INTRA-CELLULAR ACCUMULATION AND TRANS-EPITHELIAL TRANSPORT OF AIVLOSIN, TYLOSIN AND TILMICOSIN

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Summary

The macrolide antibiotics aivlosin, tilmicosin and tylosin were tested for their ability to enter and accumulate inside a number of mammalian cell types including human gut epithelial cells, pig kidney epithelial cells and pig white blood cells. Aivlosin (3-acetyl-4 isovaleryltylosin) entered and was concentrated in all three cell types and was shown to rapidly (within 10 min.) concentrate in white blood cells. This antibiotic entered polarised human gut epithelial cells (Caco-2 cells) by either the apical or baso-lateral surface, to be concentrated inside the cells and to be transported to the opposite surface. Tylosin entered all cell types relatively poorly, whilst tilmicosin was intermediate in its ability to enter and accumulate in cells. The greater uptake of aivlosin may be related to the presence of an isovaleryl group. Although the three antibiotics are all macrolides, they were shown to differ in at least one aspect of distribution, relevant to their efficacy in treating clinical disease.

Introduction

Macrolide antibiotics are used in commercial pig production. This class of antibiotic is generally effective against mycoplasma, gram +ve bacteria and some gram −ve organisms. *Mycoplasma hyopneumoniae, Brachyspira hyodysenteriae* and *Lawsonia intra cellularis* are three pig pathogens that are susceptible to macrolides.

The ability of an antibiotic to enter cells enables it to be effective against susceptible intra-cellular organisms. Macrolides are generally considered to enter cells and have intra-cellular activity, although the extent to which this happens appears to vary, depending on the particular antibiotic and cell type (Bosnar *et al.*, 2005, Labro, 1993).

The aim of this study was to investigate the uptake and concentration of three macrolide antibiotics in various cell types, representing enterocytes, epithelial cells and white blood cells (WBCs). This investigation used established human gut epithelial cell lines (HRT-18 and Caco-2) as both of these retain some of the properties of *in-vivo* enterocytes. Enterocytes are involved in the uptake of antibiotics from the gut (intestinal lumen). A pig kidney cell line (LLC PK1) was used as another example of an epithelial cell and pig WBCs were also used. The main cell type in pig blood is the neutrophil and these cells are phagocytes and are one of the first cell types to be recruited to a site of infection.

Materials and Methods

Antibiotic stock solutions

Aivlosin was obtained in the tartrate form as granules for oral solution (ECO Animal Health). Throughout this report, aivlosin refers to the active ingredient, 3-acetyl-4"isovaleryltylosin (INN: Tylvalosin). Tylosin, in the form of Tylan Soluble, (ELANCO Animal Health), was also used as the tartrate salt. Tilmicosin, in the form of Pulmotil AC (ELANCO Animal Health), was used as the phosphate salt.

Cell lines

HRT-18, Caco-2 and LLC-PK1 cells were obtained from the European Collection of Cell Cultures (ECACC). HRT-18 cells (an epithelial cell line derived from an adenocarcinoma of human colon) were maintained in RPMI 1640 media containing 10% foetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Caco-2 cells (an epithelial cell line derived from an adenocarcinoma of human colon) were maintained in DMEM supplemented with 10% foetal calf serum, 2 mM L-glutamine and 100 U/mL penicillin and 100 µg/mL streptomycin. LLC-PK1 cells were maintained in Medium 199 containing 10% foetal calf serum, 2mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

Preparation of pig white blood cells

Pig blood was collected, using Heparin-coated BD vacutainers, and the white blood cells (WBCs) were isolated, using a one step Percoll (Sigma) gradient fractionation. A 63% Percoll (v/v) solution was used. A clear band of white blood cells was seen at the Percoll interface. The white blood cells were harvested in phosphate buffered saline (PBS) and centrifuged at 500 x g for 10min. The cell pellet was re-suspended in medium (Glasgow minimum essential medium containing penicillin and streptomycin).

Time and concentration effects for the accumulation of aivlosin in HRT-18 cells

HRT-18 cells, in 6 cm^2 tissue cultures dishes, were incubated in the presence of 0.5, 5 or 50 µg/mL aivlosin. After 4 h., two dishes for each aivlosin concentration and two dishes without antibiotic were removed from the incubator. A sample of medium (about 1 mL) was removed, placed in a labelled Eppendorf tube and stored at -20°C. The rest of the medium was then removed. Each of the cell monolayers was washed twice in ice-cold PBS, using approximately 5 mL for each wash. The plunger from a 2 mL plastic syringe was used to gently scrape off the cells. PBS (500 µL) was added to the dish and the cells gently removed into this medium, using a micropipette. The sample was then placed in an Eppendorf tube and centrifuged at 8000 x g in a bench centrifuge (Biofuge pico,

Heraeus instruments) for 13 sec. An obvious cell pellet formed. The supernatant was removed, using a micropipette and the cell material was stored at -20°C in an Eppendorf tube. After approximately 24 h. the remaining tissue culture dishes were removed from the incubator and treated, as described above, for the 4 h. samples. All the samples, including the 4 h. samples, were then stored at -70 °C and later sent (frozen) for analysis.

