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Intracellular Distribution and Activity of Antibiotics

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Intracellular penetration, accumulation and disposition are important parameters governing the activity of antibiotics against intracellular bacteria. Beta-lactams diffuse into but do not accumulate in phagocytes, probably because of their acidic character. Aminoglycosides are too polar to pass across membranes and are therefore only taken up slowly by endocytosis, which results in an exclusively lysosomal localization. Lincosaminides, macrolides and fluoroquinolones all accumulate in phagocytes, the two former classes of drugs showing both a cytosolic and a lysosomal localization. Fluoroquinolones appear to be entirely soluble in cells. Analysis of their activity in a model of *Staphylococcus aureus*-infected J774 macrophages has revealed low activity of clindamycin, whereas macrolides, and even more so fluoroquinolones, easily reduce the original inoculum.

Most antibiotics available so far have been designed and/or screened primarily for their activity against extracellular bacteria. This, however, does not necessarily result in efficacy in vivo, since the overall activity of an antibiotic is the result of many factors which are only partly taken into account in the above approach. Of those, intracellular pharmacokinetics and activity have long been neglected, or studied only at a very late stage of development. This is because intracellular infection is often viewed as being of importance only for specific, obligatory intracellular parasites. However, it is now more widely recognized that intracellular survival, or even multiplication of many other bacteria referred to as facultative intracellular parasites, play a significant role in the pathogenesis of the disease these organisms cause (1). This is particularly evident in infections caused by *Salmonella* spp. or *Listeria monocytogenes*, but is also seen in the case of more common pathogens such as *Staphylococcus aureus*. Moreover, intracellular infection is also probably responsible for many of the difficulties encountered in controlling and eradicating those infections (2–4). Increased attention therefore needs to be focused on the intracellular pharmacokinetics and pharmacodynamics of antibiotics, as a complement to the evaluation of their properties in acellular systems. Figure 1 shows a schematic representation of the main cellular pharmacokinetic and pharmacodynamic properties

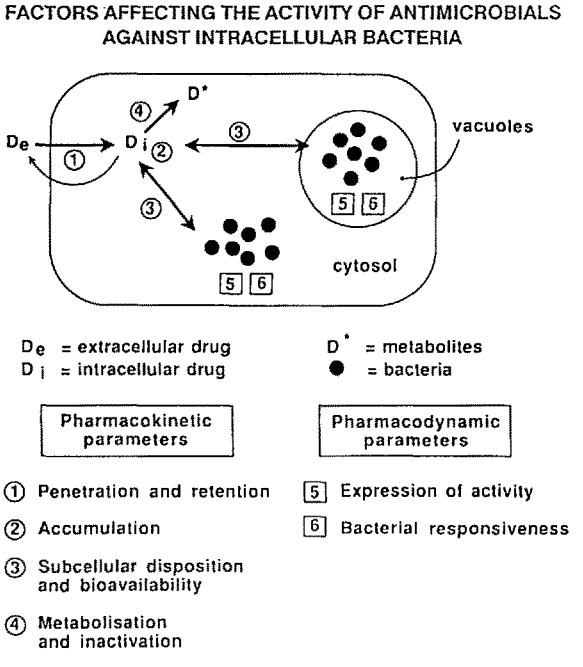

Figure 1: Pharmacokinetic and pharmacodynamic parameters involved in the activity of antimicrobial drugs against intracellular microorganisms.

which are of importance for the activity of an antibiotic against intracellular bacteria. This paper will present an overview of the present status of our knowledge on uptake and subcellular distribution of the main classes of antibiotics. It will then examine the activities of lincosaminides, macrolides and

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fluoroquinolones in a *Staphylococcus aureus*-infected J774 macrophage model in an attempt to establish whether or not pharmacokinetic properties are important for predicting intracellular efficacy.

Uptake and Subcellular Localization

Almost all studies on the uptake and subcellular localization of antibiotics have been conducted in cells maintained or grown in vitro. Until now only scanty data has been available for cells in vivo, apart from general considerations on volume of distribution or tissue accumulation. These, however, do not demonstrate that the drug is intracellular. It is therefore not known whether the in vitro data correctly describe the situation in vivo. Given this caveat, however, much basic information has been obtained in such models that has proved to be of clinical importance.

