Accumulation of trospectomycin by strains of *Salmonella typhimurium*, *Escherichia coli* and *Haemophilus influenzae*

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ABSTRACT: Trospectomycin, unlike aminoglycosidic aminocyclitols, is accumulated by a nonsaturable, energy-independent, diffusional process in *Salmonella typhimurium*, *Escherichia coli* and *Haemophilus influenzae*. A deep rough mutant of *S typhimurium* was more susceptible and accumulated the drug faster, and F porin deficient mutants of *E coli* were more resistant than parental strains. Trospectomycin likely uses both porin and nonporin pathways to cross the outer membrane. An *E coli* strain effectively accumulated the drug anaerobically, explaining its anaerobic activity. An *H influenzae* strain accumulated trospectomycin at concentrations below those for which detectable uptake could be observed with *E coli* or *S typhimurium* strains, consistent with greater activity in *Haemophilus* species.
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Key Words: *Aminocyclitols, Trospectomycin, Uptake*

TROSPECTOMYCIN OR 6′ PROPYLSPECTINOMYCIN IS A derivative of spectinomycin with four- to 32-fold increases of activity compared to spectinomycin against several bacteria including *Haemophilus influenzae*, *Branhamella catarrhalis*, *Neisseria gonorrheae*, *Proteus* species, *Bacteroides* species, *Clostridium* species (including *C difficile*) and *Chlamydia trachomatis*. The two drugs show comparable activity against the enterobacteriaceae and generally exhibit cross-resistance (1).

Trospectomycin, spectinomycin and aminoglycosides are aminocyclitols which differ in several respects. One is the much greater antibacterial activity of aminoglycosidic aminocyclitols on aerobic bacteria in general versus anaerobic organisms, whereas the spectinomycins do not show this pattern of activity (1,2). The greater activity of aminoglycosidic aminocyclitols on

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aerobic bacteria is due to their more effective accumulation by bacteria carrying out electron transport which develop an electrical potential greater than the threshold level needed for aminoglycoside uptake (3,4). Both classes of antibiotics inhibit protein synthesis, although they differ in that the spectinomycins do not cause misreading, while the aminoglycosides do (5). This is not a sufficient reason to account for the difference in activity on aerobic and anaerobic bacteria as protein synthesis proceeds in both circumstances. Earlier studies reported that mutants of *Escherichia coli* defective in aspects of electron transport showed increased resistance to aminoglycosides but not to spectinomycin (6). This finding suggests that the uptake process for the spectinomycins might be different from that for the aminoglycosides. The authors have studied the nature of the transport process for trospectomycin as a representative aminocyclitol to determine if it is significantly different from that described for aminoglycoside aminocyclitols and whether or not it could account for the reported differences in activity on anaerobic bacteria. They also examined mutations affecting porin or lipopolysaccharide structure of salmonella or *E coli* to obtain information on the pathways used by trospectomycin to cross the outer membrane. These studies on trospectomycin uptake under a variety of conditions show that the uptake of this compound is by simple diffusion, consistent with its activity in anaerobic bacteria.

MATERIALS AND METHODS

Bacterial strains: The strains used in this study were obtained from KE Sanderson, University of Calgary (*S typhimurium* LT2 SL3770, *S typhimurium* LT2 SL3769 and *S typhimurium* LT2 SL1102, *E coli* SA1306), RK Poole, University of British Columbia (*E coli* JF568, *E coli* JF694, *E coli* JF701, *E coli* JF703), BJ Bachmann, *E coli* Genetic Center, Yale University, New Haven, Connecticut (*E coli* AB2495) and from the present authors' laboratory (*H influenzae* Rd).