Accumulation of aivlosin, tylosin and tilmicosin in HRT-18 cells

HRT-18 cells were prepared, as described above. Stock solutions of antibiotics (either aivlosin, tylosin or tilmicosin) were diluted in growth media in order to give a nominal concentration of 10 µg/mL of each antibiotic in media above the confluent cell monolayers. The cells were incubated at 37°C in a 5 percent CO_2 atmosphere for either 4 h. or 24 h. The media and cells were harvested and tested for antibiotic, as described below.

Intra-cellular accumulation and trans-epithelial transport aivlosin, tylosin and tilmicosin in Caco-2 cells

Caco-2 cells were seeded at a density of 0.8 x 10^6 cells per well of a Transwell Clear filter 6 well plate, 0.4 µm pore size. The cells were incubated with 2 mL medium in both apical and baso-lateral chambers. This medium was replaced every 2 days for 10-14 days. Trans-epithelial resistance was measured, using Millicell ERS apparatus (Millipore) at day 7, 10 and/or 14, in order to identify when the cells had become polarised. Readings of >1000 Ω were indicative of polarised cells.

A first test was undertaken, using aivlosin with both apical and baso-lateral administration, at a higher concentration of 70 µg/ml antibiotic, in order to determine if there was transport to the opposite compartment. A second test was then performed with all three macrolides at 10µg/ml, using apical administration. The different antibiotics were placed in the apical chambers and at various time points, up until 240 min., the medium in both the apical and baso-lateral compartments and the cells were harvested and analysed for macrolide content.

Accumulation of aivlosin, tylosin and tilmicosin in pig kidney cells

Confluent LLC-PK1 cells in 6cm^2 plastic tissue culture dishes were incubated with 10 µg/mL of the three different macrolides. Cells and medium were harvested at 75 and 120 min. and analysed for macrolide content.

Intra-cellular accumulation of aivlosin, tylosin and tilmicosin in pig white blood cells

Pig white blood cells were incubated with 10µg/mL of each antibiotic in an Eppendorf tube. All tubes were placed on a rotary mixing machine, at 37°C. Supernatant and cell samples were harvested at the times described in the figure legends. Two samples (for both cells and medium) were taken for each antibiotic for each time point and later analysed for macrolide content. At the 20 min. time point, two samples were centrifuged and the resulting cell pellet re-suspended in media containing no antibiotic. After a further 30min. incubation at 37°C, the cells were harvested and tested for antibiotic.

Analysis of samples containing aivlosin, tylosin and tilmicosin

All the supernatant and cell samples were transported frozen (at approximately -20°C) to York Bioanalytical Solutions (York, England) for analysis. The concentration of tylosin, tilmicosin or 3-acetyl-4"isovaleryltylosin (aivlosin) was determined, using liquid chromatography with tandem mass spectrometric analysis. The method used in the GLP (good laboratory practice)-compliant laboratory included calibration standards and quality control samples.

Results

Time and concentration effects for intra-cellular accumulation of aivlosin in HRT-18 cells

Initial experiments were carried out in order to examine the effect of increasing concentration over time on the intra-cellular accumulation of aivlosin in HRT-18 cells. As seen in Table 1, there was a rapid (within 4h.) uptake of aivlosin into the HRT-18 cells directly proportional to the concentration

in the medium. The cells accumulated high concentrations of aivlosin – at least 585 µ/g of cells.

The cell:medium concentration ratio was approximately 12:1 for all three initial concentrations of aivlosin in the medium. Similar results were obtained after 24 h. incubation. However, the ratios were lower.

Two wells containing HRT-18 cells were incubated with 0.5, 5 or 50 µg/mL aivlosin for either 4 or 24 h. Samples of medium and cells were harvested. Concentrations of acetylisovalerytylosin in the medium are shown as µg/mL whilst those from the cells are µg/g cells.

