Journal of Antimicrobial Chemotherapy Advance Access published January 19, 2011

J Antimicrob Chemother
doi:10.1093/jac/dkq526

Journal of Antimicrobial Chemotherapy

The third-generation P-glycoprotein inhibitor tariquidar may overcome bacterial multidrug resistance by increasing intracellular drug concentration

I. Leitner[1], J. Nemeth[1], T. Feurstein[1], A. Abrahim[1], P. Matzneller[1], H. Lagler[2], T. Erker[3], O. Langer[1,4] and M. Zeitlinger[1]*

[1]Department of Clinical Pharmacology, Medical University Vienna, Vienna, Austria; [2]Department of Internal Medicine I, Medical University Vienna, Vienna, Austria; [3]Department of Medicinal Chemistry, University of Vienna, Vienna, Austria; [4]Molecular Medicine, AIT Austrian Institute of Technology GmbH, Seibersdorf, Austria

*Corresponding author. Tel: +43-1-40400-2981; Fax: +43-1-40400-2998; E-mail: markus.zeitlinger@meduniwien.ac.at

Received 28 October 2010; returned 4 December 2010; revised 10 December 2010; accepted 14 December 2010

Objectives: The use of efflux pump inhibitors may be a powerful strategy to overcome transporter-mediated bacterial multidrug resistance. In the present study, we set out to investigate the potency of tariquidar, a third-generation P-glycoprotein inhibitor in clinical development, for overcoming bacterial resistance towards ciprofloxacin.

Methods: *Staphylococcus aureus* 29213 (SA29213) and *S. aureus* 1199B (SA1199B), which overexpresses the multidrug transporter NorA, as well as *Pseudomonas aeruginosa* 27853 and *Stenotrophomonas maltophilia* BAA-85, which expresses SmeDEF, were exposed to ciprofloxacin in the presence and absence of tariquidar or, for comparative reasons, elacridar. Activity of both P-glycoprotein inhibitors was evaluated by determination of MICs and time–kill curves, and by quantification of uptake of ciprofloxacin into bacterial cells.

Results: Activity of tariquidar and elacridar was comparable for *S. aureus* strains, and both dose-dependently increased susceptibility towards ciprofloxacin. Highest effects were observed for SA1199B, where the addition of tariquidar resulted in a 10-fold reduction of the ciprofloxacin MIC, while no effect was observed for *P. aeruginosa*. For *S. maltophilia*, elacridar but not tariquidar improved susceptibility. Uptake of [^{14}C]ciprofloxacin and modification of susceptibility showed significant correlations ($r=0.89$, $P<0.0001$). Tariquidar had no intrinsic activity against any strain tested.

Conclusions: We conclude that tariquidar has potent inhibitory effect against certain bacterial efflux pumps *in vitro*. Their high activity at clinically achievable concentrations might yield this class of drugs promising for future applications in infectious diseases.

Keywords: MDR, ABC, ciprofloxacin, efflux pump inhibitors

Introduction

Multidrug resistance (MDR) mediated through efflux pumps has been described for various organisms, including bacteria, fungi, protozoa and mammalian tumour cells, and has been ascribed to treatment failure in infectious as well as oncological diseases.[1–3] For bacterial infections, MDR is a major problem because it limits chemotherapeutic options and has been demonstrated to result in increased costs, prolonged duration of hospital stay, and higher morbidity and mortality rates.[4–6]

Bacteria possess a wide array of drug efflux proteins, some of them sharing structural similarity to eukaryotic efflux pumps.[1,7–9] In both cases, the transporters reduce intracellular drug concentrations and thereby impede accessibility of drugs to their sites of action, ultimately leading to reduced susceptibility. Therefore, it has been speculated that efflux pump inhibitors (EPIs) developed to overcome efflux in eukaryotic cells may be also used to battle bacterial resistance.[1] The development of EPIs that could be used in conjunction with existing antibiotics could extend the useful lifetime of many antibiotics by improving therapeutic efficacy and by suppressing the emergence of resistant variants that might otherwise arise during treatment.[10–13] However, clinical use of previously described bacterial EPIs, like reserpine, verapamil, gemfibrozil or cyclosporine A, was hampered by the potential for adverse effects at concentrations

necessary to sufficiently inhibit the underlying transport protein.[1,3,7,8]

