. Cheung, G. C. Mak, C. W. Tse, T. K. Ng, C. H. Cheung, T. L. Que, R. Lam, R. W. Lai, R. W. Yung, and K. Y. Yuen**. 2007. Molecular epidemiology and household transmission of community-associated methicillin-resistant Staphylococcus aureus in Hong Kong. Diagn. Microbiol. Infect. Dis. **57**:145-151.

130. **Holden, M. T., E. J. Feil, J. A. Lindsay, S. J. Peacock, N. P. Day, M. C. Enright, T. J. Foster, C. E. Moore, L. Hurst, R. Atkin, A. Barron, N. Bason, S. D. Bentley, C. Chillingworth, T. Chillingworth, C. Churcher, L. Clark, C. Corton, A. Cronin, J. Doggett, L. Dowd, T. Feltwell, Z. Hance, B. Harris, H. Hauser, S. Holroyd, K. Jagels, K. D. James, N. Lennard, A. Line, R. Mayes, S. Moule, K. Mungall, D. Ormond, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, S. Sharp, M. Simmonds, K. Stevens, S. Whitehead, B. G. Barrell, B. G. Spratt, and J. Parkhill**. 2004. Complete genomes of two clinical Staphylococcus aureus strains: evidence for the rapid evolution of virulence and drug resistance. Proc. Natl. Acad. Sci. U. S. A **101**:9786-9791.

131. **Holt, J. G., R. N. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams**. 1994. Bergey's Manual of Determinative Bacteriology, 9th edition..

132. **Hudson, M. C., W. K. Ramp, N. C. Nicholson, A. S. Williams, and M. T. Nousiainen**. 1995. Internalization of Staphylococcus aureus by cultured osteoblasts. Microb. Pathog. **19**:409-419.

133. **Hulten, K., R. Rigo, I. Gustafsson, and L. Engstrand**. 1996. New pharmacokinetic in vitro model for studies of antibiotic activity against intracellular microorganisms. Antimicrob. Agents Chemother. **40**:2727-2731.

134. **Jacobs, R. F. and C. B. Wilson**. 1983. Intracellular penetration and antimicrobial activity of antibiotics. J. Antimicrob. Chemother. **12 Suppl C**:13-20.

135. **Jones, R. N., M. G. Stilwell, H. S. Sader, T. R. Fritsche, and B. P. Goldstein**. 2006. Spectrum and potency of dalbavancin tested against 3322 Gram-positive cocci isolated in the United States Surveillance Program (2004). Diagn. Microbiol. Infect. Dis. **54**:149-153.

136. **Kaatz, G. W., S. M. Seo, J. R. Aeschlimann, H. H. Houlihan, R. C. Mercier, and M. J. Rybak**. 1998. Efficacy of LY333328 against experimental methicillin-resistant Staphylococcus aureus endocarditis. Antimicrob. Agents Chemother. **42**:981-983.

137. **Kahl, B., M. Herrmann, A. S. Everding, H. G. Koch, K. Becker, E. Harms, R. A. Proctor, and G. Peters**. 1998. Persistent infection with small colony variant strains of Staphylococcus aureus in patients with cystic fibrosis. J. Infect. Dis. **177**:1023-1029.

138. **Kaneko, J. and Y. Kamio**. 2004. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. Biosci. Biotechnol. Biochem. **68**:981-1003.

139. **Kaneko, J., T. Kimura, Y. Kawakami, T. Tomita, and Y. Kamio**. 1997. Panton-valentine leukocidin genes in a phage-like particle isolated from mitomycin C-treated Staphylococcus aureus V8 (ATCC 49775). Biosci. Biotechnol. Biochem. **61**:1960-1962.

140. **Kazakova, S. V., J. C. Hageman, M. Matava, A. Srinivasan, L. Phelan, B. Garfinkel, T. Boo, S. McAllister, J. Anderson, B. Jensen, D. Dodson, D. Lonsway, L. K. McDougal, M. Arduino, V. J. Fraser, G. Killgore, F. C. Tenover, S. Cody, and D. B. Jernigan**. 2005. A clone of methicillin-resistant Staphylococcus aureus among professional football players. N. Engl. J. Med. **352**:468-475.

141. **Khatib, R., L. B. Johnson, M. G. Fakih, K. Riederer, A. Khosrovaneh, T. M. Shamse, M. Sharma, and S. Saeed**. 2006. Persistence in Staphylococcus aureus bacteremia: Incidence, characteristics of patients and outcome. Scand. J. Infect. Dis. **38**:7-14.

142. **King, A., I. Phillips, and K. Kaniga**. 2004. Comparative in vitro activity of telavancin (TD-6424), a rapidly bactericidal, concentration-dependent anti-infective with multiple mechanisms of action against Gram-positive bacteria. J. Antimicrob. Chemother. **53**:797-803.

143. **Kintarak, S., S. A. Whawell, P. M. Speight, S. Packer, and S. P. Nair**. 2004. Internalization of Staphylococcus aureus by human keratinocytes. Infect. Immun. **72**:5668-5675.