Aminoglycoside Antibiotics. Many authors have claimed that aminoglycosides do not penetrate mammalian cells. However, studies in macrophages or in fibroblasts (5, 6) have shown that cells incubated for several days in the presence of aminoglycosides accumulate these drugs to an apparent intracellular/extracellular concentration ratio of 2 to 4. Aminoglycosides also are known to accumulate in proximal tubular cells of kidney. Intracellular aminoglycosides remain potentially bioactive (i.e. they can be recovered in a microbiologically active form), which is in line with the lack of metabolism of these antibiotics in mammals. Further studies disclosed that intracellular aminoglycosides are not homogeneously distributed within cells, but localize largely if not exclusively within lysosomes. In cultured macrophages or fibroblasts, the concentration of aminoglycosides in lysosomes was estimated to reach values at least 10- to 20-fold higher than in the extracellular fluid (6). The concentration is considerably higher in the lysosomes of kidney proximal tubular cells (7). The mechanism of cell uptake of aminoglycosides is probably endocytosis (8), of the 'fluid' type in macrophages or fibroblasts, and of the absorbtive type in kidney. This explains the slow rate of uptake in the former type of cells (9, 10). Much of the literature describing the 'non-penetration' of aminoglycosides in phagocytic cells reports use of short-term (1–6 h) experiments, which is not sufficient to obtain detectable intracellular levels in most cases.

Beta-Lactam Antibiotics. As with aminoglycosides, many authors have reported a lack of accumulation of beta-lactams in phagocytic cells exposed for a few hours to these antibiotics (11–14), and have also concluded that beta-lactams do not penetrate cells. Contrary to aminoglycosides, however, the lack of accumulation of beta-lactams (i.e. an apparent intracellular concentration lower than the extracellular concentration) is genuine and is observed even if cells are maintained in the presence of these drugs for several days. However, this does not mean that *beta-lactams do not penetrate cells.* In fact, these antibiotics diffuse through membranes, as evidenced by their volume of distribution in vivo, which is considerably larger than the volume of extracellular water, and by the fact that most of them are absorbed, albeit to variable extents, after oral administration. Moreover, intracellular beta-lactams are found free in the cell cytosol. Thus, their behaviour is in sharp contrast to that of aminoglycosides which do not diffuse through membranes and, when taken up by cells, are found in lysosomes. The lack of intracellular accumulation of beta-lactams could result from an active outward transport. Studies using a basic derivative of penicillin G, N-(3-dimethylaminopropyl)benzylpenicillinamide (ABP), however, have suggested that the lack of accumulation of beta-lactams may merely result from their character as weak organic acids (15). In contrast to penicillin G, ABP accumulates 5- to 6-fold in cells and, moreover, localizes in part in lysosomes. Yet, ABP diffuses in and out of macrophages at almost the same rate as penicillin G (t 1/2 approximately 20–30 min), which would not be anticipated if penicillin G underwent active, specific outward transport. Assuming that the key difference between the two substances is their acidobasic character, the 'classical' models of unequal distribution of weak organic acids and bases between compartments of different pH bounded by biological membranes (16–18) may explain their contrasting behaviour. This model, as applied to ABP, is represented in Figure 2.

Lincosaminides. Lincosaminide antibiotics such as clindamycin have been shown to accumulate in phagocytes (12, 19). Evidence has been presented suggesting that clindamycin could use the membrane transport system for nucleosides (20). This mechanism, however, does not explain why lincomycin, which closely resembles clindamycin, is not accumulated by phagocytes. It also fails to explain why cell-associated lincosaminides (clindamycin, lincomycin or pirlimycin) are found almost equally distributed in absolute values between lysosomes and cytosol, irrespective of their level of cellular accumulation (unpublished observation). Since lysosomes represent only a small proportion of the total cell volume (2–5 % in unstimulated macrophages), it can easily be seen by calculation that the intralysosomal concentration of clindamycin greatly exceeds

