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Cellular uptake and subcellular distribution of roxithromycin and erythromycin in phagocytic cells

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The intracellular accumulation and subcellular distribution of ^{14}C-labelled roxithromycin and erythromycin has been studied in macrophages and polymorphonuclear neutrophils of both human and animal origin. Roxithromycin was consistently and significantly more accumulated than erythromycin, reaching intracellular/extracellular concentration ratios between 14 (in polymorphonuclear neutrophils) and 190 (in alveolar macrophages from smokers). Uptake was reversible, insensitive to anaerobiosis and to the presence of an aminoglycoside, but inhibited by acid pH. Upon subcellular fractionation by isopycnic centrifugation in sucrose gradients, half the roxithromycin or erythromycin recovered in cell homogenates was found associated with the lysosomes in macrophages, and about one third with azurophil granules in polymorphonuclear leucocytes. Inasmuch as cellular uptake is a necessary, albeit not sufficient, condition for antimicrobials to kill or inhibit the growth of intracellular bacteria the properties of roxithromycin may give it a distinct advantage over other antimicrobial agents.

Introduction

Few antibiotics markedly accumulate in eukaryotic cells (Tulkens, 1985). The intracellular concentration of β-lactam antibiotics consistently remains lower than their extracellular concentration, probably because of their acidic character (Renard *et al.*, 1987). Aminoglycoside antibiotics are too polar and therefore accumulate too slowly to achieve significant intracellular concentrations during conventional therapy in most cells, except kidney proximal tubular cells (Tulkens & Trouet, 1978; Giurgea-Marion *et al.*, 1986). Accordingly, neither β-lactams nor aminoglycosides are rapidly efficacious against intracellular bacteria. Conversely, erythromycin, rifampicin and clindamycin have been reported to accumulate rapidly in cells (Johnson *et al.*, 1980; Prokesch & Hand, 1982; Martin, Johnson & Miller, 1985). For clindamycin, this accumulation has not been associated with significant activity against potentially sensitive bacteria such as *Staphylococcus aureus* (Sanchez, Ford & Yancey, 1986; Steinberg & Hand, 1986). Conversely, intracellular *S. aureus* is killed by rifampicin, and infections caused by *Legionella* spp., *Listeria* spp. or *Chlamydia* spp., which are intracellular parasites, are effectively treated with erythromycin (Abramowicz, 1984). This effect is, at least partially, related to the drug uptake. We have examined the uptake of roxithromycin by various phagocytic cells in comparison with erythromycin. Because intracellular bacteria are often found in the vacuolar apparatus of phagocytes

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(Moulder, 1985), we have also investigated the subcellular distribution of roxithromycin, along with that of erythromycin since the latter has not yet been examined by quantitative cell fractionation techniques.

Methods

Cells

The following types of macrophages were obtained by standard techniques: (i) *J774* macrophages, a continuous reticulosarcoma cell line of murine origin (Snyderman *et al.*, 1977), which possess many features of mouse macrophages, including the ability to harbour intracellular bacteria and toxoplasma; (ii) mouse peritoneal macrophages stimulated by infection of tryptic soy broth; (iii) human alveolar macrophages obtained by bronchoalveolar lavage in volunteers (smokers and non-smokers).

Polymorphonuclear neutrophils were obtained from (i) hypersideraemic patients undergoing plasmapheresis; (ii) human volunteers; (iii) bovine blood (isolated by the technique of Carlson & Kaneko 1973). Unless stated otherwise, all cells were cultured or maintained at pH 7·2, in appropriate culture medium containing 10% serum and in a humidified 5% CO_2–95% air atmosphere.