144. **Kipp, F., B. C. Kahl, K. Becker, E. J. Baron, R. A. Proctor, G. Peters, and C. von Eiff**. 2005. Evaluation of two chromogenic agar media for recovery and identification of Staphylococcus aureus small-colony variants. J. Clin. Microbiol. **43**:1956-1959.

145. **Kluytmans, J. A. and H. F. Wertheim**. 2005. Nasal carriage of Staphylococcus aureus and prevention of nosocomial infections. Infection **33**:3-8.

146. **Kluytmans-Vandenbergh, M. F. and J. A. Kluytmans**. 2006. Community-acquired methicillin-resistant Staphylococcus aureus: current perspectives. Clin. Microbiol. Infect. **12 Suppl 1**:9-15.

147. **Kohler, C., C. von Eiff, G. Peters, R. A. Proctor, M. Hecker, and S. Engelmann**. 2003. Physiological characterization of a heme-deficient mutant of Staphylococcus aureus by a proteomic approach. J. Bacteriol. **185**:6928-6937.

148. **Krut, O., O. Utermohlen, X. Schlossherr, and M. Kronke**. 2003. Strain-specific association of cytotoxic activity and virulence of clinical Staphylococcus aureus isolates. Infect. Immun. **71**:2716-2723.

149. **Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu**. 2001. Whole genome sequencing of meticillin-resistant Staphylococcus aureus. Lancet **357**:1225-1240.

150. **Leadbetter, M. R., S. M. Adams, B. Bazzini, P. R. Fatheree, D. E. Karr, K. M. Krause, B. M. Lam, M. S. Linsell, M. B. Nodwell, J. L. Pace, K. Quast, J. P. Shaw, E. Soriano, S. G. Trapp, J. D. Villena, T. X. Wu, B. G. Christensen, and J. K. Judice**. 2004. Hydrophobic vancomycin derivatives with improved ADME properties: discovery of telavancin (TD-6424). J. Antibiot. (Tokyo) **57**:326-336.

151. **Lee, C. G., Y. C. Fu, and C. H. Wang**. 2005. Simulation of gentamicin delivery for the local treatment of osteomyelitis. Biotechnol. Bioeng. **91**:622-635.

152. **Lemaire, S., Bogdanovitch, T., Chavez-Bueno, S., Savage, P. D., Van Bambeke, F., Appelbaum, P. C., and Tulkens, P. M**. Bactericidal activity of ceragenin CSA-13 against intracellular MSSA, hospital-acquired (HA) and Community-acquired (CA) MRSA, and VISA in THP-1 macrophages: relation to cellular toxicity ? 46th Intescience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Ca , A-0633. 2006.

153. **Lemaire, S., Van Bambeke, F., Mingeot-Leclercq, M. P., Glupzynski, Y., and Tulkens, P. M.** Carbapenems (ETP, MEM) are Active against Intraphagocytic MRSA in a THP-1 Macrophage Model. 45th Intescience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC , A1832. 2005.

154. **Leuthner, K. D., C. M. Cheung, and M. J. Rybak**. 2006. Comparative activity of the new lipoglycopeptide telavancin in the presence and absence of serum against 50 glycopeptide non-susceptible staphylococci and three vancomycin-resistant Staphylococcus aureus. J. Antimicrob. Chemother. **58**:338-343.

155. **Leuthner, K. D., C. M. Cheung, and M. J. Rybak**. 2006. Comparative activity of the new lipoglycopeptide telavancin in the presence and absence of serum against 50 glycopeptide non-susceptible staphylococci and three vancomycin-resistant Staphylococcus aureus. J. Antimicrob. Chemother.

156. **Lew, D. P. and F. A. Waldvogel**. 1997. Osteomyelitis. N. Engl. J. Med. **336**:999-1007.

157. **Lewis, R. E., M. E. Klepser, E. J. Ernst, B. C. Lund, D. J. Biedenbach, and R. N. Jones**. 1999. Evaluation of low-dose, extended-interval clindamycin regimens against Staphylococcus aureus and Streptococcus pneumoniae using a dynamic in vitro model of infection. Antimicrob. Agents Chemother. **43**:2005-2009.

158. **Lin, S. W., P. L. Carver, and D. D. DePestel**. 2006. Dalbavancin: a new option for the treatment of gram-positive infections. Ann. Pharmacother. **40**:449-460.

159. **Liu, C. and H. F. Chambers**. 2003. Staphylococcus aureus with heterogeneous resistance to vancomycin: epidemiology, clinical significance, and critical assessment of diagnostic methods. Antimicrob. Agents Chemother. **47**:3040-3045.

160. **Liu, P., M. Muller, and H. Derendorf**. 2002. Rational dosing of antibiotics: the use of plasma concentrations versus tissue concentrations. Int. J. Antimicrob. Agents **19**:285-290.

161. **Livermore, D. M.** 2005. Tigecycline: what is it, and where should it be used? J. Antimicrob. Chemother. **56**:611-614.

162. **Livermore, D. M.** 2006. Can beta-lactams be re-engineered to beat MRSA? Clin. Microbiol. Infect. **12 Suppl 2**:11-16.

163. **Looney, W. J.** 2000. Small-colony variants of Staphylococcus aureus. Br. J. Biomed. Sci. **57**:317-322.