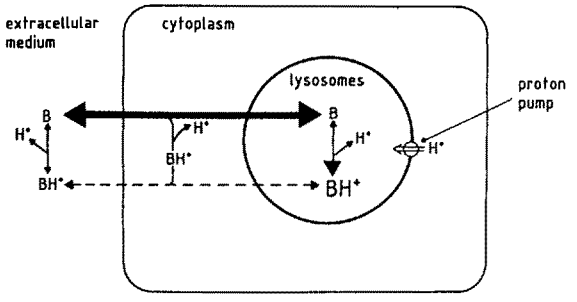

Figure 2: Proposed mechanism for the accumulation of basic drugs in cells, and for their partition between cytosol and lysosomes. Biological membranes are more permeable to the unprotonated form of organic bases (B) than their protonated forms (BH^+). The cytoplasmic pH is usually 0.5 units, and the lysosomal pH 2 units lower than the extracellular milieu. This results in the BH^+ forms of basic drugs accumulating in the corresponding compartments in relation to the proton concentration difference (see reference 18 for a complete discussion and analysis of the salient drug parameters responsible for the rate and extent of accumulation). Conversely, organic acids will tend to be excluded from acidic compartments because their unprotonated, charged form (A^-) diffuses much more readily than their protonated form (AH) which is uncharged. From reference 15.

the cytosolic one. This would imply that the putative transport system not only operates at the level of the pericellular membrane, pumping the extracellular drug into cytosol, but also at the level of the lysosomes, further pumping the drug from cytosol into these organelles. Finally, one would expect a carrier-mediated transport to show saturation, but this was not observed for lincosaminides. Since lincosaminides are all organic bases, non-ionic diffusion/segregation, as explained above for the basic derivative of penicillin G (see also Figure 1) could actually account for both their accumulation and subcellular distribution. It has been suggested that PMNs which take up large amounts of clindamycin could actually deliver this drug at their site of migration, e.g. in abscesses. In our opinion, such a transport would probably be very ineffective because of the rapid efflux of lincosaminides. Thus, PMNs maintain a gradient, but not an actual concentration of drug, and it is likely that PMNs will arrive at their tissue location having lost most of the antibiotic, unless its extracellular concentration is kept constant throughout, also in the abscess itself. In such a case, migrating cells would have no advantage over the resident cells.

Macrolides. A marked intracellular accumulation of macrolide antibiotics has been observed in several types of cultured cells. Thus, polymorphonuclear leukocytes, macrophages and various cells in culture were shown to concentrate erythromycin 2- to 10-fold over the extracellular concentration (21–23). As with lincosaminides, marked differences are observed among apparently closely related derivatives, and new compounds such as roxithromycin usually show (or have been selected for ?) a larger accumulation (23). Accumulation of macrolides is, however, severely decreased upon incubation in acid pH. Conversely, it is considerably increased in activated macrophages, namely those collected by broncho-alveolar lavage of smokers (21, 23). Uptake of macrolides is non-saturable and usually rapid, but so is efflux (half-life approximately 15–20 min at most). A marked recent exception is azithromycin (24), the peculiar behaviour of which (slow penetration, very high intracellular levels, slow release) definitely deserves further mechanistic studies. A useful consequence of the slow release of azithromycin, in contrast to other macrolides or to lincosaminides, could be the effective use of drug-laden PMNs to deliver the antibiotic at the site of infection (see discussion above).

Fractionation studies have shown that at equilibrium cell-associated macrolides distribute almost equally between the cell soluble fraction and lysosomes. The latter association is stable as long as lysosomes are kept intact during the homogenization and fractionation procedures. As with lincosaminides, the overall pharmacokinetic and distribution properties of macrolides strongly suggest that uptake and disposition of these drugs in phagocytes occur by non-ionic diffusion and segregation of the ionized species of the antibiotic in the acidic compartments of the cell. Physicochemical studies comparing series of macrolides with different uptake and release rates and accumulation levels are, however, required before this supposition can be further substantiated.