Antibiotic accumulation and release

Antibiotic accumulation and release were measured exactly as described by Renard *et al.* (1987). In brief, after suitable incubation in the presence of the antibiotics (followed by further incubation in drug-free medium for release experiments), the cells were quickly washed with ice-cold phosphate buffered saline and the amount of cell-associated radiolabelled antibiotic measured by reference to the cell protein content—determined by the technique of Lowry *et al.* (1951). Unless explicitly stated otherwise, the extracellular concentration of the antibiotics was set at 10 mg/l. The ratio of intracellular to extracellular drug concentration was calculated, with the consideration that 1 mg of cell protein is equivalent to a cellular volume of 5 μl, as reported for peritoneal macrophages (Steinman, Brodie & Cohn, 1976) or cultured fibroblasts (Tulkens, Beaufay & Trouet, 1974). This technique gave similar results to the silicon oil barrier technique (Johnson *et al.*, 1980), while being more reproducible in our hands for adhering cells such as macrophages (Tatzber *et al.*, 1983).

Subcellular fractionation

Cells were homogenized in ice-cold 0·25 M sucrose (macrophages) or 0·34 M sucrose, 0·15 M NaCl (neutrophils) and the homogenates fractionated by differential and isopycnic centrifugation, by the procedures described in detail in Beaufay & Amar-Costesec (1976) and Renard *et al.* (1987). We checked that the homogenization did not cause marked destruction of the cytoplasmic granules such as the lysosomes. Marker-enzymes of lysosomes and azurophil granules constituents were assayed as described in Renard *et al.* (1987) or Steinman, Silver & Cohn (1974).

Antibiotics

Roxithromycin and ^{14}C-labelled roxithromycin (55·4 mCi/mmol) were supplied by Roussel-Uclaf, Paris, France. Erythromycin and ^{14}C-labelled erythromycin

(9 mCi/mmol) were a gift of Abbott Laboratories, North Chicago, Ill. Labelled antibiotic was mixed with the unlabelled drug and used at a specific radioactivity of 1–4 mCi/g. We checked by thin layer chromatography, in two independent systems, that the radiolabelled compounds supplied and the radiolabelled material accumulated by the cells consisted of intact roxithromycin or erythromycin, of more than 95% purity.

Results

Uptake and release

Figure 1 shows that roxithromycin is quickly and avidly accumulated by J774 macrophages upon incubation in the presence of the drug at 37°C. Its steady-state intracellular concentration reaches values approximately ten-fold larger than erythromycin. No significant uptake occurred at 4°C (data not shown). As shown in Figure 2, a similarly larger uptake of roxithromycin, compared with erythromycin, was observed in human alveolar macrophages obtained from smokers. This figure also reveals that the absolute accumulation of both roxithromycin and erythromycin was considerably greater in these macrophages than in J774 macrophages. The ratios between the intracellular concentration (C_i) and the extracellular concentration (C_e) of roxithromycin and erythromycin after 30 min incubation at 37°C in the various cells studied are shown in Table I. Whereas in absolute values the intracellular concentrations of both roxithromycin and erythromycin widely varied from one cell type to another (and were considerably lower in polymorphonuclear neutrophils

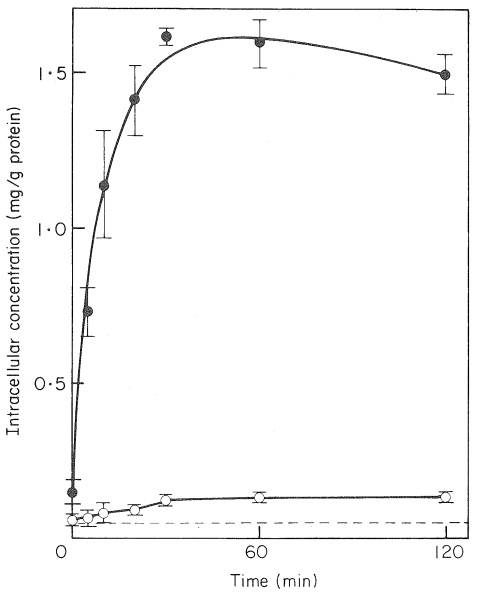

Figure 1. Accumulation of roxithromycin (●) and erythromycin (○) in J774 macrophages. The extracellular concentration of either antibiotic was 10 mg/l. The intracellular content is expressed by reference to the cell protein. Values are the mean (±S.D.) of three independent experiments. The dotted line indicates the ordinate value above which the intracellular concentration of the antibiotics would be equal to their extracellular concentration, assuming a homogeneous subcellular distribution.