164. **Loveday, H. P., C. M. Pellowe, S. R. Jones, and R. J. Pratt**. 2006. A systematic review of the evidence for interventions for the prevention and control of meticillin-resistant Staphylococcus aureus (1996-2004): report to the Joint MRSA Working Party (Subgroup A). J. Hosp. Infect. **63 Suppl 1**:S45-S70.

165. **Lowdin, E., I. Odenholt, S. Bengtsson, and O. Cars**. 1996. Pharmacodynamic effects of sub-MICs of benzylpenicillin against Streptococcus pyogenes in a newly developed in vitro kinetic model. Antimicrob. Agents Chemother. **40**:2478-2482.

166. **Lowy, F. D.** 1998. Staphylococcus aureus infections. N. Engl. J. Med. **339**:520-532.

167. **Lowy, F. D.** 2000. Is Staphylococcus aureus an intracellular pathogen? Trends Microbiol. **8**:341-343.

168. **Lowy, F. D.** 2003. Antimicrobial resistance: the example of Staphylococcus aureus. J. Clin. Invest **111**:1265-1273.

169. **Lowy, F. D., J. Fant, L. L. Higgins, S. K. Ogawa, and V. B. Hatcher**. 1988. Staphylococcus aureus--human endothelial cell interactions. J. Ultrastruct. Mol. Struct. Res. **98**:137-146.

170. **Lu, P. L., L. C. Chin, C. F. Peng, Y. H. Chiang, T. P. Chen, L. Ma, and L. K. Siu**. 2005. Risk factors and molecular analysis of community methicillin-resistant Staphylococcus aureus carriage. J. Clin. Microbiol. **43**:132-139.

171. **Lynch, A. S.** 2005. Antibacterial Drug Discovery and Development Summit - Tenth Annual SRI Summit. IDrugs. **8**:395-398.

172. **Lyon, B. R. and R. Skurray**. 1987. Antimicrobial resistance of Staphylococcus aureus: genetic basis. Microbiol. Rev. **51**:88-134.

173. **Ma, X. X., T. Ito, P. Chongtrakool, and K. Hiramatsu**. 2006. Predominance of clones carrying Panton-Valentine leukocidin genes among methicillin-resistant Staphylococcus aureus strains isolated in Japanese hospitals from 1979 to 1985. J. Clin. Microbiol. **44**:4515-4527.

174. **Madgwick, L., S. Mayer, and P. Keen**. 1989. Penetration of antibiotics into bovine neutrophils and their activity against intracellular Staphylococcus aureus. J. Antimicrob. Chemother. **24**:709-718.

175. **Madrigal, A. G., L. Basuino, and H. F. Chambers**. 2005. Efficacy of Telavancin in a rabbit model of aortic valve endocarditis due to methicillin-resistant Staphylococcus aureus or vancomycin-intermediate Staphylococcus aureus. Antimicrob. Agents Chemother. **49**:3163-3165.

176. **Malabarba, A., R. Ciabatti, R. Scotti, B. P. Goldstein, P. Ferrari, M. Kurz, B. P. Andreini, and M. Denaro**. 1995. New semisynthetic glycopeptides MDL 63,246 and MDL 63,042, and other amide derivatives of antibiotic A-40,926 active against highly glycopeptide-resistant VanA enterococci. J Antibiot. (Tokyo) **48**:869-883.

177. **Manfredi, R., S. Sabbatani, and F. Chiodo**. 2005. Severe staphylococcal knee arthritis responding favourably to linezolid, after glycopeptide-rifampicin failure: a case report and literature review. Scand. J. Infect. Dis. **37**:513-517.

178. **Mastouri, M., M. Nour, M. Ben Nejma, O. Bouallegue, M. Hammami, and M. Khedher**. 2006. [Antibiotics resistance of meticilline-resistant Staphylococcus aureus: detection of the first glycopeptides low sensibility strains in Tunisia]. Pathol. Biol. (Paris) **54**:33-36.

179. **McCormick, J. K., J. M. Yarwood, and P. M. Schlievert**. 2001. Toxic shock syndrome and bacterial superantigens: an update. Annu. Rev. Microbiol. **55**:77-104.

180. **McNamara, P. J. and R. A. Proctor**. 2000. Staphylococcus aureus small colony variants, electron transport and persistent infections. Int. J. Antimicrob. Agents **14**:117-122.

181. **Melles, D. C., W. B. van Leeuwen, H. A. Boelens, J. K. Peeters, H. A. Verbrugh, and A. van Belkum**. 2006. Panton-Valentine leukocidin genes in Staphylococcus aureus. Emerg. Infect. Dis. **12**:1174-1175.

182. **Mempel, M., C. Schnopp, M. Hojka, H. Fesq, S. Weidinger, M. Schaller, H. C. Korting, J. Ring, and D. Abeck**. 2002. Invasion of human keratinocytes by Staphylococcus aureus and intracellular bacterial persistence represent haemolysin-independent virulence mechanisms that are followed by features of necrotic and apoptotic keratinocyte cell death. Br. J. Dermatol. **146**:943-951.

183. **Menzies, B. E. and I. Kourteva**. 1998. Internalization of Staphylococcus aureus by endothelial cells induces apoptosis. Infect. Immun. **66**:5994-5998.

