

Influence of outer membrane mutations on susceptibility of *Escherichia coli* to the dibasic macrolide azithromycin

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Azithromycin differs chemically from erythromycin by having an extra positive charge created by the presence of a methyl-substituted nitrogen in the 15-membered macrolide ring. This results in substantially increased potency against Gram-negative bacteria. Therefore, the possibility was considered that azithromycin was taken across the outer membrane of *Escherichia coli* by the self-promoted uptake route, which is utilized by other cationic antibiotics including polymyxins and aminoglycosides. Azithromycin, like polymyxin B and gentamicin, demonstrated equal activity against porin-sufficient and porin-deficient *E. coli* strains but its MIC was increased eight-fold by magnesium supplementation. Nevertheless, an outer membrane-altered mutant DC2 was eight-fold more susceptible than its parent strain UB1005 to azithromycin, indicating that the outer membrane was a permeability barrier to this macrolide. A mutant SC9252 which had an alteration in the self-promoted uptake of polymyxin and gentamicin, was more resistant to azithromycin, polymyxin and gentamicin compared to its parent SC9251. Further azithromycin, like polymyxin B and gentamicin, was capable of weakly permeabilizing cells to the hydrophobic fluorophor 1-*N*-phenyl-naphthylamine, a process antagonized by Mg^{2+} . The monobasic macrolide erythromycin on the other hand was less affected by the SC9252 mutation, less effectively antagonized by Mg^{2+} , and was a far less effective permeabilizer than dibasic azithromycin. These data are consistent with the hypothesis that the improved efficacy of azithromycin compared to erythromycin against *E. coli* reflects its better access to the self-promoted uptake pathway due to its additional positive charge.

Introduction

Azithromycin is a recently developed 15-membered-ring macrolide. The addition of a methyl substituted nitrogen in the macrolide ring makes it dibasic and results in significantly improved Gram-negative potency, while retaining the classical erythromycin spectrum of activity, inhibiting the majority of Gram-positive organisms (Retsema *et al.*, 1987). It has been reported that the mechanism of action of azithromycin is similar to that of erythromycin since both inhibit protein synthesis to a similar extent in a cell free system, are ineffective against altered ribosomes from erythromycin-resistant mutants of *Staphylococcus aureus* and compete for binding to susceptible ribosomes (Retsema *et al.*, 1987). Therefore the 8–32-fold increase in minimal inhibitory concentrations (MICs) of azithromycin compared to those of erythromycin for most Gram-negative bacteria, despite only slightly lower potencies against Gram-positive bacteria, is probably due to differences in uptake. However, to date little

is known about the mechanism of azithromycin (or erythromycin) uptake into bacterial cells.

It is now well understood that hydrophilic antibiotics below a certain size limit can cross the outer membranes of Gram-negative bacteria through the water-filled channels of porins (Hancock, 1987; Hancock & Bell, 1988). However, azithromycin is bulky and has a molecular weight of 747 that exceeds the exclusion limit of *Escherichia coli* porins suggesting that it would be unlikely that azithromycin is efficiently taken up via the porin pathway. Furthermore, although azithromycin has limited water solubility and might be described as moderately hydrophobic, both *E. coli* and *Salmonella* spp. which lack a hydrophobic antibiotic uptake pathway (Nikaido, 1976; Hancock & Bell, 1988), are moderately susceptible to azithromycin (MICs of 1–4 mg/L; Retsema *et al.*, 1987). Therefore, since the increased Gram-negative potency of azithromycin correlates with increased basic character (Retsema *et al.*, 1987), in this study a variety of outer membrane mutants were examined in an attempt to clarify the possible mechanism of uptake.

Methods

Strains and culture conditions

All *E. coli* strains are listed in Table I and in Hancock *et al.* (1991) except for parent strain UB1005 and its antibiotic supersusceptible derivatives DC1 and DC2 (Clark, 1984; Rocque *et al.*, 1988) obtained from Dr Estelle McGroarty, Michigan State University. *E. coli* UB1636 *trp his rpsL lac lamB*(RP1) was provided by Dr P. M. Bennett (Department of Bacteriology, University Bristol, Bristol, UK). All strains were grown in Luria broth (1% bacto-tryptone, 0.5% yeast extract) with or without the additions stated in the text.