Sample no.	Incubation period	Aivlosin medium conc. (ug/mL)	Aivlosin cell conc. (ug/g)	Cell: medium ratio
1	4 hrs	0	0	–
2	4 hrs	0	0	–
3	4 hrs	0.49	6.0	12.24
4	4 hrs	0.53	5.37	10.13
5	4 hrs	5.03	61.6	12.24
6	4 hrs	4.41	57.2	12.97
7	4 hrs	45.4	580	12.77
8	4 hrs	45.4	585	12.88
9	24 hrs	0	0	
10	24 hrs	0	0	
11	24 hrs	0.37	3.36	9.08
12	24 hrs	0.35	2.97	8.48
13	24 hrs	3.94	29.8	7.56
14	24 hrs	4.08	31.6	7.74
15	24 hrs	37.8	270	7.14
16	24 hrs	38.3	307	8.01

Table 1 – Effect of time and concentration on the intra-cellular accumulation of aivlosin in HRT-18 cells

Intra-cellular accumulation of aivlosin, tylosin and tilmicosin in HRT-18 cells

The intra-cellular accumulation of the three macrolides in HRT-18 cells is shown in Fig. 1. Aivlosin entered HRT-18 gut epithelial cells more rapidly than the other two macrolides. An average value of 61.8 µg aivlosin/g cells was detected at 4 h., whilst only an average of 7.24 µg tilmicosin/g cells was detected at the same time point. At 24 h., an average of 57.6 µg aivlosin/g cells was found and

32.45 μg tilmicosin/g cells. At both time points, tylosin did not readily enter HRT-18 cells. After 4 h., aivlosin accumulation resulted in an average value for the concentration within the cells compared to the media of 5.13 fold. Only one of the two tilmicosin cells samples was concentrated (1.32 fold compared to the medium). After 24 h., mean concentrations ratios were similar for aivlosin (5.34 fold) and tilmicosin (4.64 fold). The inability of tylosin to enter HRT-18 epithelial cells meant that the cell:medium concentration ratios were less than 1.
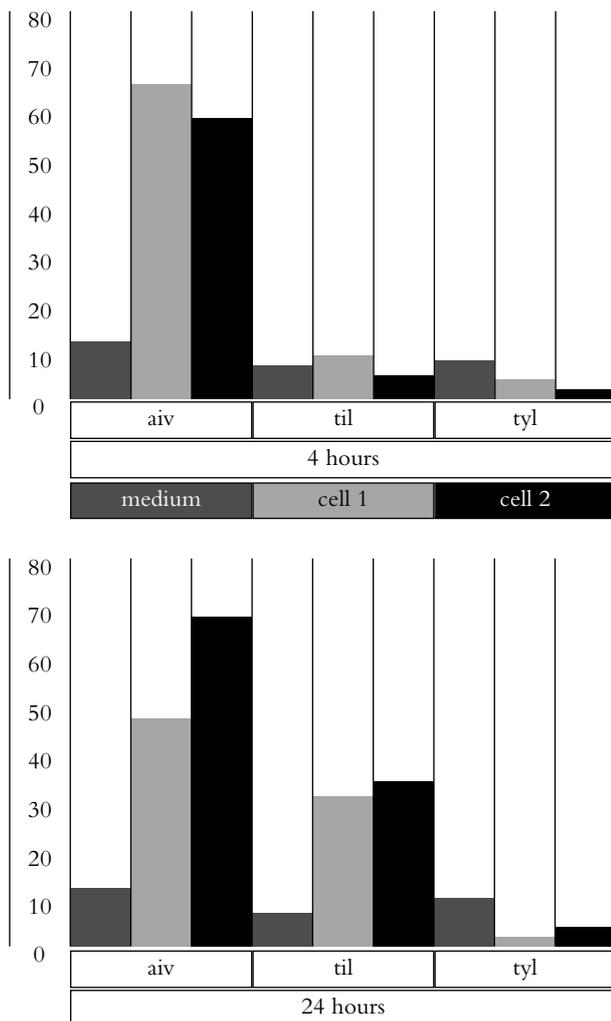

Fig. 1 – Intra-cellular accumulation of aivlosin, tylosin and tilmicosin in HRT-18 cells

(HRT-18 cells were incubated with 10 μg/mL each antibiotic for 4 or 24 h. Two samples of cells were harvested at each time point (light grey and black bars) together with the corresponding two samples medium (average value shown) (dark grey bars). Concentrations in the medium are shown as μg macrolide/ml medium whilst those from the cells are μg macrolide/g cells. Aiv = aivlosin, til = tilmicosin, tyl = tylosin).