184. **Metzler, K., G. M. Hansen, P. Hedlin, E. Harding, K. Drlica, and J. M. Blondeau**. 2004. Comparison of minimal inhibitory and mutant prevention drug concentrations of 4 fluoroquinolones against clinical isolates of methicillin-susceptible and -resistant Staphylococcus aureus. Int. J. Antimicrob. Agents **24**:161-167.

185. **Michot, J. M., C. Seral, F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens**. 2005. Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. Antimicrob. Agents Chemother. **49**:2429-2437.

186. **Moreillon, P., Y.-A. Que, and M. P. Glauser**. 2005. Staphylococcus aureus, p. 2321-2351. *In*: G. E. Mandell, J. E. Bennett, and R. Dolin (eds.), Principles and practice of infectious diseases. Elsevier, Philadelphia, PA.

187. **Moreillon, P. and Y. A. Que**. 2004. Infective endocarditis. Lancet **363**:139-149.

188. **Mork, T., T. Tollersrud, B. Kvitle, H. J. Jorgensen, and S. Waage**. 2005. Comparison of Staphylococcus aureus genotypes recovered from cases of bovine, ovine, and caprine mastitis. J. Clin. Microbiol. **43**:3979-3984.

189. **Mylonakis, E., E. T. Ryan, and S. B. Calderwood**. 2001. Clostridium difficile--Associated diarrhea: A review. Arch. Intern. Med. **161**:525-533.

190. **Needham, A. J., M. Kibart, H. Crossley, P. W. Ingham, and S. J. Foster**. 2004. Drosophila melanogaster as a model host for Staphylococcus aureus infection. Microbiology **150**:2347-2355.

191. **Nguyen, H. A., J. Grellet, D. Paillard, V. Dubois, C. Quentin, and M. C. Saux**. 2006. Factors influencing the intracellular activity of fluoroquinolones: a study using levofloxacin in a Staphylococcus aureus THP-1 monocyte model. J. Antimicrob. Chemother. **57**:883-890.

192. **Nielsen, S. L. and F. T. Black**. 1999. Extracellular and intracellular killing in neutrophil granulocytes of Staphylococcus aureus with rifampicin in combination with dicloxacillin or fusidic acid. J. Antimicrob. Chemother. **43**:407-410.

193. **Noguchi, N., T. Okihara, Y. Namiki, Y. Kumaki, Y. Yamanaka, M. Koyama, K. Wakasugi, and M. Sasatsu**. 2005. Susceptibility and resistance genes to fluoroquinolones in methicillin-resistant Staphylococcus aureus isolated in 2002. Int. J. Antimicrob. Agents **25**:374-379.

194. **Norden, C. W.** 1983. Experimental chronic staphylococcal osteomyelitis in rabbits: treatment with rifampin alone and in combination with other antimicrobial agents. Rev. Infect. Dis. **5 Suppl 3**:S491-S494.

195. **Nuzzo, I., M. R. Sanges, A. Folgore, and C. R. Carratelli**. 2000. Apoptosis of human keratinocytes after bacterial invasion. FEMS Immunol. Med. Microbiol. **27**:235-240.

196. **O'Reilly, T., S. Kunz, E. Sande, O. Zak, M. A. Sande, and M. G. Tauber**. 1992. Relationship between antibiotic concentration in bone and efficacy of treatment of staphylococcal osteomyelitis in rats: azithromycin compared with clindamycin and rifampin. Antimicrob. Agents Chemother. **36**:2693-2697.

197. **Olivier, A., Lemaire, S., Mingeot-Leclercq, M. P., Van Bambeke, F., and Tulkens, P.M.** Intracellular infection of THP-1 human macrophages by Staphylococcus aureus and role of haemolysins. 12th International Symposium on Staphylococci & Staphylococcal InfectionsMaastricht, The Netherlands , P-021. 2006.

198. **Onyeji, C. O., C. H. Nightingale, and M. N. Marangos**. 1994. Enhanced killing of methicillin-resistant Staphylococcus aureus in human macrophages by liposome-entrapped vancomycin and teicoplanin. Infection **22**:338-342.

199. **Pace, J. L. and J. K. Judice**. 2005. Telavancin (Theravance). Curr. Opin. Investig. Drugs **6**:216-225.

200. **Pace, J. L., K. Krause, D. Johnston, D. Debabov, T. Wu, L. Farrington, C. Lane, D. L. Higgins, B. Christensen, J. K. Judice, and K. Kaniga**. 2003. In vitro activity of TD-6424 against Staphylococcus aureus. Antimicrob. Agents Chemother. **47**:3602-3604.

201. **Pace, J. L. and G. Yang**. 2006. Glycopeptides: Update on an old successful antibiotic class. Biochem. Pharmacol.

202. **Paillard, D., J. Grellet, V. Dubois, M. C. Saux, and C. Quentin**. 2002. Discrepancy between uptake and intracellular activity of moxifloxacin in a Staphylococcus aureus-human THP-1 monocytic cell model. Antimicrob. Agents Chemother. **46**:288-293.

203. **Pan, E. S., B. A. Diep, H. A. Carleton, E. D. Charlebois, G. F. Sensabaugh, B. L. Haller, and F. Perdreau-Remington**. 2003. Increasing prevalence of methicillin-resistant Staphylococcus aureus infection in California jails. Clin. Infect. Dis. **37**:1384-1388.