Fluoroquinolones. Available data show that pefloxacin, ciprofloxacin, and all other fluoroquinolones tested so far, accumulate in phagocytic cells (25, 26; MB Carlier et al, 27th ICAAC, Abstract no. 622; MB Carlier, PM Tulkens, 28th ICAAC, Abstract no. 52). This observation has been extended to non-phagocytic cells, such as fibroblasts; erythrocytes, however, do not accumulate fluoroquinolones (MB Carlier et al, data to be published). As with macrolides, uptake of fluoroquinolones is rapid. The accumulated drugs are also quickly released when cells are transferred to drug-free medium. Intracellular accumulation of fluoroquinolones is similar to that of erythromycin (approximately 2- to 8-fold), and therefore lower than that of new macrolides such as roxithromycin. In contrast to macrolides, accumulation of fluoroquinolones is slightly enhanced by incubation in acidic medium. No influence of the activation of macrophages has been observed. In contrast to macrolides or lincosaminides, no major differences have been observed among fluoroqui-

nolones available for clinical use to date with respect to cell accumulation. A most intriguing observation is that the 'accumulation' of fluoroquinolones does not require cell viability and is actually enhanced by exposure of cells to formaldehyde or preincubation at 56 °C. Nevertheless, fluoroquinolones that become cell-associated under these conditions are still recoverable in a fully active form. Whether there is subcellular localization of fluoroquinolones is not known for certain, since cell fractionation studies fail to reveal significant association of accumulated fluoroquinolones with specific subcellular organelles, the drugs being almost entirely recovered in the so-called 'soluble fractions' (MB Carlier et al, data to be published). This could be interpreted as a genuinely cytosolic localization, or as an elution from a storage compartment. However, there is no indication as to what this compartment could be.

The mechanism(s) whereby fluoroquinolones accumulate in cells is not yet known, and no simple model can be presented in view of the findings presented above. It must also be noted that all fluoroquinolones display a free carboxyl group which should make them ionize at physiological pH.

Other Antibiotics. Ansamycins, rifampicin in particular, have been reported to accumulate in phagocytes. However, the actual accumulation ratios measured in several cell types are relatively low, being in the order of 2 to 3 (12). This indicates that liposolubility per se is not necessarily a favourable property for intracellular accumulation, even though it certainly plays a major role in determining the rate of uptake. Little is known about the subcellular distribution of ansamycins in cells, but available data suggests that rifampicin is distributed in the cytosol (27). Tetracyclines and chloramphenicol have been reported to accumulate in cells, to a moderate extent. Trimethoprim accumulates only transiently. Pleuromutilins (a class of antichlamydial agents) accumulate to a large extent. Nitroimidazoles diffuse easily through membranes and actually need to be metabolized in sensitive bacteria and parasites to express their activity, but do not accumulate in cells. Polypeptidic and glycopeptidic antibiotics show no or only very low accumulation.

Activity of Intracellular Antibiotics

The activity of antibiotics against intracellular bacteria has been examined in a very large number of models both in vitro and in vivo, with many apparently conflicting results. Van den Broek (3) has published a review of these studies and commented on the difficulties in comparing data generated by different laboratories. Clearly, the duration of incubation, the extracellular drug concentration, the type of pathogen (including its subcellular location), the potential enhancement or impairment of host bactericidal capabilities by the antibiotic or other chemicals present in the assay system are all variables which must be carefully taken into account. Moreover, the determination of antibiotic activity against intracellular organisms is often associated with methodological problems, especially when dealing with species that can grow both extra- and intracellularly. Another problem which concerns both facultative and obligatory intracellular parasites is related to the quiescent character of many intracellular bacteria. This prevents many antibiotics from expressing their activity, making them ineffective in the corresponding models, whereas the same drugs show distinct effects in models in which intracellular bacteria actively multiply.