Intra-cellular accumulation and trans-epithelial transport of aivlosin in polarised Caco-2 cells

The intra-cellular accumulation of aivlosin in another gut epithelial cell line, Caco-2, was examined. These cells, when grown on permeabilised supports, can differentiate and become polarised: in that they form tight junctions between the cells and this results in the formation of an apical (upper) and baso-lateral (sides and lower) membrane. Fig. 2a shows the concentration of aivlosin in the cells and the medium of the apical and baso-lateral chambers when aivlosin was added to the medium of the apical chamber. Fig. 2b shows the results when aivlosin was added to the baso-lateral chamber. Aivlosin demonstrates rapid entry in polarised Caco-2 cells. An average intra-cellular concentration of 428 μg aivlosin/g cells was detected 30 min. after apical administration (dark grey bars in Fig. 2a) and reached a maximum (590 μg aivlosin/g cells) at 120 min. and then decreased (to 375 μg aivlosin/g cells) at 240 min. A value of 493 μg aivlosin/g cells was obtained 30 min. after antibiotic administration in the baso-lateral compartment (dark grey bars in Fig. 2b). After this time, the intra-cellular concentrations decreased to 155.5 μg aivlosin/mg cells at 240 min.
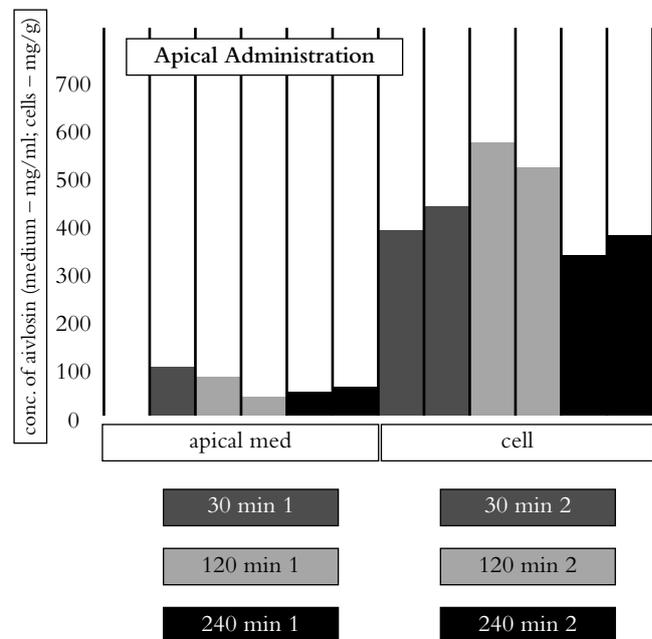

Fig. 2a – Intra-cellular accumulation and trans-epithelial transport of aivlosin in polarised Caco-2 cells showing the concentration of aivlosin in the cells and the medium of the apical and baso-lateral chambers when aivlosin was added to the medium of the apical chamber
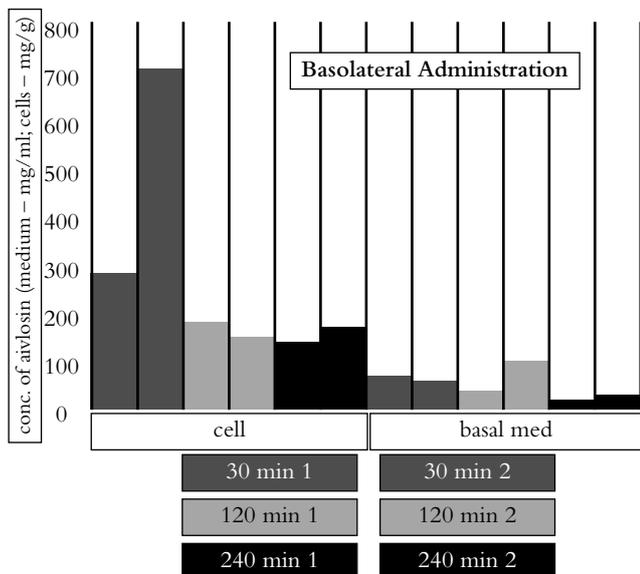
Fig. 2b – Intra-cellular accumulation and trans-epithelial transport of aivlosin in polarised Caco-2 cells showing the results when aivlosin was added to the baso-lateral chamber

(Caco-2 cells were incubated with 70 μg/mL aivlosin in either the apical (a) or baso-lateral (b) chambers for 30 (dark grey bars), 120 (light grey bars) and 240 (black bars) minutes. Two samples of cells and medium from both chambers were harvested at each time point. Concentrations in the medium are shown as μg aivlosin/ml medium whilst those from the cells are μg aivlosin/g cells).

These values relate to a cell:medium ratio of 4.64 after 30 min., 15.58 after 120 min. and 5.85 after 240 min., for apical administration. Aivlosin also demonstrated rapid concentration after baso-lateral administration – an average cell:medium ratio of 7 was observed after only 30 min. After this time though, the ratio decreased to 3.57 after 240 min.

The medium in the chamber opposite to the administration site (i.e. the baso-lateral chamber when aivlosin was added to the apical chamber) was also analysed in order to investigate whether aivlosin could be transported across the polarised epithelial cells. Similar levels of aivlosin were detected, after 240 min, in the opposite chamber – reaching levels of 19% or 22% of the initial concentration of aivlosin present in the administration chamber (apical and baso-lateral, respectively). The increased concentration of aivlosin in the medium of opposite chamber coincides with the decreased levels of aivlosin detected within the cells, suggesting that the decreased intra-cellular concentrations may be due to the aivlosin passing out into the medium on the opposite side of the cells.