204. **Pascual, A., I. Garcia, S. Ballesta, and E. J. Perea**. 1999. Uptake and intracellular activity of moxifloxacin in human neutrophils and tissue-cultured epithelial cells. Antimicrob. Agents Chemother. **43**:12-15.

205. **Pascual, A., D. Tsukayama, J. Kovarik, G. Gekker, and P. Peterson**. 1987. Uptake and activity of rifapentine in human peritoneal macrophages and polymorphonuclear leukocytes. Eur. J. Clin. Microbiol. **6**:152-157.

206. **Patti, J. M., B. L. Allen, M. J. McGavin, and M. Hook**. 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu. Rev. Microbiol. **48**:585-617.

207. **Patti, J. M., T. Bremell, D. Krajewska-Pietrasik, A. Abdelnour, A. Tarkowski, C. Ryden, and M. Hook**. 1994. The Staphylococcus aureus collagen adhesin is a virulence determinant in experimental septic arthritis. Infect. Immun. **62**:152-161.

208. **Pechere, J. C.** 2001. New perspectives on macrolide antibiotics. Int. J. Antimicrob. Agents **18 Suppl 1**:S93-S97.

209. **Peck, O. M., H. Fan, G. E. Tempel, G. Teti, P. V. Halushka, and J. A. Cook**. 2004. Staphylococcus aureus and lipopolysaccharide induce homologous tolerance but heterologous priming: role of interferon-gamma. Shock **21**:254-260.

210. **Pinho, M. G., S. R. Filipe, H. de Lencastre, and A. Tomasz**. 2001. Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in Staphylococcus aureus. J. Bacteriol. **183**:6525-6531.

211. **Proctor, R. A., B. Kahl, C. von Eiff, P. E. Vaudaux, D. P. Lew, and G. Peters**. 1998. Staphylococcal small colony variants have novel mechanisms for antibiotic resistance. Clin. Infect. Dis. **27 Suppl 1**:S68-S74.

212. **Proctor, R. A. and G. Peters**. 1998. Small colony variants in staphylococcal infections: diagnostic and therapeutic implications. Clin. Infect. Dis. **27**:419-422.

213. **Prokesch, R. C. and W. L. Hand**. 1982. Antibiotic entry into human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **21**:373-380.

214. **Que, Y. A., P. Francois, J. A. Haefliger, J. M. Entenza, P. Vaudaux, and P. Moreillon**. 2001. Reassessing the role of Staphylococcus aureus clumping factor and fibronectin-binding protein by expression in Lactococcus lactis. Infect. Immun. **69**:6296-6302.

215. **Que, Y. A., J. A. Haefliger, L. Piroth, P. Francois, E. Widmer, J. M. Entenza, B. Sinha, M. Herrmann, P. Francioli, P. Vaudaux, and P. Moreillon**. 2005. Fibrinogen and fibronectin binding cooperate for valve infection and invasion in Staphylococcus aureus experimental endocarditis. J. Exp. Med. **201**:1627-1635.

216. **Raisanen, S. R., J. Halleen, V. Parikka, and H. K. Vaananen**. 2001. Tartrate-resistant acid phosphatase facilitates hydroxyl radical formation and colocalizes with phagocytosed Staphylococcus aureus in alveolar macrophages. Biochem. Biophys. Res. Commun. **288**:142-150.

217. **Rayner, C. and W. J. Munckhof**. 2005. Antibiotics currently used in the treatment of infections caused by Staphylococcus aureus. Intern. Med. J. **35 Suppl 2**:S3-16.

218. **Reilly, S. S., M. C. Hudson, J. F. Kellam, and W. K. Ramp**. 2000. In vivo internalization of Staphylococcus aureus by embryonic chick osteoblasts. Bone **26**:63-70.

219. **Reynolds, P. E.** 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur. J. Clin. Microbiol. Infect. Dis. **8**:943-950.

220. **Roberts, R. L., M. A. Barbieri, J. Ullrich, and P. D. Stahl**. 2000. Dynamics of rab5 activation in endocytosis and phagocytosis. J. Leukoc. Biol. **68**:627-632.

221. **Roberts, S. and S. Chambers**. 2005. Diagnosis and management of Staphylococcus aureus infections of the skin and soft tissue. Intern. Med. J. **35 Suppl 2**:S97-105.

222. **Ruef, C.** 2004. Epidemiology and clinical impact of glycopeptide resistance in Staphylococcus aureus. Infection **32**:315-327.

223. **Sabath, L. D.** 1982. Mechanisms of resistance to beta-lactam antibiotics in strains of Staphylococcus aureus. Ann. Intern. Med. **97**:339-344.

224. **Sabath, L. D., S. J. Wallace, and D. A. Gerstein**. 1972. Suppression of intrinsic resistance to methicillin and other penicillins in Staphylococcus aureus. Antimicrob. Agents Chemother. **2**:350-355.

225. **Sabath, L. D., N. Wheeler, M. Laverdiere, D. Blazevic, and B. J. Wilkinson**. 1977. A new type of penicillin resistance of Staphylococcus aureus. Lancet **1**:443-447.