Reagents: Tritiated [^{3}H]-trospectomycin sulphate (63.6 mCi/mmol) and trospectomycin sulphate were provided by Dr Donald Batts at the Upjohn Company (Kalamazoo, Michigan). Unlabelled and [^{3}H]-trospectomycin were confirmed to be identical in bioactivity by identical zone sizes produced by 30.5 µg of each material spotted to 6.5 mm paper discs and incubated overnight with a lawn of *E coli* SA1306. Labelled and unlabelled compounds produced identical patterns on thin layer chromatography with CEL300-25 cellulose plates using *n*-butanol:water:acetic acid in ratios of 12:5:3 and iodine vapour as the developing reagent. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was supplied by the Sigma Chemical Co (St Louis, Missouri).

Media and MICs: Media used were brain heart infusion broth from Difco Laboratories (Detroit, Michigan) and nutrient broth from BBL Microbiology Systems (Cockeyville, Maryland). Minimum inhibitory concentrations (MICs), were determined in brain heart infusion broth, nutrient broth and nutrient broth with 10 mM magnesium chloride using an inoculum of 10^5 cells/mL and incubating tubes without shaking at 35°C for 16 h. All studies on *H influenzae* used supplemented brain heart infusion broth containing CVA enrichment (10 mL/L) and hemin (10 µg/mL).

Uptake studies: The basal medium used for uptake studies was nutrient broth. Cells were grown at 37°C overnight, a 1:10 dilution subcultured into fresh media and the incubation continued in 50 mL volumes until the A_{600} reading reached 0.4. Cells were collected by centrifugation and resuspended at a concentration of 30 A_{600} units/mL. The suspension was incubated in a block heater at the temperature to be used for uptake for 7 mins with shaking, and thereafter the appropriate amount of trospectomycin was added using a range of one part labelled drug to nine parts unlabelled, and one part labelled to 19 parts unlabelled, for final concentrations of 64 µg/mL or greater. For lower concentrations the ratio was between 1:3 and 1:6.

At the various times specified, 80 µL samples were added to 2 mL of the same broth as rapidly as possible and filtered through Whatman GF/F microfibre glass filters, and a further 2 mL of broth was pipetted over the area of the filter occupied by the filtered cells. Filters were dried and counted in Beckman Ready Safe liquid scintillation cocktail in a Beckman LS6800 scintillation counter. Uptake is reported as ng trospectomycin/mg dry weight of cells. For uptakes using dead cells the cells were held at 70°C for 30 mins before concentration to 30 A_{600} units/mL. No survivors were detected after this treatment using a dilution series capable of detecting 10 bacteria/mL. Uptake of *H influenzae* was performed using supplemented brain heart infusion broth. Anaerobic uptake was carried out with *E coli* SA1306 grown at 37°C using an atmosphere of 95% nitrogen and 5% carbon. Cells were collected by centrifugation at 4°C and resuspended in nutrient broth through which 100% nitrogen gas was bubbled.

Samples were taken as above under an atmosphere of 100% nitrogen.

Other methods: All strains were confirmed to carry their lipopolysaccharide or porin phenotype using the methods for analysis of lipopolysac-