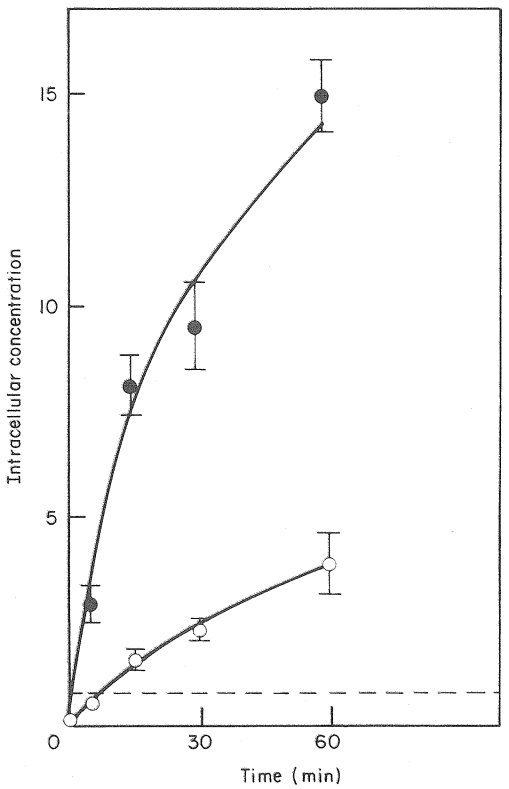

Figure 2. Accumulation of roxithromycin (●) and erythromycin (○) in human alveolar macrophages obtained by broncho-alveolar lavage of smokers. The extracellular concentration of the either antibiotic was 10 mg/l. The intracellular content is expressed as in Figure 1. Values are the mean (±S.D.) of four independent experiments. The dotted line indicates the ordinate value above which the intracellular concentration of the antibiotics exceeds *ten-fold* the extracellular concentration, assuming a homogeneous subcellular distribution.

compared with macrophages), roxithromycin was consistently found more accumulated than erythromycin. The ratios shown in Table I remained largely constant over a wide range of extracellular concentration (up to 50 mg/l), demonstrating no saturability of the transport process (data not shown).

We also investigated a series of clinically-relevant situations which might have influenced the accumulation of macrolides. Anaerobiosis or the presence of an aminoglycoside (tobramycin, up to 50 mg/l) had no effect. Conversely, acid pH significantly decreased both roxithromycin and erythromycin uptake. As shown in Figure 3, this decrease was particularly prominent between pH 8·0 and 6·5. Most noticeably, however, the intracellular concentration or roxithromycin remained higher than its extracellular concentration even at pH 6·0, whereas the intracellular/extracellular concentration ratio of erythromycin fell close to 1, indicating no net accumulation at acid pH.

Roxithromycin and erythromycin accumulated by J774 macrophages were quickly lost from the cells upon reincubation in drug-free medium at 37°C. Such release, however, was almost completely inhibited if reincubation was performed at 4°C.

Table I. Intracellular/extracellular concentration ratios of roxithromycin and erythromycin in phagocytic cells, after 30 min of incubation at 37°C in the presence of 10 mg/l of either antibiotic.

Cell	Intracellular/extracellular concentration ratio[a] roxithromycin	erythromycin
J774	25±5 (12)	4±2 (6)
Mouse peritoneal macrophages	27±2 (3)	—
Human alveolar macrophages (non-smoker)	61±7 (3)	38±10 (3)
Human alveolar macrophages (smoker)	190±21 (4)	46±4 (4)
Human polymorphonuclear neutrophils	14±3 (19)	8±1 (19)
Bovine polymorphonuclear neutrophils	11±3 (6)	—

[a] ±S.D. (n = no. of experiments). The calculation assumes that the drug is homogenously distributed in the cell, which is incorrect (see Figure 5). The real values are therefore higher at the site(s) of storage, and lower in other compartments, but the ratios between cell types and between erythromycin and roxithromycin are probably correct.