The synthetic compound tariquidar belongs to the most promising representatives of the third generation of MDR modulators developed so far, and was discovered during screening for inhibitors of mammalian ATP-binding cassette (ABC)-type transporters, particularly P-glycoprotein (P-gp, ABCB1) and breast cancer resistance protein (BCRP, ABCG2).[14] Like elacridar, another third-generation P-gp inhibitor, tariquidar has been introduced into clinical development up to Phase II/III studies, where it showed clinical activity to restore susceptibility of cancer cells towards chemotherapy.[15–17]

Efflux-mediated fluoroquinolone resistance has been reported in a number of Gram-negative and -positive pathogenic bacteria, and may be encoded by various efflux pumps, including, but not limited to, members of the major facilitator, ABC and resistance nodulation division families.[1] Indeed, elacridar was previously found to be equipotent to reserpine in enhancing the *in vitro* activity of ciprofloxacin and norfloxacin against strains of *Staphylococcus aureus* expressing efflux-related MDR pumps.[3] However, the clinical development of elacridar was stopped.[18]

In the present study, we set out to investigate the potency of tariquidar to overcome bacterial resistance towards ciprofloxacin in Gram-positive and -negative strains. Tariquidar and elacridar were directly compared by investigating efflux pump-overexpressing and standard strains. The effect of tariquidar or elacridar was evaluated pharmacodynamically by determination of MICs and time–kill curves, and by quantification of uptake of ciprofloxacin into bacterial cells.

Materials and methods

Bacterial strains

S. aureus ATCC 29213 (SA29213), *Pseudomonas aeruginosa* ATCC 27853 and *Stenotrophomonas maltophilia* ATCC BAA-85 were obtained from the ATCC (USA). *S. aureus* 1199B (SA1199B), which overexpresses the major *S. aureus* multidrug transporter NorA, was generously provided by the Centre for Pharmacognosy and Phytotherapy, University of London.[3] *S. maltophilia* ATCC BAA-85 (also described as D457R) expresses an efflux-mediated MDR system (SmeDEF) against a range of antibacterials, including quinolones.[19,20]

Antibiotics, chemicals and growth media

Mueller–Hinton broth (MHB; Merck, Germany) was adjusted with cations to concentrations of 25 mg/L calcium and 12.5 mg/L magnesium, according to the CLSI. Ciprofloxacin was obtained from Bayer AG (Germany), and [^{14}C]ciprofloxacin (specific activity 370 MBq/mmol) was obtained from Moravek Biochemicals and Radiochemicals (USA). Tariquidar hydrochloride and elacridar hydrochloride were synthesized at the Department of Medicinal Chemistry, University of Vienna (Austria). All stock solutions were stored at −80°C.

In vitro susceptibility tests

Susceptibility towards ciprofloxacin was tested for the four test strains in the presence and absence of the two EPIs in analogy to the CLSI guidelines for susceptibility testing. The strains were pre-cultured overnight on Columbia agar plates and were then introduced at an initial inoculum of ~5×10^5 cfu/mL into MHB. MHB contained defined concentrations of ciprofloxacin and the two EPIs in decreasing steps. The lowest concentration of the ciprofloxacin/EPI mixture that inhibited visible bacterial growth after 20 h of incubation at 37°C was defined as the MIC.

Time–kill curves

Bacterial killing curves for SA1199B were performed in pure MHB and MHB containing 2.5 mg/L tariquidar for ciprofloxacin concentrations equal to the MIC of ciprofloxacin against SA1199B, i.e. 8 mg/L, and two and four times above and below the MIC, respectively. The tubes containing 5 mL aliquots with an initial inoculum of ~5×10^5 cfu/mL were kept in a water bath at 37°C. After vortexing of the culture tubes, 100 μL samples were pulled and serially diluted with aqueous sodium chloride (NaCl) solution (0.9%, w/v). After each dilution step, 20 μL aliquots were plated onto Columbia agar plates, which were incubated for 24 h at 37°C. The colonies of bacteria were counted at 0, 2, 4, 6 and 8 h after the incubation, and were back extrapolated to the original volume to determine cfu/mL. All simulations were performed in triplicate.