Antibiotics and determination of susceptibility

Azithromycin was supplied by Dr J. Retsema, Pfizer Central Research, Groton, CT. Gentamicin, erythromycin and polymyxin B were purchased from Sigma Chemical Co. (St. Louis, MO). Ceftazidime was provided by Glaxo Group Research, Greenford,

Table I. Susceptibility of strains of *E. coli* with altered outer membranes

Strain	Relevant characteristics	Reference	MIC (mg/L)				
			AZM	ERY	PX	GM	CAZ
UB1005	parental	Clark (1984)	4	64	0.25	1	0.5
DC1	antibiotic supersusceptible	Clark (1984)	2*	16*	0.25	0.5	1.0
DC2	antibiotic supersusceptible	Clark (1984)	0.5*	2*	0.25	2	0.5
CGSC 6043	parental	CGSC ^b	8	64	0.25	2	0.125
CGSC 6047	OmpC ⁻	CGSC	4	64	0.25	2	0.125
CGSC 6044	OmpF ⁻	CGSC	4	32	0.25	2	0.5*
SC 9251	parental	Peterson <i>et al.</i> (1987)	4	64	0.25	1	0.5
SC 9252	polymyxin resistant	Peterson <i>et al.</i> (1987)	16*	128	16*	4*	0.5

*Indicates significantly different from parental strain ($P < 0.05$ by Fisher's exact test).

AZM, azithromycin; ERY, erythromycin; PX, polymyxin B; GM, gentamicin; CAZ, ceftazidime.

^bObtained from the Coli Genetic Stock Centre, Yale University, CT.

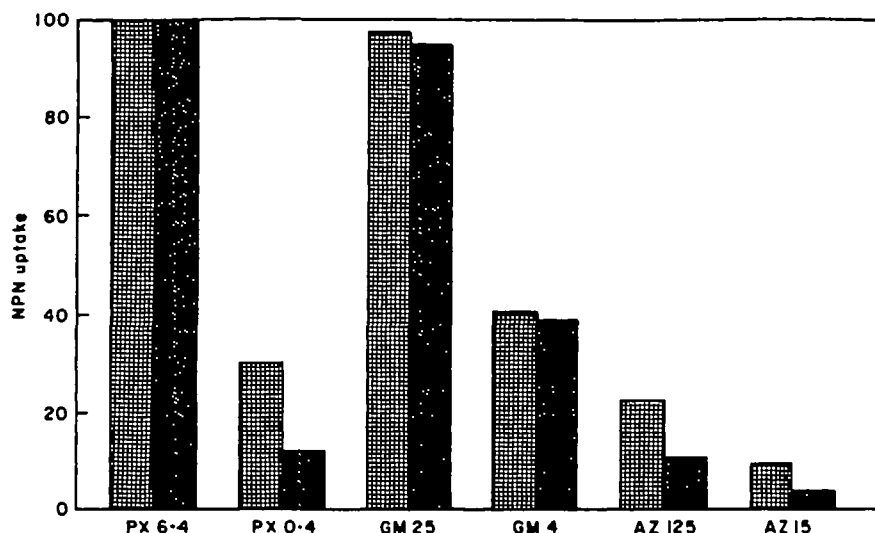


Figure. Uptake of 1-*N*-phenyl-naphthylamine (NPN) into *E. coli* strains UB1636 (▨) and SC9251 (■) after treatment with various concentrations (given in mg/L on the x-axis) of polymyxin B (PX), gentamicin (GM) or azithromycin (AZ). Results presented are the means of 2–3 independent experiments with standard deviations between 7% (for the higher results) and 50% (for the lower results). Uptake was recorded as fluorescence enhancement in arbitrary units due to entry of NPN into the hydrophobic interior of the cell membranes after a stable level of fluorescence was achieved (typically 10–15 sec after antibiotic addition). Background due to weak fluorescence of NPN in the aqueous phase and intrinsic cell fluorescence (usually around ten units on the y axis scale) was subtracted.