226. **Saiman, L. and J. Siegel**. 2003. Infection control recommendations for patients with cystic fibrosis: Microbiology, important pathogens, and infection control practices to prevent patient-to-patient transmission. Am. J. Infect. Control **31**:S1-62.

227. **Saleh-Mghir, A., N. Ameur, C. Muller-Serieys, F. Ismael, F. Lemaitre, L. Massias, C. Feger, R. Bleton, and A. C. Cremieux**. 2002. Combination of quinupristin-dalfopristin (Synercid) and rifampin is highly synergistic in experimental Staphylococcus aureus joint prosthesis infection. Antimicrob. Agents Chemother. **46**:1122-1124.

228. **Salgado, C. D., B. M. Farr, and D. P. Calfee**. 2003. Community-acquired methicillin-resistant Staphylococcus aureus: a meta-analysis of prevalence and risk factors. Clin. Infect. Dis. **36**:131-139.

229. **Sandberg, A., Hessler, J. H. R., Skov, R. L., and Frimodt-Moller, N**. PK/PD of dicloxacillin against extra- and intracellular S. aureus *in vivo*. 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA , A-647. 2006.

230. **Schaad, H. J., C. Chuard, P. Vaudaux, F. A. Waldvogel, and D. P. Lew**. 1994. Teicoplanin alone or combined with rifampin compared with vancomycin for prophylaxis and treatment of experimental foreign body infection by methicillin-resistant Staphylococcus aureus. Antimicrob. Agents Chemother. **38**:1703-1710.

231. **Scheffers, D. J. and M. G. Pinho**. 2005. Bacterial cell wall synthesis: new insights from localization studies. Microbiol. Mol. Biol. Rev. **69**:585-607.

232. **Schentag, J. J.** 1999. Antimicrobial action and pharmacokinetics/pharmacodynamics: the use of AUIC to improve efficacy and avoid resistance. J. Chemother. **11**:426-439.

233. **Schentag, J. J., A. K. Meagher, and A. Forrest**. 2003. Fluoroquinolone AUIC break points and the link to bacterial killing rates. Part 1: In vitro and animal models. Ann. Pharmacother. **37**:1287-1298.

234. **Schito, G. C.** 2006. The importance of the development of antibiotic resistance in Staphylococcus aureus. Clin. Microbiol. Infect. **12 Suppl 1**:3-8.

235. **Schmidt-Ioanas, M., A. de Roux, and H. Lode**. 2005. New antibiotics for the treatment of severe staphylococcal infection in the critically ill patient. Curr. Opin. Crit Care **11**:481-486.

236. **Schrenzel, J., S. Harbarth, G. Schockmel, D. Genne, T. Bregenzer, U. Flueckiger, C. Petignat, F. Jacobs, P. Francioli, W. Zimmerli, and D. P. Lew**. 2004. A randomized clinical trial to compare fleroxacin-rifampicin with flucloxacillin or vancomycin for the treatment of staphylococcal infection. Clin. Infect. Dis. **39**:1285-1292.

237. **Seligman, S. J.** 2006. Small-colony variants of Staphylococcus aureus. Clin. Infect. Dis. **42**:155-156.

238. **Seral, C., S. Carryn, P. M. Tulkens, and 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.

239. **Seral, C., J. M. Michot, H. Chanteux, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke**. 2003. Influence of P-glycoprotein inhibitors on accumulation of macrolides in J774 murine macrophages. Antimicrob. Agents Chemother. **47**:1047-1051.

240. **Seral, C., F. Van Bambeke, and P. M. Tulkens**. 2003. Quantitative analysis of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, and oritavancin (LY333328) activities against intracellular Staphylococcus aureus in mouse J774 macrophages. Antimicrob. Agents Chemother. **47**:2283-2292.

241. **Shaw, J. P., J. Seroogy, K. Kaniga, D. L. Higgins, M. Kitt, and S. Barriere**. 2005. Pharmacokinetics, serum inhibitory and bactericidal activity, and safety of telavancin in healthy subjects. Antimicrob. Agents Chemother. **49**:195-201.

242. **Shuter, J., V. B. Hatcher, and F. D. Lowy**. 1996. Staphylococcus aureus binding to human nasal mucin. Infect. Immun. **64**:310-318.

243. **Sifri, C. D., J. Begun, F. M. Ausubel, and S. B. Calderwood**. 2003. Caenorhabditis elegans as a model host for Staphylococcus aureus pathogenesis. Infect. Immun. **71**:2208-2217.

244. **Sinha, B., P. P. Francois, O. Nusse, M. Foti, O. M. Hartford, P. Vaudaux, T. J. Foster, D. P. Lew, M. Herrmann, and K. H. Krause**. 1999. Fibronectin-binding protein acts as Staphylococcus aureus invasin via fibronectin bridging to integrin alpha5beta1. Cell Microbiol. **1**:101-117.

245. **Smith, R. P., A. L. Baltch, M. A. Franke, P. B. Michelsen, and L. H. Bopp**. 2000. Levofloxacin penetrates human monocytes and enhances intracellular killing of Staphylococcus aureus and Pseudomonas aeruginosa. J. Antimicrob. Chemother. **45**:483-488.