Taking these remarks in consideration, it can be understood why ampicillin can be used to treat listeriosis, or under which circumstances aminoglycosides will be effective against tuberculosis, as demonstrated by their clinical use, although both diseases are characterized by an important intracellular component. Since beta-lactams actually have access, albeit limited, to cytosol, and since infective *Listeria monocytogenes* also have access to that cell compartment, a large extracellular concentration of ampicillin will allow the intracytosolic concentration to reach the necessary threshold value for acting against these bacteria (i.e. above the MICs). With respect to aminoglycosides, activity can be observed if the intracellular drug overcomes two major defeating effects, namely its almost exclusive localization in lysosomes and its consequent exposure to acid pH. Thus, it can be predicted that aminoglycosides will have very poor activity against organisms that stay away from lysosomes; in addition, the concentrations in lysosomes needed to impair bacterial growth may be up to 2 to 3 times greater than those measured in broth. In most short-term experiments (see below, for instance), the latter condition is not met due to the slow penetration of aminoglycosides described earlier, and results are therefore systematically negative. Conversely, in long-term experiments or in the case of high extracellular concentrations, aminoglycosides become effective against several sensitive bacteria (28–32).

The activity of macrolides and fluoroquinolones is less subject to controversy. Macrolides, and more recently fluoroquinolones, have been shown to be effective agents against typical obligatory intracellular pathogens such as *Legionella* spp. (33–35). Moreover, both classes of drug have been shown to be effective in macrophages against *Staphylococcus*

aureus (26, and results shown below), and fluoroquinolones against *Salmonella* spp. (unpublished data). No activity of erythromycin, however, has been observed against resting *Staphylococcus aureus* in PMNs (36). The activity of macrolides and fluoroquinolones undoubtedly relates to their capacity to enter phagocytes and not to suffer too much a defeating effect of the intracellular environment on their antibacterial properties. Compared to all antibiotics discussed so far, however, rifampicin is the 'golden standard' for intracellular activity, in all studies having consistently shown a high degree of efficacy against all sensitive bacteria (3), even resting bacteria in PMNs, against which erythromycin is ineffective (36). As explained in the first section, efficacy of rifampicin does not primarily result from a higher accumulation of this drug compared to other antibiotics. The most widely accepted hypotheses is that the activity of rifampicin is somehow enhanced by the intracellular physicochemical conditions prevailing at the site of infection.

With the aim of comparing the intracellular activity of antibiotics in a reproducible manner, we designed a model of J774 macrophages that can be infected by *Staphylococcus aureus*, which is based on similar approaches using peritoneal macrophages or polymorphonuclear phagocytes (26, 36). These cells were choosen since we already had a good understanding of antibiotic uptake and distribution in them, and since they allow rapid growth of *Staphylococcus aureus*, apparently because of their weak or hampered bactericidal mechanisms. This model has enabled us to draw several conclusions from some of the key data summarized in Table 1. Firstly, it is clear that clindamycin is effective, whereas lincomycin is not, for controlling the intracellular multiplication of *Staphylococcus aureus*, despite the fact that, by manipulating the extracellular concentration, it is possible to obtain intracellular concentrations of lincomycin exceeding the MIC 100-fold. Yet, compared to macrolides, or more so fluoroquinolones, clindamycin remains only moderately efficacious on

Table 1: Correlation between accumulation and effectiveness of antibiotics in *Staphylococcus aureus* infected J774 macrophages. Cells were allowed to phagocytose opsonized *Staphylococcus aureus* (from a bovine clinical isolate), washed with lysostaphin and then reincubated for 24 h at 37 °C in the absence (control) or the presence of ^{14}C-labelled antibiotics.

Drug	Extracellular concentration[a] (mg/l)	Intracellular concentration[b] (mg/l)	Variation from original inoculum[c] (%)	Intrinsic activity[d]
None (controls)			8,722 ± 48	
Lincomycin	10 (10)	23.5 ± 3.2	13,750 ± 267	−910
	50 (50)	118.7 ± 8.1	8,769 ± 185	−792
Clindamycin	2.5 (10)	19.5 ± 2.4	110 ± 7	−5.2
	5.0 (20)	49.2 ± 1.4	60 ± 2	22.6
Erythromycin	0.5 (1)	4.2 ± 0.1	81 ± 4	11.5
	5.0 (10)	50.9 ± 1.7	43 ± 2	35.9
Roxithromycin	0.5 (1)	8.7 ± 1.3	64 ± 2	11.1
	5.0 (10)	107.8 ± 18.2	23 ± 2	30.4
Pefloxacin	1 (1)	10.9 ± 0.2	37 ± 3	53.6
	10 (10)	109.5 ± 10.2	19 ± 3	85.3
Ciprofloxacin	0.5 (1)	3.9 ± 0.2	22 ± 2	80.0
	5.0 (10)	45.2 ± 6.5	3 ± 2	140.0

[a]Figures in parentheses show the value in multiples of the MIC determined in broth against the strain of *S. aureus* used.