England. Azithromycin and erythromycin stock solutions were prepared in 95% ethanol. The minimum inhibitory concentrations (NK) of each agent for each strain was determined by the standard agar dilution or broth micro-dilution methods using inocula of 10^4 cells per spot or well, as described previously (Angus, Fyfe & Hancock, 1987). All MIC determinations were performed between seven and nine times and the data presented as the median MIC. Fisher's exact test (2×2 contingency table chi-square analysis) was used for comparing discrete data.

Permeabilization

A slight modification of the procedure of Hancock *et al.* (1991) was utilized. *E. coli* strain UB1636 was grown to an absorbance of 0.5 at 600 nm, then centrifuged at 3000 g for 10 min and resuspended in 5 mM sodium hydroxyethylpiperazine ethanesulphonate (HEPES) buffer pH 7.2, containing 50 μ M carbonyl cyanide *m*-chlorophenylhydrazine, at the same absorbance, then washed again by centrifugation and resuspended at the same absorbance in the same buffer solution. One millilitre of this bacterial suspension was placed in a glass cuvette and inserted into the cuvette holder of a Perkin-Elmer 650 10S spectrofluorometer. 1-*N*-phenyl-naphthylamine (NPN) from a 500 μ M stock solution in acetone was added to a final concentration of 10 μ M, and the background fluorescence, due to binding of NPN to the cell surface, intrinsic bacterial fluorescence and the intrinsic low fluorescence of NPN in aqueous solution, was recorded at an excitation wavelength of 350 nm (slit width = 5 nm) and an emission wavelength of 420 nm (slit width = 5 nm). The background fluorescence, which

remained at a stable level for at least 10 min was typically approximately ten arbitrary units on the scale utilized in the Figure. Subsequently, a potential permeabilizing compound was added to the cuvette and the increased fluorescence emission recorded as a function of time until a plateau was reached. Control experiments were performed to demonstrate that enhanced fluorescence was due to uptake of NPN into cells, as described previously (Loh, Grant & Hancock, 1984). Briefly, cells were removed by centrifugation as above and the residual NPN in the supernatant determined by the fluorescence yield after addition of 3% Triton X-100. Experiments in which the effect of magnesium ions was measured were performed by the pre-addition of $MgCl_2$ to a final concentration of 1 mM before addition of the permeabilizing antibiotic.

Results

Effect of outer membrane mutations and magnesium ions upon antibiotic susceptibility

There was no significant difference in the MIC of azithromycin, erythromycin, polymyxin or gentamicin for the OmpC-mutant (CGSC6047) or the OmpF-mutant (CGSC6044) compared to their parent strain (CGSC6043) (Table I). In contrast the OmpF-mutant showed the expected four-fold increase in the MIC of the β -lactam ceftazidime. Conversely, the outer membrane-altered, antibiotic supersusceptible mutants, DC1 and DC2, were two- to eight-fold more susceptible to azithromycin and four- to 32-fold more susceptible to erythromycin than their parent (Table I). The LPS-altered mutant SC9252 had MICs of azithromycin and gentamicin that were four-fold higher and of polymyxin 64-fold higher than the MICs for its parent strain SC9251 (Table I).

Inclusion of 5 mM $MgCl_2$ in the broth used for the determination of MICs caused an eight-fold increase in the MIC of azithromycin for *E. coli*, like strain UB1636 (Table II). This was due to Mg^{2+} , and not due to the anion Cl^- or the effect of increased ionic strength, since 80 mM NaCl only increased the MICs of azithromycin two-fold. $MgCl_2$ had a similar effect on MICs of polymyxin B, whereas MICs of gentamicin and erythromycin were increased four-fold. $MgCl_2$ and NaCl had little or no effect upon susceptibility to the β -lactam ceftazidime.