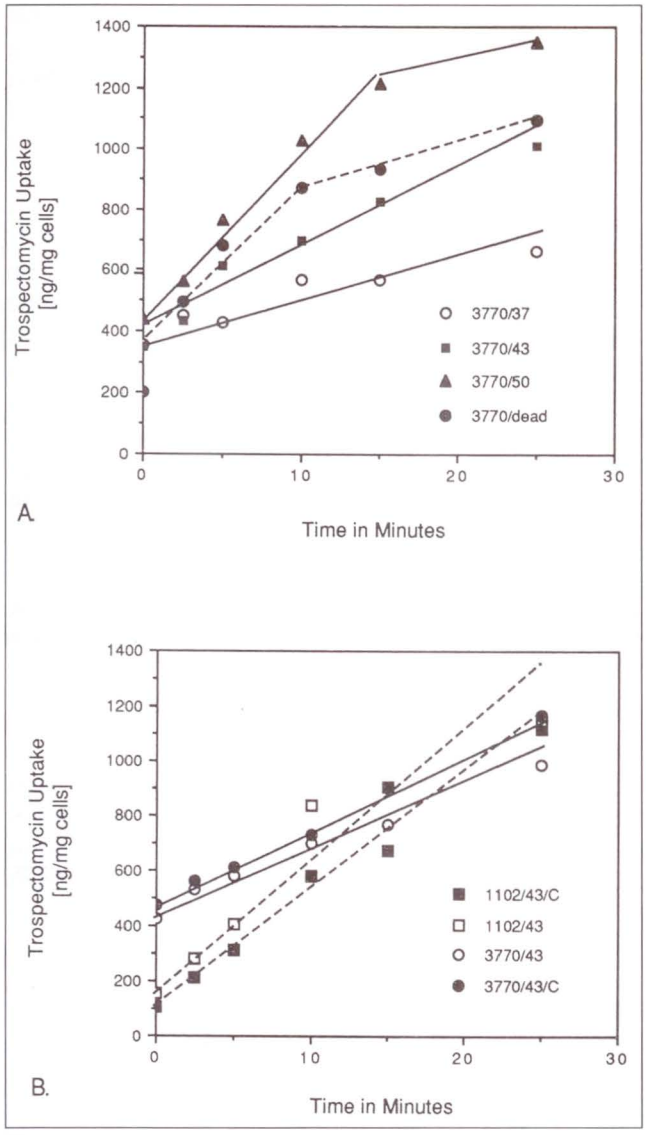

Figure 1) *Uptake of trospectomycin at a concentration of 128 µg/mL in nutrient broth of* **A** Salmonella typhimurium *LT2 SL3770 at 37°C (open circles), 43°C (closed squares) and 50°C (closed triangles) and of heat-killed SL3770 at 37°C (closed circles, broken line) and* **B** S typhimurium *LT2 SL3770 (circles) and SL1102 [deep rough] (squares, broken lines) at 43°C with 40 µM carbonyl cyanide* m-chlorophenylhydrazone *(CCCP) added 2 mins before addition of trospectomycin (closed circles or squares) or without CCCP (open circles or squares)*

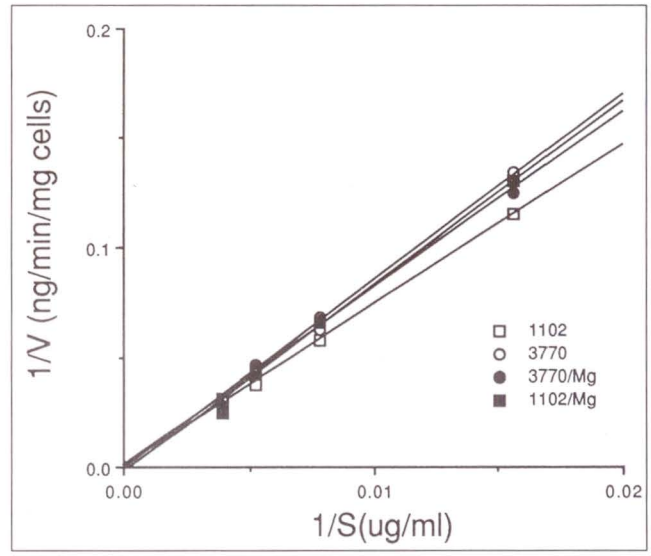

Figure 2) *Lineweaver-Burk plot of 1/V (ng trospectomycin/min/mg dry weight of cells) based on the initial 10 mins of uptake in nutrient broth at 43°C with 10 mM magnesium chloride (closed circles or squares) or without any addition (open circles or squares) versus 1/S (concentration of trospectomycin in mg/mL) for* Salmonella typhimurium *LT2 SL3770 (circles) [parent] and LT2 SL1102 (squares) [deep rough mutant]. Lines were drawn using the least squares method. As this is a reciprocal plot, faster uptake is shown as the line with the lower slope*

charide and outer membrane proteins described by Bryan et al (7).