The intra-cellular accumulation and trans-epithelial transport of aivlosin, tylosin and tilmicosin in Caco-2 cells

A similar experiment was carried out in order to compare the cellular accumulation and trans-epithelial transport of aivlosin with tylosin and tilmicosin, although only the effect after apical administration was examined. As shown in Fig. 3, aivlosin again showed rapid entry and accumulation, reaching an average concentration of 42.2 μg aivlosin/g cells after 120 min. The intra-cellular concentration of aivlosin at the 240 min. time point was an average value of 34.3 μg aivlosin/g cells. Tylosin did not readily enter polarized Caco-2 cells – after 240 min. only 1.86 μg tylosin/g cells was detected. Tilmicosin demonstrated slower entry and accumulation than aivlosin. The average intra-cellular concentration at 120 min. was 8.6 μg tilmicosin/g cells (compared to 42.2 μg aivlosin/g cells at the same time point). At 240 min. tilmicosin reached an average concentration of 16.05 μg tilmicosin/g cells.
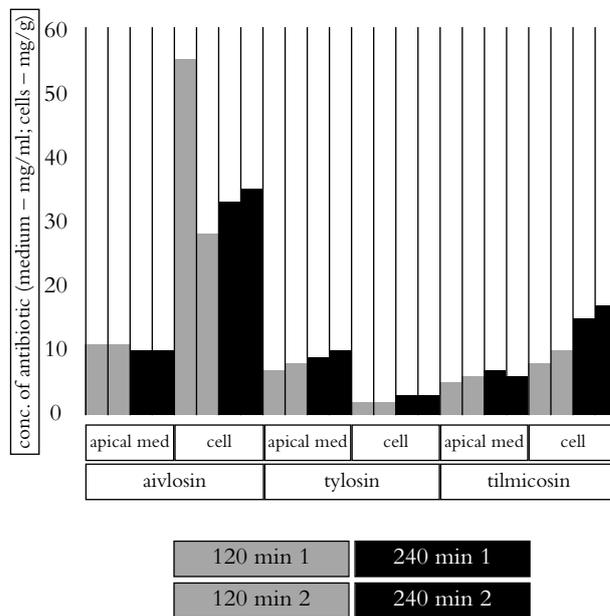

Fig. 3 – Intra-cellular accumulation and trans-epithelial transport of aivlosin, tylosin and tilmicosin in polarised Caco-2 cells

(Caco-2 cells were incubated with 10 μg/mL aivlosin, tylosin or tilmicosin in the apical for 120 (light grey bars) and 240 (black bars) min. Two samples of cells and medium from both chambers were harvested at each time point. Concentrations in the apical medium are shown as μg macrolide/ml medium whilst those from the cells are μg macrolide/g cells)

The cell:medium ratios show that aivlosin rapidly becomes concentrated in the polarized Caco-2 cells. Tylosin did not concentrate in these cells. Tilmicosin showed an intermediate effect, with slower concentration kinetics than aivlosin. After 240 min., there was a 3.49 fold concentration for aivlosin and a 2.5 fold concentration for tilmicosin.

The data indicate a more efficient transport of aivlosin to the baso-lateral fluid than either tylosin or tilmicosin. After 240 min. incubation with aivlosin, the baso-lateral chamber contained an average of 10.48% of the apical chamber value. This compares with an average of 0.52% of the value for tylosin and 1.12% for tilmicosin.

Intra-cellular accumulation of aivlosin, tylosin and tilmicosin in pig kidney cells