Azithromycin as a permeabilizer

Addition of polymyxin B, gentamicin or azithromycin to bacterial cells, subsequent to NPN addition, led to increased NPN fluorescence (Figure) due to the enhanced uptake

Table II. Influence of added magnesium ions or salt on susceptibility of *E. coli* UB1636 to selected antibiotics in Luria broth

Antibiotics	MIC (mg/L)		
	no addition	+ 5 mM $MgCl_2$	+ 80 mM NaCl
Azithromycin	2	16	4
Erythromycin	32	128	64
Polymyxin B	0.5	4	1
Gentamicin	1	4	2
Ceftazidime	0.25	0.25	0.5

of NPN into cells. For each of these agents, higher concentrations of antibiotics caused a greater increase in fluorescence. Since the extent of NPN uptake increased with increasing antibiotic concentration but the rate of uptake did not, it was assumed that the kinetics of permeabilization was rapid and that the extent of NPN uptake reflected either accessibility to certain binding sites or an equilibrium between NPN uptake and excretion from cells as discussed previously (Loh *et al.*, 1984). Regardless of the explanation, it must be stressed that the known outer membrane permeabilizer polymyxin B caused similar effects (Figure). Addition of 1 mM Mg²⁺ decreased the fluorescence enhancement due to addition of 100 mg/L azithromycin, 25 mg/L gentamicin or 6.4 mg/L polymyxin B by 74%, 100% and 15% respectively. Erythromycin failed to enhance NPN uptake at concentrations less than 500 mg/L.

Discussion

The data described in this paper indicate that the activity of the macrolides azithromycin and erythromycin is diminished by the outer membrane permeability barrier of *E. coli*, since mutants DC1 and DC2, which have an altered LPS (Rocque *et al.*, 1988) resulting in increased permeability, were more susceptible than their parent strain, UB1005 (Table I). This result was consistent with the hydrophobic nature of these antibiotics since DC1 and DC2 show relatively greater increases in susceptibility to more hydrophobic compounds (Clark, 1984) (cf. the minimal effects for the water-soluble antibiotics gentamicin, polymyxin B and ceftazidime in Table I). Since specific mutations in the *E. coli* outer membrane resulted in increased susceptibility, this implied that the outer membrane acted as a permeability barrier to azithromycin and erythromycin uptake. These data together with the higher activity (lower MICs) of azithromycin than erythromycin against *E. coli*, despite similar abilities to inhibit protein synthesis (Retsema *et al.*, 1987), suggest that the *E. coli* outer membrane serves as a great barrier towards erythromycin, or conversely that azithromycin passes across the *E. coli* outer membrane more efficiently than erythromycin.

Thus, it was of interest to determine the basis for this differential activity, i.e. the mode of uptake of these antibiotics in *E. coli*. Azithromycin, like the related antibiotic erythromycin, appeared to be rather bulky relative to the estimated size (approximately 1.16 nm in diameter; Hancock, 1987) of *E. coli* OmpF and OmpC porin pores. To confirm this, a space-filling molecular model of azithromycin was constructed using the program Alchemy (TRIPOS Associates, St. Louis, Mo). This revealed that azithromycin had a cuboid structure of approximate dimensions 1.3 × 1.0 × 0.92 nm and would thus be expected to pass extremely slowly through OmpF or OmpC porin channels. Similar dimensions were predicted for erythromycin. In addition, the poor water solubility of azithromycin and erythromycin would argue against utilization of the porin pathway. Consistent with this, porin deficient mutants showed no decrease in susceptibility to these macrolides (Table I). Thus these data strongly imply that azithromycin and erythromycin are unable to pass efficiently through the channels of the major porins of *E. coli*. In addition, it has been proposed that most *E. coli* strains do not have an uptake pathway for hydrophobic compounds (Hancock & Bell, 1988), a result compatible with the inability of *E. coli* K-12 wild type strains to take up the hydrophobic fluorophore NPN in the absence of permeabilizers (Hancock *et al.*, 1991) and the high resistance of such strains to hydrophobic antibiotics (Clark, 1984; Hancock & Bell, 1988; Rocque *et al.*, 1988).