Subcellular localization

The latter observation allowed us to prepare cell homogenates at 4°C with reasonable confidence concerning the lack of diffusion of the antibiotics from subcellular storage site(s). Figure 4 shows that the subcellular distribution of both roxithromycin and erythromycin in J774 macrophages was bimodal, with about half of the amount of accumulated antibiotics being soluble (and presumably free in the cytosol) and the other half associated with a subcellular component equilibrating at densities around 1·13–1·18. When a granule fraction, i.e., a homogenate freed of the soluble components, was centrifuged, it became clearly apparent that both roxithromycin and erythromycin distribution patterns were coincident with those of two marker-enzymes of the lysosomes, *N*-acetyl-β-hexosaminidase and cathepsin B (Figure 5). This association was stable and specific, as similar patterns were observed if the homogenates were kept several hours at 4°C before fractionation. Conversely, addition of roxithromycin to a homogenate of cells cultivated in drug-free medium showed the antibiotic to be entirely soluble. Disruption of the lysosomes in homogenates from cells cultivated with roxithromycin (by exposure to hypotonic media or to detergents) solubilized the granule-associated antibiotic. When homogenates of polymorphonuclear neutrophils incubated with roxithromycin were fractionated, about one third of the intracullular drug was associated with the azurophil granules, shown by peroxidase as marker-enzyme (Spitznagel *et al.*, 1974) (data not shown).

Discussion

The present data extend the previous observations made by Martin *et al.* (1985) concerning the uptake, accumulation and egress of erythromycin in tissue culture cells. We have shown that macrolides are significantly accumulated by various phagocytic

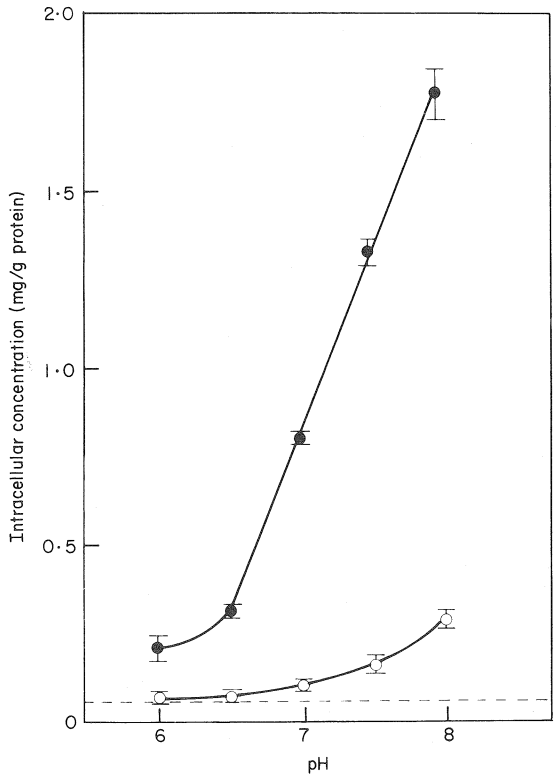

Figure 3. Influence of the pH of the culture medium on the accumulation of roxithromycin (●) or erythromycin (○) in J774 macrophages (30 min incubation; 37°C). The culture medium was prepared without bicarbonate and buffered at each pH with 5 mM sodium monohydrogeno/dihydrogenophosphate. Incubation was carried out in a normal, humidified atmosphere (i.e. without CO_2), in the presence of 10 mg/l of either antibiotic. The intracellular drug content is expressed as in Figure 1. Values are the means (±S.D.) of three independent experiments. The dotted line indicates the ordinate value at which the intracellular concentration of the antibiotics would be equal to their extracellular concentration assuming a homogeneous subcellular distribution.