The same pattern of antibiotic accumulation was shown, using a pig kidney cell line (LLC-PK 1). Aivlosin rapidly entered and accumulated in these cells – after 75 min., an average concentration of 53.95 µg aivlosin/g cells were detected, compared with 2.41 µg tylosin/g cells and 4.98 µg tilmicosin/g cells (see Fig. 4). Aivlosin reached a maximum cell:medium ratio of 8.9 at 120 min. Tylosin was not concentrated in the LLC-PK1 cells at all and tilmicosin showed 1.7 fold concentration at 120 min.
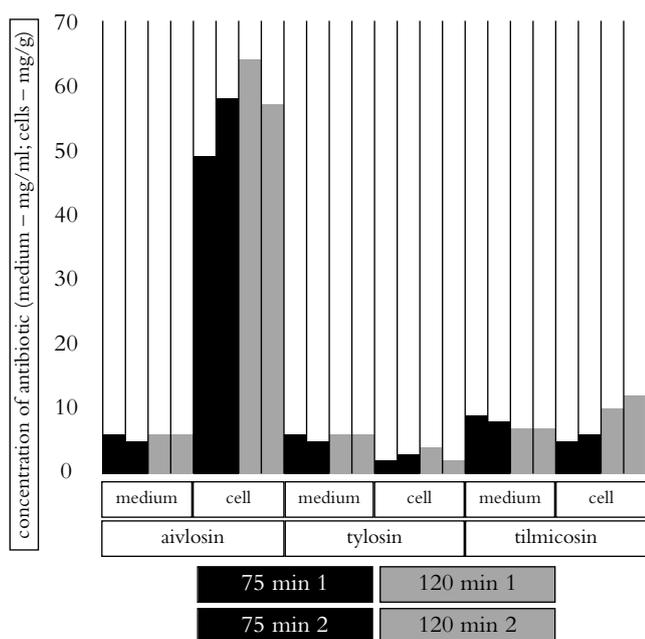
(LLC-PK1 cells were incubated with 10 µg/mL each antibiotic for the times shown in the key. Two samples of cells and medium were harvested at each time point (light grey bars for 75 min. and black bars for 120 min. samples). Concentrations in the medium are shown as µg macrolide/mL medium, whilst those from the cells are µg macrolide/g cells.)

Intra-cellular accumulation of aivlosin and tilmicosin in pig white blood cells

The intra-cellular accumulations of aivlosin and tilmicosin in pig white blood cells were compared. After 10 min., an average concentration of 57.4 µg aivlosin/g cells was detected compared to 31.25 µg tilmicosin/g cells (Fig. 5). Little increase in these values was detected at 20 min. – when 61.45 µg aivlosin/g cells and 36 µg tilmicosin/g cells were detected. The average intra-cellular to extra-cellular ratio was 8.68 for aivlosin, compared to 4.33 for tilmicosin. Similar ratios were found for both antibiotics after 20 min. When the cells laden with antibiotic were left in media without antibiotic, there was a transport out of the cell. The amount retained within the cell was similar for both antibiotics at about 14 to 15%. Hence, the majority of antibiotic was lost (excreted) from the cells under these conditions.
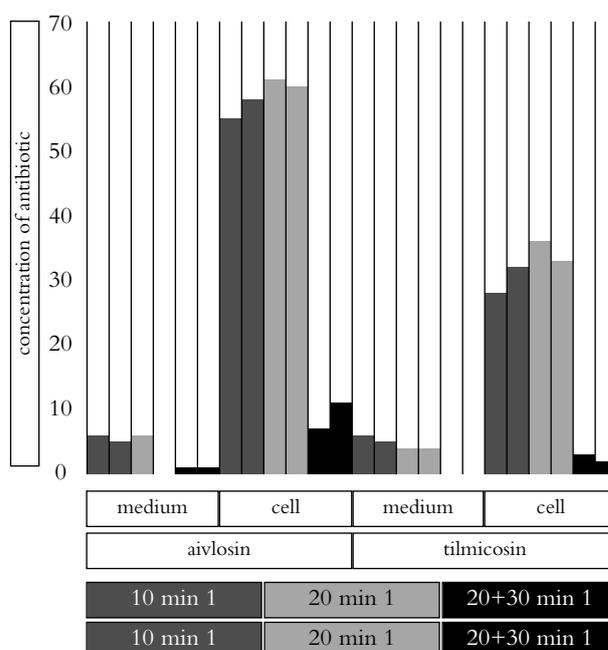

Fig. 4 – Intra-cellular accumulation of aivlosin, tylosin and tilmicosin in pig kidney epithelial cells

Fig. 5 – Intra-cellular accumulation of macrolide antibiotics in pig white blood cells

(Pig white blood cells were incubated with 10 μg/mL aivlosin or tilmicosin for 10 or 20 min. Cells loaded with macrolide after 20 min. incubation were transferred to medium and incubated for a further 30 min. Two samples of cells and medium were harvested at each time point (dark grey bars for 10 min., light grey bars for 20 min. and black bars for 30 min. washout). Concentrations in the medium are shown as μg macrolide/mL medium, whilst those from the cells are μg macrolide/g cells).

Discussion

In this study, the uptake and intra-cellular accumulation of three macrolides – aivlosin, tylosin and tilmicosin was investigated. Aivlosin was able to enter and concentrate to a greater degree than either tylosin or tilmicosin in all cells types. However, the authors' data clearly underestimated the degree of intra-cellular concentration because of the presence of medium between the cells (inter-cellular fluid) (Scorneaux and Shryock, 1999). In addition, macrolides have been shown to localise within acidic cellular compartments, particularly lysosomes (Tulkens, 1991; Carbon, 1995). These compartments constitute a relatively small proportion of the cytosolic volume in epithelial cell lines and localized concentrations of antibiotic will thus be higher.