The observation that the extra positive charge on azithromycin compared to erythromycin correlated with increased Gram-negative potency (Retsema *et al.*, 1987), suggested the possibility that azithromycin was utilizing the self-promoted uptake pathway to cross the outer membrane. Self-promoted uptake has been shown to be the route of uptake of numerous polycations including the aminoglycosides and polymyxins in *E. coli* and *Pseudomonas aeruginosa* (Hancock & Bell, 1988; Hancock *et al.*, 1991). The self-promoted uptake hypothesis proposes that subsequent to the initial interaction of polycationic antibiotics with divalent cation binding sites on LPS, that these compounds competitively displace divalent cations and bind to the LPS. This is then proposed to increase the permeability of the outer membrane to the polycationic antibiotic (hence the name self-promoted uptake). There is substantial support for this hypothesis based on studies with mutants, and biophysical and biochemical assays (Hancock & Bell, 1988).

The data from three types of experiments in this study were consistent with the proposal that azithromycin crossed the outer membrane by the self-promoted uptake route. Firstly, the MIC of azithromycin was increased four-fold (Table I) by a mutation in SC9252 influencing the LPS, such that it had reduced affinity for polymyxin B and gentamicin, compared to the parental strain SC9251 (Peterson *et al.*, 1987). Secondly, magnesium ions were able to considerably decrease the MICs of azithromycin (Table II), consistent with magnesium ions and azithromycin competing for divalent cation binding sites on LPS molecules at the surface of *E. coli*. Thirdly, azithromycin was able, albeit weakly, to permeabilize the outer membrane of *E. coli* to NPN, an effect that could be inhibited by addition of magnesium ions (Figure). Similar results were obtained with polymyxin B and gentamicin, compounds known to utilize the self promoted uptake pathway in *E. coli* (Hancock *et al.*, 1984; Hancock & Bell, 1988).

Azithromycin was clearly less effective than polymyxin or gentamicin at increasing NPN uptake, since higher concentrations were required and a lower maximal uptake was observed (Figure). Although the data in the Figure were obtained for concentrations four-fold or more higher than the MIC, it should be noted that small but reproducible effects were seen at the MIC. In addition the time span of these experiments (conducted within 1 min or less) was substantially lower than the times required to kill cells at the MIC concentration (e.g. Retsema *et al.*, 1987), since it was necessary to ensure that initial interaction kinetics were being observed rather than some secondary effect of antibiotic action on cells. Nevertheless, the order of effectiveness at permeabilizing the outer membrane was the same as the order of effectiveness in killing *E. coli* (Table I) i.e. polymyxin B > gentamicin > azithromycin.

It must be noted, however, that although azithromycin permeabilized the outer membrane weakly to NPN, this does not *per se* imply that it is equivalently weak at promoting its own uptake across the outer membrane, as required by the self-promoted uptake hypothesis. For example a molecule of azithromycin might bind at an LPS site then be displaced by a second azithromycin molecule such that the first molecule crosses the hydrophobic barrier of the outer membrane. Thus azithromycin molecules might promote their own uptake but not necessarily efficiently promote uptake of other molecules.

The proposal that the dibasic macrolide azithromycin is taken up by the self-promoted uptake pathway is consistent with its greater activity against *E. coli* compared to its monobasic parent compound erythromycin (Retsema *et al.*, 1987). Thus, erythromycin was less affected by the mutation affecting the LPS of *E. coli*

SC9252, less effectively antagonized by magnesium ions, and was a far less effective permeabilizer than azithromycin. Thus, it is proposed that the added positive charge on azithromycin permits better interaction than that of erythromycin with the self-promoted uptake pathway. Interestingly, a tribasic compound C11, synthesized at the same time as azithromycin, has even lower MICs than azithromycin (Bright *et al.*, 1988). Preliminary experiments (since this compound was in very short supply) indicated that it was a more effective permeabilizer than azithromycin.

Acknowledgements

This work was supported in its initial stages by the Medical Research Council of Canada, and subsequently by the Networks of Centres of Excellence in the form of a grant to the Canadian Bacterial Diseases Network. The authors wish to acknowledge Jim Retsema, who first suggested that azithromycin might be taken up via the self-promoted uptake route.

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(Received 21 June 1991; revised version accepted 23 August 1991)