cells, especially alveolar macrophages. Furthermore, we have demonstrated that these drugs partly localize in lysosomes, the remaining intracellular antibiotic being apparently distributed in the cell cytosol. The accumulation of macrolides in cells is reversible and is inversely proportional to the pH in a physiological range. This behaviour is consistent with the model of intracellular diffusion/intralysosomal segregation of the weak organic bases proposed by de Duve *et al.* (1974) and which also explained the uptake and subcellular distribution of a basic derivative of penicillin G (Renard *et al.*, 1987). This model is illustrated in Figure 6. The larger accumulation observed in macrophages from smokers, and which has also been observed for other basic lipophilic antimicrobials (Hand, Boozer & King-Thomson, 1985) could be related to the marked functional and morphogical alterations of these cells, including the occurrence of very much enlarged lysosomal inclusions (Hocking & Golde, 1979*a*,*b*). The lack of influence of anaerobiosis does not necessarily imply that the accumulation of macrolides is not energy-dependent, since enough ATP could be

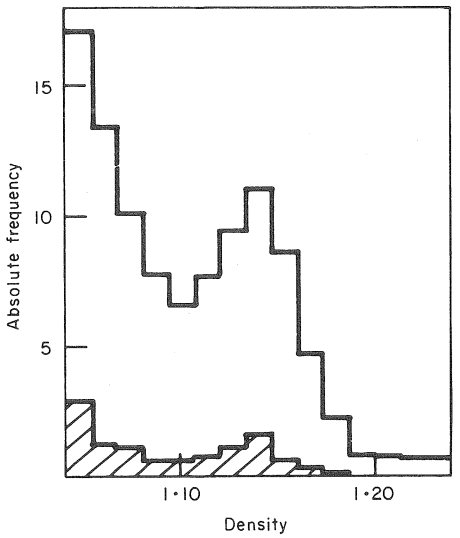

Figure 4. Distribution patterns of roxithromycin (open) and erythromycin (hatched) after isopycnic centrifugation (sucrose gradient) of homogenates (freed from the nuclear fraction) prepared from J774 macrophages incubated 30 min with 10 mg/l of either antibiotic. The fractions to the left correspond to the zone of the gradient where the sample has been deposited, and the antibiotic collected in these fractions is therefore thought to be freely soluble. The ordinate gives the absolute frequency (in mg antibiotic/g cell protein in the homogenate x density increment of each fraction), as defined in Bulychev, Trouet & Tulkens (1978), so that the total area of each histogram is proportion to the actual content of each homegenate in roxithromycin or erythromycin, respectively.

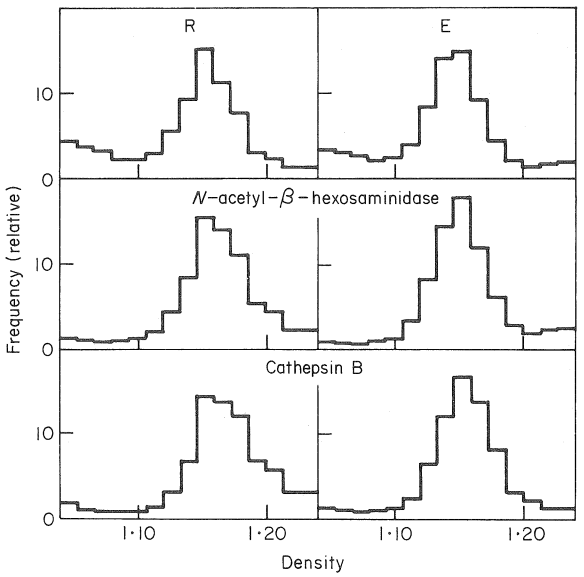

Figure 5. Distribution patterns of roxithromycin (R) and erythromycin (E), and two marker-enzymes of lysosomes after isopycnic centrifugation (sucrose gradient) of a granule fraction prepared from homogenates of J774 macrophages incubated with either antibiotic (10 mg/l). The ordinate is the relative frequency (i.e., the fractional amount of each constituent recovered in each fraction/density increment) as defined in Beaufay & Amar-Costesec (1976) and Renard *et al.* (1987), so that the total *area* of each histogram is equal to 1.