[^{14}C]ciprofloxacin accumulation

Changes in intracellular concentrations of ciprofloxacin in the presence of different concentrations of tariquidar or elacridar were determined by use of [^{14}C]ciprofloxacin. Strains were pre-cultured overnight on a Columbia agar plate, introduced into 1000 mL of MHB and incubated overnight at 37°C. Broth containing an inoculum of ~10^9 cfu/mL was then centrifuged at 2500 **g** at room temperature for 10 min. The pellets were resuspended in NaCl and the pooled bacterial solution was introduced into MHB to achieve final concentrations of 10^{10} cfu/mL. After 15 min of incubation at 37°C, EPIs were added at predefined concentrations. After another 15 min of incubation, [^{14}C]ciprofloxacin and unlabelled ciprofloxacin were added to achieve a final radioactivity concentration of 1.9 kBq/mL and a mass concentration of 2 mg/L. After incubation for 60 min, this mixture was centrifuged at 4°C, the supernatant was discarded and the pellet was resuspended in ice-cold aqueous NaCl solution (0.9%, w/v). This procedure was repeated, and then liquid scintillation cocktail (Ultima Gold solution; PerkinElmer, USA) was added and the radioactivity was measured in a liquid scintillation counter (WALLAC 1410; Finland). All simulations were performed in triplicate.

Results

In vitro susceptibility tests

MIC values of ciprofloxacin were 0.5 and 8 mg/L for SA29213 and SA1199B, respectively, and 0.25 and 8 mg/L for *P. aeruginosa* and *S. maltophilia*, respectively. All values were within CLSI ranges. For tariquidar and elacridar, no intrinsic activity was observed at the investigated concentrations (MIC values >64 mg/L).

The addition of tariquidar and elacridar increased susceptibility towards ciprofloxacin of both *S. aureus* strains in a dose-dependent manner whereas no effect was observed for *P. aeruginosa* (Figure 1, reduction is presented as ratio versus baseline). For both inhibitors, reduction of MIC versus baseline was higher for SA1199B than for SA29213. The presence of tariquidar and elacridar resulted in an up to 10-fold reduction in ciprofloxacin MICs for SA1199B. For *S. maltophilia*, only elacridar was able to improve susceptibility towards ciprofloxacin in a dose-dependent manner, while no effect was observed for tariquidar.

Time–kill curves

Figure 2 presents bacterial time–kill profiles of SA1199B after exposure to ciprofloxacin at 0–32 mg/L alone or combined

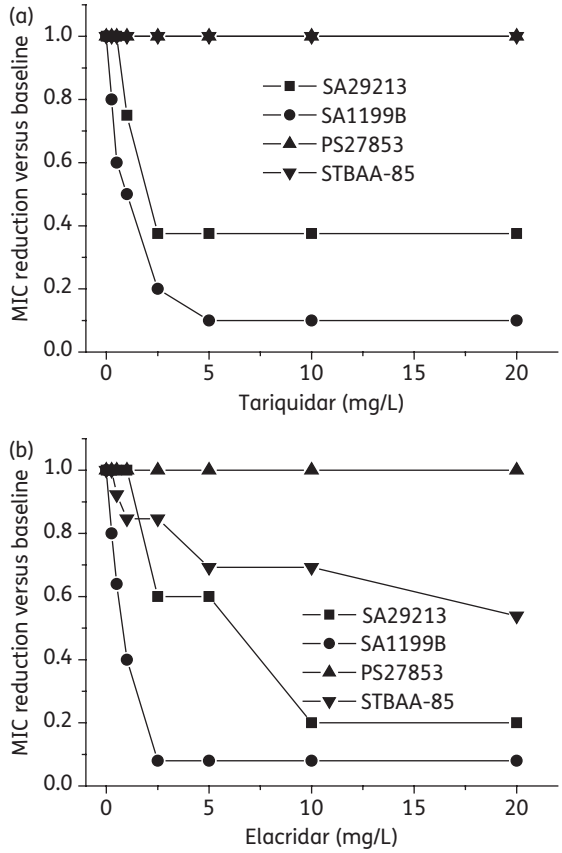

Figure 1. Reduction of MIC of ciprofloxacin for Gram-positive and -negative bacteria by tariquidar (a) and elacridar (b). MIC reduction is presented as the ratio versus baseline for SA29213, SA1199B, *P. aeruginosa* 27853 (PS27853) and *S. maltophilia* BAA-85 (STBAA-85).