RESULTS

The patterns of trospectomycin accumulation at 37 or 43°C by *S typhimurium*, *E coli* and *H influenzae* were linear with time for at least 20 to 25 mins. More extensive studies using *S typhimurium* LT2 strain SL3770 showed that the rate of accumulation increased with the temperature used for uptake determination, although the rate levelled off after 15 mins when uptake was done at 50°C (Figure 1). If it is assumed that all of the drug accumulated after zero time was intracellular, and that there is 1.6 µL cell water/mg cells (8), the intracellular concentrations at 37, 43 and 50°C were 180, 360 and 560 µg/mL cell water, respectively, or about 1.4, 2.8 and 4.4 times the extracellular concentration. Heat-killed cells showed excellent uptake at 37°C with the initial uptake rate faster than in viable cells at 37 or 43°C, demonstrating that an energy source was not necessary for accumulation. This was confirmed by the failure of CCCP to reduce uptake in *S typhimurium* LT2 SL3770 or SL1102 (Figure 1). Accumulation of trospectomycin by *S typhimurium* LT2 strains 3770 or 1102 at different drug concentrations gave a pattern of nonsaturability when the data were plotted by the Lineweaver-Burk method (Figure 2). Other tested circumstances where uptake was also not saturable were with *E coli* SA1306 at 43°C and *H influenzae* Rd at 37°C (data not shown).

Comparison of uptake by smooth *S typhimurium* SL3770 which possesses repeating o-somatic side chain subunits of lipopolysaccharide (Ra chemotype) compared to the deep rough mutant *S typhimurium* SL1102 (Re chemotype),

TABLE 1
Minimal inhibitory concentrations (MICs) of trospectomycin for bacterial mutants in lipopolysaccharide chain length and porins

Bacteria	MIC (μg/mL) Brain heart infusion broth	Nutrient broth	Nutrient broth with 10 mM magnesium chloride
Salmonella typhimurium			
SL3770 –Ra*	32	32	128
SL 3769 –Rd1*	16	16	128
SL1102 –Re*	16	16	64
Escherichia coli			
JF568	16	8	32
JF701 OmpC⁻	16	16	32
JF703 OmpF⁻	16	32	128
JF694 OmpF⁻C⁻	16	32	128

Chemotype of lipopolysaccharide

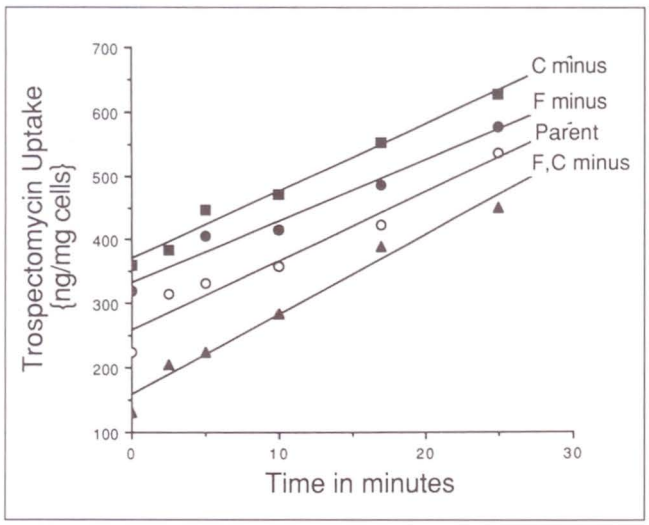

Figure 3) *Uptake of trospectomycin at a concentration of 128 μg/mL in nutrient broth at 43°C of* Escherichia coli *strains JF568 (parent, open circles), JF701 (Omp C deficient, closed squares), JF703 (Omp F deficient, closed circles) and JF694 (Omp F and C deficient, closed triangles)*