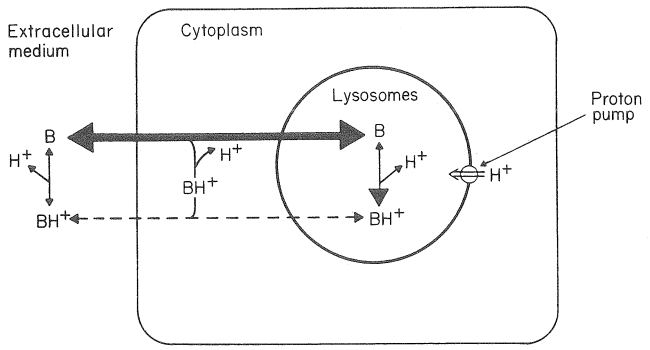

Figure 6. Proposed mechanism of accumulation of macrolides in phagocytic cells (diagram reproduced from Renard *et al.*, 1987, with permission). Macrolides are weak organic bases, and the pericellular and lysosomal membranes are more permeable to their unprotonated (B) than to their protonated (BH^+) form. The cytoplasmic pH is usually 0·5, and the lysosomal pH 2 units of lower than the extracellular milieu (Waddel & Bates, 1969; Ohkuma & Poole, 1978). Thus, weak organic bases tend to be concentrated in lysosomes, and to a lesser extent in the cytosol, under their protonated forms. The ratio between the absolute lysosomal and cytosolic contents depend upon (i) the relative permeabilities of the pericellular and lysosomal membranes to either forms of the base; (ii) the lysosomal/cytosolic pH gradient; (iii) the lysosomal/cytosolic volume ratio; see de Duve *et al.* (1974) for further discussion.

derived from glycolysis for proper maintenance of the acid pH of lysosomes. Experiments which metabolic inhibitors may help to clarify this point. Conversely, the decrease in the extracellular/intralysosomal pH ratio, obtained by cultivating the cells in an acid medium, is anticipated to cause a corresponding decrease in accumulation, as is observed with several other weak bases (Wibo & Poole, 1974).

Further to these observations common to both erythromycin and roxithromycin, the present data also show that roxithromycin is consistently and most significantly more accumulated by all types of phagocytic cells investigated so far. Such constant behaviour indicates that this property is an intrinsic characteristic of the drug. It is probably related both to its slightly higher pK_a (9·2 *vs.* 8·8 for erythromycin) and perhaps also a lower permeability of its protonated form—see discussion in de Duve *et al.* (1974) and in Milne, Scribner & Crawford (1958). More detailed comparative characterization of the diffusion properties of roxithromycin and erythromycin, however, would be needed in this respect.

Intraphagocytic accumulation is a necessary requirement for antibiotics to express their activity against intracellular, sensitive organisms. The constantly larger accumulation of roxithromycin should therefore confer a distinct advantage to this drug over erythromycin in this respect, all other parameters being identical. Since lysosomes do not account for much more than 10% of the cell volume, it can be calculated that the intralysosomal concentration of roxithromycin may reach values as high as 400 to 9000 mg/l for polymorphonuclear neutrophils and human alveolar macrophages, respectively, which is much above the MBC of the drug towards sensitive organisms. The acidic pH, however, and lysosomal chemical and enzymatic environment may decrease the day's activity. Further studies will, however, need to examine whether and to what extent the intracellular roxithromycin enters into contact with the offending organisms, and how much of its antibacterial activity can be expressed in this environment. Of particular interest would be to establish whether roxithromycin or erythromycin or both gain access to phagosomes that contain

pathogenic micro-organisms, which have been reported to fail to fuse with lysosomes (Moulder, 1985).

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