246. **Sompolinsky, D., Z. Samra, W. W. Karakawa, W. F. Vann, R. Schneerson, and Z. Malik**. 1985. Encapsulation and capsular types in isolates of Staphylococcus aureus from different sources and relationship to phage types. J. Clin. Microbiol. **22**:828-834.

247. **Srinivasan, A., J. D. Dick, and T. M. Perl**. 2002. Vancomycin resistance in staphylococci. Clin. Microbiol. Rev. **15**:430-438.

248. **Stefani, S. and P. E. Varaldo**. 2003. Epidemiology of methicillin-resistant staphylococci in Europe. Clin. Microbiol. Infect. **9**:1179-1186.

249. **Struelens, M. J. and O. Denis**. 2006. Rapid molecular detection of methicillin-resistant Staphylococcus aureus: a cost-effective tool for infection control in critical care? Crit Care **10**:128.

250. **Stryjewski, M. E., W. D. O'Riordan, W. K. Lau, F. D. Pien, L. M. Dunbar, M. Vallee, V. G. Fowler, Jr., V. H. Chu, E. Spencer, S. L. Barriere, M. M. Kitt, C. H. Cabell, and G. R. Corey**. 2005. Telavancin versus standard therapy for treatment of complicated skin and soft-tissue infections due to gram-positive bacteria. Clin. Infect. Dis. **40**:1601-1607.

251. **Stucki, A., P. Gerber, F. Acosta, M. Cottagnoud, and P. Cottagnoud**. 2006. Efficacy of Telavancin against Penicillin-Resistant Pneumococci and Staphylococcus aureus in a Rabbit Meningitis Model and Determination of Kinetic Parameters. Antimicrob. Agents Chemother. **50**:770-773.

252. **Suter, F., R. Maserati, G. Carnevale, P. Marone, C. Filice, and E. Concia**. 1984. Management of staphylococcal endocarditis in drug addicts. Combined therapy with oral rifampicin and aminoglycosides. J. Antimicrob. Chemother. **13 Suppl C**:57-60.

253. **Takeuchi, F., S. Watanabe, T. Baba, H. Yuzawa, T. Ito, Y. Morimoto, M. Kuroda, L. Cui, M. Takahashi, A. Ankai, S. Baba, S. Fukui, J. C. Lee, and K. Hiramatsu**. 2005. Whole-genome sequencing of staphylococcus haemolyticus uncovers the extreme plasticity of its genome and the evolution of human-colonizing staphylococcal species. J. Bacteriol. **187**:7292-7308.

254. **Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada**. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer **26**:171-176.

255. **Tucker, K. A., S. S. Reilly, C. S. Leslie, and M. C. Hudson**. 2000. Intracellular Staphylococcus aureus induces apoptosis in mouse osteoblasts. FEMS Microbiol. Lett. **186**:151-156.

256. **Tulkens, P. M.** 1991. Intracellular distribution and activity of antibiotics. Eur. J. Clin. Microbiol. Infect. Dis. **10**:100-106.

257. **Ubukata, K., R. Nonoguchi, M. Matsuhashi, and M. Konno**. 1989. Expression and inducibility in Staphylococcus aureus of the mecA gene, which encodes a methicillin-resistant S. aureus-specific penicillin-binding protein. J. Bacteriol. **171**:2882-2885.

258. **Ueda, Y. and M. Sunagawa**. 2003. In vitro and in vivo activities of novel 2-(thiazol-2-ylthio)-1beta-methylcarbapenems with potent activities against multiresistant gram-positive bacteria. Antimicrob. Agents Chemother. **47**:2471-2480.

259. **Van Amersfoort, E. S., T. J. Van Berkel, and J. Kuiper**. 2003. Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. Clin. Microbiol. Rev. **16**:379-414.

260. **Van Bambeke, F.** 2004. Glycopeptides in clinical development: pharmacological profile and clinical perspectives. Curr. Opin. Pharmacol. **4**:471-478.

261. **Van Bambeke, F., M. Barcia-Macay, S. Lemaire, and P. M. Tulkens**. 2006. Cellular pharmacodynamics and pharmacokinetics of antibiotics: current views and perspectives. Curr. Opin. Drug Discov. Devel. **9**:218-230.

262. **Van Bambeke, F., S. Carryn, C. Seral, H. Chanteux, D. Tyteca, M. P. Mingeot-Leclercq, and 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.

263. **Van Bambeke, F., J. Saffran, M. P. Mingeot-Leclercq, and 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. **49**:1695-1700.

264. **Van Bambeke, F., Y. Van Laethem, P. Courvalin, and P. M. Tulkens**. 2004. Glycopeptide antibiotics: from conventional molecules to new derivatives. Drugs **64**:913-936.

265. **Van de Velde, S., Carryn, S., Van Bambeke, F., and Tulkens, P. M**. Mise au point de l'isolation de *Listeria monocytogenes* intracellulaires pour étude protéomique. 3ème Colloque International Francophone de Bactériologie Vétérinaire . 2006.

266. **van den Broek, P. J.** 1989. Antimicrobial drugs, microorganisms, and phagocytes. Rev. Infect. Dis. **11**:213-245.