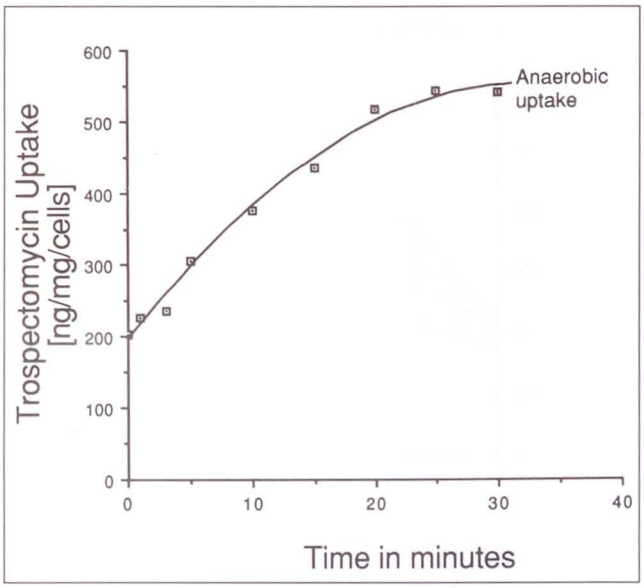

Figure 4) *Uptake of trospectomycin under anaerobic conditions at a concentration of 128 μg/mL in nutrient broth at 43°C of* Escherichia coli *SA1306*

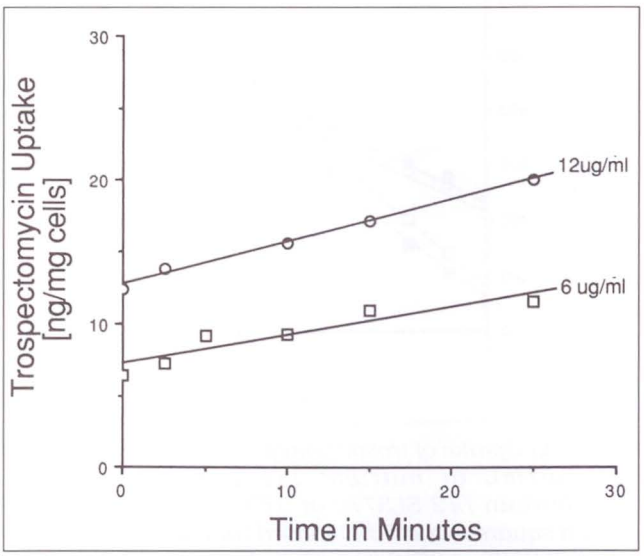

Figure 5) *Uptake of trospectomycin at 37°C in brain heart infusion broth by* Haemophilus influenzae *Rd at concentrations of 6 μg/mL (open squares) and 12 μg/mL (closed squares)*

showed that there was a small but consistently greater uptake by the rough strain (Figures 1,2). This difference correlated with increased susceptibility to trospectomycin as demonstrated by a consistent twofold lower MIC value in three media for the deep rough mutant (Table 1). Strain 3769 with the intermediate Rdl chemotype of lipopolysaccharide had a twofold lower MIC under two of the three testing conditions. Uptake and susceptibility of strains of *E coli* with differences in quantity of the F, C or both outer membrane porins were examined. Strain JF694 deficient in both porins and strain JF703 deficient in the F porin were more resistant by fourfold in nutrient broth with or without added magnesium. However, no appreciable consistent difference in uptake among the four strains could be detected (Figure 3). The inclusion of 10 mM magnesium chloride in nutrient broth increased MICs of both *E coli* and *S typhimurium* strains fourfold. *S typhimurium* LT2 strain SL1102 showed somewhat greater uptake in nutrient broth than in the same medium with 10 mM magnesium chloride, but no difference was detected with strain SL3770 (Figure 2). The authors also examined uptake in *E coli* AB2495 and its *rpsE*⁻ ribosomally spectinomycin-resistant derivative KL252 and in *E coli*

SA1306 and *E coli* SA1306 containing aminoglycoside nucleotidyl transferase (3") capable of inactivating spectinomycin (9). Both resistant isolates conferred resistance raising the MICs by four- and eightfold, respectively, but neither caused any difference in uptake of trospectomycin.