Initial experiments to study aivlosin accumulation in gut epithelial (HRT 18) cells showed that the antibiotic was concentrated efficiently to levels that depended directly on those in the medium. Further experiments comparing the uptake and concentration of aivlosin, tylosin and tilmicosin in HRT-18 and LLC-PK1 cells demonstrated marked differences in behaviour. The most striking difference was that tylosin entered both cell lines inefficiently and was not concentrated. From the HRT-18 data it might therefore be expected that the uptake into the host through gut epithelial cells is slower. *In vivo* data (Okamoto *et al.*, 1981) have shown that orally administered aivlosin produced higher blood levels than tylosin. There is clearly a marked difference in behaviour between aivlosin and tylosin, despite their closely related molecular structures. The observation (unpublished data) that 3-acetyltylosin, a major metabolite of aivlosin, and tylosin behave in the same way supports the view that the hydrophobic isovaleryl group present in aivlosin (3-acetyl-4"isovaleryltylosin) is important in its efficient uptake and subsequent accumulation

in acidic compartments. Tilmicosin is intermediate in terms of rate of cell entry/accumulation. This may reflect the intermediate hydrophobicity of tilmicosin. The reported pKa values of the drugs are all consistent with protonation and accumulation in acidic compartments such as lysosomes. The failure of tylosin accumulation, even over 24 hours, probably reflects its limited ability to cross both the plasma membrane and intra-cellular organelle membranes.

Investigation of the uptake and excretion of macrolide antibiotics using Caco-2 cells extended the HRT-18 cell work. Caco-2 cells are epithelial cells that exhibit the characteristics of mature enterocytes (Varilek *et al.*, 1994, Kaiserlian *et al.*, 1991; Zweibaum *et al.*; 1983, Sood *et al.*, 1992; Molmenti *et al.*; 1993) and these features make them well suited to evaluate the ability of drugs to pass through epithelia in a directional manner. The results in the present investigation demonstrate a rapid concentration of aivlosin at 30 min. (the first time point used) for both apical and baso-lateral administration. Similar values were obtained using either route of administration, suggesting similar mechanisms of uptake at both surfaces. Aivlosin moved across the cell to the opposite surface and was released into the medium. Thus, antibiotic administered at the apical surface could be detected in the basal medium. This effect increased over time, so that after 4 h. about 20% of the total amount of aivlosin had been transported. This appears to be an efficient process, taking into account the small number of cells involved in the transport. The movement of aivlosin is down a concentration gradient. The mechanism by which this occurs is unknown. Tylosin was not concentrated in Caco-2 cells, tilmicosin showed intermediate behaviour – rapid entry did not take place but, over time, the antibiotic was concentrated intra-cellularly and a modest amount was transported across the epithelium. The *in vitro* work using gut epithelial cells suggests that *in vivo* antibiotic could be transported from the lumen of the gut into the body and also from the body into the gut lumen. This has been shown for the macrolides clarithromycin and azithromycin. Nightingale (1997) reported that azithromycin is eliminated by the hepatic route with some biliary excretion, and is also eliminated directly by secretion into the lumen of the intestine. The latter trans-intestinal route is believed to account for the elimination of 30% to 35% of the total administered dose. The same author stated that clarithromycin undergoes both hepatic and renal

elimination and, in addition, trans-intestinal elimination, which accounts for excretion of about 10% of the total dose of this macrolide.

Previous studies have highlighted the increased uptake and greater accumulation of macrolides by neutrophils and cells of the monocyte/macrophage lineage. Aivlosin was rapidly taken up by pig white blood cells (the majority of which are neutrophils) and after just 10 min. incubation a cell:medium ratio of about nine was obtained. Tilmicosin also rapidly entered porcine WBCs, although the ratio attained was less (about four). A major function of neutrophils is to phagocytose pathogenic organisms. Cells laden with antibiotic may be better able to rid the host of susceptible organisms by increasing the kill capacity within the cell. Also, by releasing antibiotic into the immediately surrounding medium, the cells enable high concentrations of antibiotic to be produced locally, which allows extra-cellular killing to take place. Macrolides have been shown to have effects on the innate immune system (Labro, 2000; Sunazuka *et al.*, 2003) which include reducing inflammation by causing increased apoptosis of neutrophils (Chin *et al.*, 1998) and reducing the recruitment of neutrophils to the site of infection (Ianaro *et al.*, 2000). These effects could also assist the host in preventing the pathology that can be associated with inflammation.