267. **Vann, J. M. and R. A. Proctor**. 1987. Ingestion of Staphylococcus aureus by bovine endothelial cells results in time- and inoculum-dependent damage to endothelial cell monolayers. Infect. Immun. **55**:2155-2163.

268. **Vazifeh, D., H. Abdelghaffar, and M. T. Labro**. 2002. Effect of telithromycin (HMR 3647) on polymorphonuclear neutrophil killing of Staphylococcus aureus in comparison with roxithromycin. Antimicrob. Agents Chemother. **46**:1364-1374.

269. **Vercellotti, G. M., D. Lussenhop, P. K. Peterson, L. T. Furcht, J. B. McCarthy, H. S. Jacob, and C. F. Moldow**. 1984. Bacterial adherence to fibronectin and endothelial cells: a possible mechanism for bacterial tissue tropism. J. Lab Clin. Med. **103**:34-43.

270. **Verhoef, J., P. Peterson, Y. Kim, L. D. Sabath, and P. G. Quie**. 1977. Opsonic requirements for staphylococcal phagocytosis. Heterogeneity among strains. Immunology **33**:191-197.

271. **Vesely, J. J., F. D. Pien, and B. C. Pien**. 1998. Rifampin, a useful drug for nonmycobacterial infections. Pharmacotherapy **18**:345-357.

272. **Vesga, O., M. C. Groeschel, M. F. Otten, D. W. Brar, J. M. Vann, and R. A. Proctor**. 1996. Staphylococcus aureus small colony variants are induced by the endothelial cell intracellular milieu. J. Infect. Dis. **173**:739-742.

273. **von Eiff, C., K. Becker, K. Machka, H. Stammer, and G. Peters**. 2001. Nasal carriage as a source of Staphylococcus aureus bacteremia. Study Group. N. Engl. J. Med. **344**:11-16.

274. **von Eiff, C., K. Becker, D. Metze, G. Lubritz, J. Hockmann, T. Schwarz, and G. Peters**. 2001. Intracellular persistence of Staphylococcus aureus small-colony variants within keratinocytes: a cause for antibiotic treatment failure in a patient with darier's disease. Clin. Infect. Dis. **32**:1643-1647.

275. **von Eiff, C., C. Heilmann, R. A. Proctor, C. Woltz, G. Peters, and F. Gotz**. 1997. A site-directed Staphylococcus aureus hemB mutant is a small-colony variant which persists intracellularly. J. Bacteriol. **179**:4706-4712.

276. **von Eiff, C., P. McNamara, K. Becker, D. Bates, X. H. Lei, M. Ziman, B. R. Bochner, G. Peters, and R. A. Proctor**. 2006. Phenotype microarray profiling of Staphylococcus aureus menD and hemB mutants with the small-colony-variant phenotype. J. Bacteriol. **188**:687-693.

277. **von Eiff, C., G. Peters, and K. Becker**. 2006. The small colony variant (SCV) concept -- the role of staphylococcal SCVs in persistent infections. Injury **37 Suppl 2**:S26-S33.

278. **von Eiff, C., R. A. Proctor, and G. Peters**. 2000. Staphylococcus aureus small colony variants: formation and clinical impact. Int. J. Clin. Pract. Suppl44-49.

279. **Vouillamoz, J., J. M. Entenza, P. Hohl, and P. Moreillon**. 2004. LB11058, a new cephalosporin with high penicillin-binding protein 2a affinity and activity in experimental endocarditis due to homogeneously methicillin-resistant Staphylococcus aureus. Antimicrob. Agents Chemother. **48**:4322-4327.

280. **Weinrick, B., P. M. Dunman, F. McAleese, E. Murphy, S. J. Projan, Y. Fang, and R. P. Novick**. 2004. Effect of mild acid on gene expression in Staphylococcus aureus. J. Bacteriol. **186**:8407-8423.

281. **Wesson, C. A., L. E. Liou, K. M. Todd, G. A. Bohach, W. R. Trumble, and K. W. Bayles**. 1998. Staphylococcus aureus Agr and Sar global regulators influence internalization and induction of apoptosis. Infect. Immun. **66**:5238-5243.

282. **Yancey, R. J., M. S. Sanchez, and C. W. Ford**. 1991. Activity of antibiotics against Staphylococcus aureus within polymorphonuclear neutrophils. Eur. J. Clin. Microbiol. Infect. Dis. **10**:107-113.

283. **Yao, L., V. Bengualid, F. D. Lowy, J. J. Gibbons, V. B. Hatcher, and J. W. Berman**. 1995. Internalization of Staphylococcus aureus by endothelial cells induces cytokine gene expression. Infect. Immun. **63**:1835-1839.

284. **Yao, L., F. D. Lowy, and J. W. Berman**. 1996. Interleukin-8 gene expression in Staphylococcus aureus-infected endothelial cells. Infect. Immun. **64**:3407-3409.

285. **Yeaman, M. R., A. S. Bayer, S. P. Koo, W. Foss, and P. M. Sullam**. 1998. Platelet microbicidal proteins and neutrophil defensin disrupt the Staphylococcus aureus cytoplasmic membrane by distinct mechanisms of action. J. Clin. Invest **101**:178-187.