Trospectomycin has antibacterial activity against anaerobic bacteria and facultative bacteria grown anaerobically (1,10) and thus it was of interest to demonstrate that uptake occurs anaerobically. Figure 4 shows that *E coli* SA1306 accumulates the drug under anaerobic growth and uptake conditions at a similar rate to other *E coli* strains (Figure 3). *H influenzae* strains have been reported more susceptible to trospectomycin than *E coli*. Figure 5 shows that uptake can be demonstrated in *H influenzae* Rd at 37°C using 6 or 12 μg/mL, whereas no uptake was detectable in *E coli* under these conditions at either concentration.

DISCUSSION

The present studies show that the aminocyclitol trospectomycin is accumulated by the bacteria examined through a process different from aminoglycosides. The latter require electron transport and a threshold electrical potential to be taken up (3), but trospectomycin undergoes simple energy-independent diffusion into the bacterial cells. This mode of accumulation is consistent with the observation that trospectomycin has equivalent activity under anaerobic and aerobic conditions. The present studies show that trospectomycin is effectively accumulated by *E coli* anaerobically and aerobically. There appears to be a modest cellular concentration of the drug over that in the extracellular medium. Such concentration is likely due to a substantial fraction bound either at the cell surface in spite of washing the cells, or intracellularly at target sites. However the ribosomal site modified in the *rpsE*$^{-}$ mutant does not contribute a significant amount to total cell binding, as no difference in total accumulation was observed compared to the parental strain. These results were supported by the fact that there was no change in accumulation by a strain adenylating spectinomycin, which also interferes with ribosomal binding (9). The increase in rate of uptake with temperature is characteristic of a diffusional process. Examination of uptake at 43°C was selected for many of the authors' studies because reproducibility was higher than at 37°C where the relative contribution of background zero time binding was greater.

Heat-killed dead cells took up trospectomycin faster than living cells. One explanation for this could have been inactivation of an active efflux system which has been described for other antibiotics (11,12). However, the use of the protonophore CCCP did not produce a consistent increase in accumulation which would have been expected for an energy-driven efflux process. The most probable explanation for the increased rate of accumulation at 37°C in heat-killed cells is that the outer membrane was modified, perhaps by release of lipopolysaccharide, thus enhancing uptake. A deep rough mutant of salmonella was more susceptible than a smooth strain, and the rate of uptake was also somewhat faster, supporting either a modest barrier function or an interaction with the polysaccharide portion of the lipopolysaccharide. The outer membrane has been shown to function as a barrier to relatively hydrophobic antimicrobial agents (13). Deficiency of F porin in an isogenic set of *E coli* strains also increased resistance in nutrient broth but not in brain heart infusion broth. These observations on the effects of lipopolysaccharide and porins indicate that both porin and nonporin routes of cell entry through the outer membranes of two enteric bacteria may be used so that mutations in either do not produce major effects on susceptibility or uptake.

Magnesium chloride reduced the activity of trospectomycin for the salmonella or *E coli* strains tested. Although this compound reduced the rate of uptake in the rough salmonella strain it had little or no effect in the smooth strain SL3770 nor in several *E coli* strains examined (data not shown). These results are consistent with the view that magnesium antagonizes uptake to a small extent through an effect deep in lipopolysaccharides. However, they also suggest that some of the magnesium effect is not being exerted at the level of uptake but possibly at the ribosomal site of action.

Accumulation of trospectomycin by *H influenzae* at concentrations much lower than those at which accumulation by *E coli* could be demonstrated indicates that one of the factors accounting for the better activity of this compound in the former bacterium is better penetration. The present studies do not exclude inhibition of ribosomal function at lower concentrations as an explanation for the enhanced activity alone or, more likely, in association with better uptake. *H influenzae* has a much rougher type of lipopolysaccharide which could account in part for its better uptake (14).

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