There are several different mechanisms by which a molecule may enter cells. These include passive diffusion (for lipid-soluble molecules) down a concentration gradient, active transport and endocytosis in its various forms (clathrin-mediated, caveolae-mediated, pinocytosis, macro-pinocytosis and phagocytosis, as described by Pelkmans and Helenius, 2003, Stuart and Ezekowitz, 2005). The movement of macrolide antibiotics into cells has been studied, but the information is rather scant. Many of the studies fail to distinguish between the process of crossing the plasma membrane into the cytosol and the process of accumulation in acidified vesicles. It is likely that entry into the cytosol is a passive, concentration gradient-dependent process. The uptake of macrolides has been shown to be greater at alkaline pH. This confirms the view that passive diffusion of uncharged molecules is favoured. Accumulation of macrolides does not take place at 4°C. This is possibly because the proton pumps that acidify endosomes and lysosomes are not active at this temperature, rather than that an active process at the plasma membrane is inhibited, but the observation

that azithromycin is still concentrated in cells pre-treated with metabolic inhibitors (Gladue and Snider, 1990) suggests that a pre-existing pH gradient should be sufficient to allow macrolide accumulation in endosomes and lysosomes. The effect of low temperature may thus be due to a dissipation of the low pH in these organelles or, more probably, to a re-organisation of plasma membrane structure that slows diffusion more dramatically than would be predicted from the normal effect of temperature on diffusion rates. The ability of molecules to cross membranes in the absence of specific channels or transporters is strongly dependent on their ability to 'dissolve' in the lipid phase of the membrane. Hydrophobicity is thus likely to be the key determinant of the efficiency of macrolide entry and accumulation, with pKa values also playing a role in some cases. The properties of aivlosin reported in this publication are consistent with its possession of an isovaleryl group and a pKa of 7.6.

The issues surrounding the relationship between antibacterial activity and efficient cellular accumulation are complex. Carbon (1995) stated that it is very difficult to predict, for a given type of cell and a given type of intra-cellular infection, the actual intra-cellular concentrations which are necessary for an antimicrobial drug to be effective *in vivo*.

A key consideration is, however, the spatial relationship between the bacterium and the cell accumulating the antibiotic. Three types of interaction are of particular interest in the context of macrolides such as aivlosin, tylosin and tilmicosin. The first involves those intra-cellular bacteria, which are capable of survival in the low pH environment of the endosome/phagolysosome. In this case, accumulation of the drug in the acidic compartment should make it highly effective.

The second type of interaction involves escape from a vesicle into the cytosol. In this case, the accumulation of antibiotic in the vesicle will only be of relevance prior to the bacterium's escape, but the vesicle-accumulated material may provide a reservoir of antibiotic for release into the cytosol.

The third form of interaction involves close extra-cellular interaction in which the bacterium typically binds to the plasma membrane of the 'host' cell. In this case also, accumulation will be of relevance only if the cell acts as a reservoir of drug that is slowly released into the surrounding milieu. The properties of aivlosin described in this paper are consistent with efficacy against bacteria that

enter into any of these types of relationships with cells.

The difficulty in extrapolating from *in vitro* properties is illustrated by experiences with other efficiently accumulated, relatively hydrophobic macrolides. Thus, the cellular uptake of azithromicin is relatively high, with uptake slower than that for other macrolides, but this is not paralleled by an increase in efficacy (Labro, 1996). Similarly, high intra-cellular to extra-cellular ratios have been reported for tilmicosin (Scorneaux and Shryock, 1998a,b;1999) but the efficacy is not exceptional, especially at the commercial dosage used, as demonstrated by Reeve-Johnson *et al.*, (1997) for mycoplasmosis in chickens. This antibiotic, that is found in high intra-cellular concentrations and that moves across the cell membrane relatively easily, might have been expected to be highly effective against mycoplasma.

The authors' results using epithelial cells from the pig (PK) gave similar results to those using human (HRT-18, Caco-2). In fact, the results were very clear-cut and once again aivlosin was rapidly concentrated in the cells, whereas tylosin was not concentrated and tilmicosin had intermediate properties.

3-acetyl-4"isovaleryltylosin differs from 3-acetyltylosin (3AT) because it has the isovaleryl group. The ability of aivlosin to rapidly penetrate cells appears to be due to the isovaleryl group, which is shown in the data from this study and also from a previous study (Tsuchiya *et al.*, 1981) that suggested that aivlosin is more lipophilic than either tylosin or 3AT. 3AT is a major metabolite of aivlosin, and is generated within the cell. Presumably, the metabolite has reduced ability to exit the cell, compared to aivlosin. The fact that 3AT is still metabolically active and is likely to remain in the cell longer than the parent molecule might be advantageous clinically.

In conclusion, the data presented in this study show that, even though the antibiotics share a common macrocyclic lactone ring structure, there are marked differences with regards to cellular uptake of the molecules.

Acknowledgements

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