[b]Calculated concentration, based on the measurement of antibiotic/cellular protein ratio (15), and assuming the drug is homogenously distributed in the cell.

[c]The original inoculum amounted to approx 1–2 bacteria/cell. Cells incubated in the presence of oxacillin or gentamicin (10 × MIC) did not show significant difference from cells incubated without drug.

[d]Intrinsic activity is defined as the effectiveness of the drug disregarding its level of accumulation. The unit used is: log (CFU at time 0 h/CFU at time = 24 h)/Ci/Ce X 10^3, where CFU is the number of viable bacteria per mg of cell protein, Ce the extracellular concentration and Ci the calculated intracellular concentration.

decreasing the inoculum. Secondly, the model allows comparison of drugs with respect to their intrinsic intracellular activity. This is defined as the activity of the antibiotic divided by its accumulation ratio. In actual fact, the intrinsic activity of each drug studied increases more rapidly than its concentration, suggesting threshold effects. Thirdly, it is clear that within the class of macrolides and at a given extracellular concentration, activity immediately correlates to accumulation. Thus, the intrinsic intracellular activity of erythromycin and roxithromycin are similar, but for all practical purposes roxithromycin will offer advantages because of its greater accumulation. A similar effect has been observed for other macrolides with high tissue penetration (B Scorneaux, PM Tulkens, 29th ICAAC, Abstract no. 157). Finally, the model also shows that the intrinsic activity of fluoroquinolones is greater – at the same intracellular/extracellular concentration ratio – than that of macrolides (for instance of ciprofloxacin versus erythromycin). Thus, it can be anticipated that fluoroquinolones exhibiting greater accumulation than those presently available would be very efficacious since they would combine both pharmacodynamic (intrinsic intracellular activity) and pharmacokinetic (accumulation) advantages. In contrast to macrolides, fluoroquinolones have shown differences in intrinsic intracellular activity within the class, but results are still too few to yield useful information on structure-activity relationships. Interesting subjects for future research would be the evaluation of susceptible factors modulating the intrinsic intracellular activity of antibiotics and the analysis of mechanisms that may suppress or enhance it.

Concluding Remarks

The present review has tried to establish some links between intracellular pharmacokinetic parameters of antibiotics and their efficacy against intracellular bacteria. Determining which of these are critical is probably useful for rational analysis of the respective merits of the antibiotics available, but another challenging goal could also be the more effective development of new strategies aimed at better control of bacterial infection. Drug-related parameters such as those shown in Figure 1 obviously are of critical importance in the early stages of choosing or designing appropriate compounds. Whereas it is clear that a large cellular accumulation per se is not essential for activity, it definitely will help. Prodrugs may allow cellular delivery of diffusible but otherwise non-accumulated substances. The same could be achieved by coupling antibiotics to appropriate non-diffusible carriers, provided this does not result in an irreversible loss of activity. A better knowledge of where the drug needs to be targeted to is also necessary. Contrary to what we and others have often claimed (2, 4, 18, 27), it now becomes increasingly clear that specific subcellular localization may not necessarily be an optimal property of an antimicrobial, given the necessity to track down bacteria in different subcellular compartments. We do not know if this is the reason for the greater intrinsic intracellular activity of fluoroquinolones compared to macrolides. Two key parameters related to intracellular disposition certainly are crucial: the ability of the drug to reach its target and to act in the corresponding environment. There is little we can do in the case of the available antibiotics concerning the microorganism-related parameters, except record whether these microorganisms are accessible and sensitive to the drugs under study. However, recognition of these parameters may provide us with more rational choices and help in designing new strategies.

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