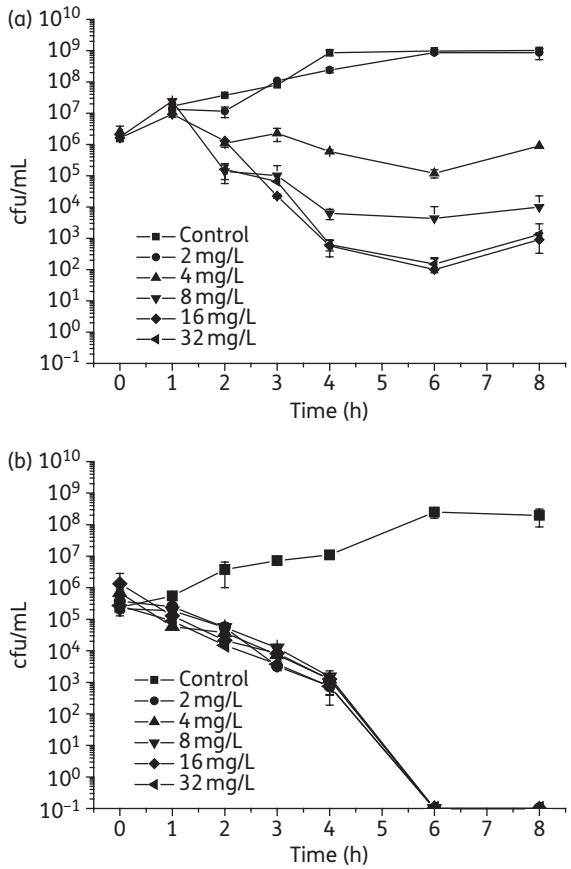

Figure 2. Time–killing of various concentrations of ciprofloxacin (2–32 mg/L) against SA1199B in the absence (a) and presence (b) of tariquidar at a concentration of 2.5 mg/L.

with tariquidar at 2.5 mg/L over 8 h. Bacterial killing by ciprofloxacin combined with tariquidar was markedly increased for all ciprofloxacin concentrations.

[^{14}C]ciprofloxacin accumulation

Figure 3 shows accumulation of [^{14}C]ciprofloxacin at increasing tariquidar or elacridar concentrations for all four investigated strains. In analogy with results from susceptibility tests, the highest impact was observed for SA1199B and virtually no impact was seen for *P. aeruginosa*. In Figure 4, the Spearman rank order correlation between the modification of susceptibility and the uptake of [^{14}C]ciprofloxacin by tariquidar and elacridar (pooled data) is shown for SA1199B ($r=0.89$, $P<0.0001$).

Discussion

To date, no EPI has been licensed for use in the treatment of bacterial infections.[21] The present study indicates that tariquidar, a potent inhibitor of human P-gp, may overcome resistance towards ciprofloxacin in staphylococci. Although P-gp and NorA both act by pumping drug out of the cell, the first is energized by ATP while the second is driven by a proton gradient. Therefore, the mode of action of substances known to inhibit P-gp, i.e. suppression of drug efflux, should not be transferred to bacteria without proof of concept. By the strong correlation of MIC reduction and increase of intracellular radioactivity in our experiments (Figure 4), and the absence of intrinsic activity of tariquidar at the employed concentrations, we were able to demonstrate that tariquidar improves susceptibility towards ciprofloxacin by increasing its intracellular concentration. MICs of ciprofloxacin were reduced in a dose-dependent manner, with maximal effects for SA1199B, i.e. the strain that overexpresses NorA (Figure 1). This effect corresponded to a reduction of the baseline MIC of 8 mg/L to values <1 mg/L, transforming a highly resistant strain into one susceptible to ciprofloxacin. However, also for *a priori* susceptible strains like SA29213, further reduction of the MIC could be beneficial in the case of difficult-to-target compartments, such as diabetic foot or brain infections.[22,23]

Currently available potent inhibitors of efflux pumps, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), are too toxic to be safely used in patients.[24] In comparison to the early generation of chemosensitizers, like cyclosporine A or verapamil, which were not optimized for MDR reversal, third-generation MDR inhibitors have higher specificity for P-gp and a lower cellular toxicity.[25] The difference in activity of tariquidar against different strains further underlines its specificity for inhibition of

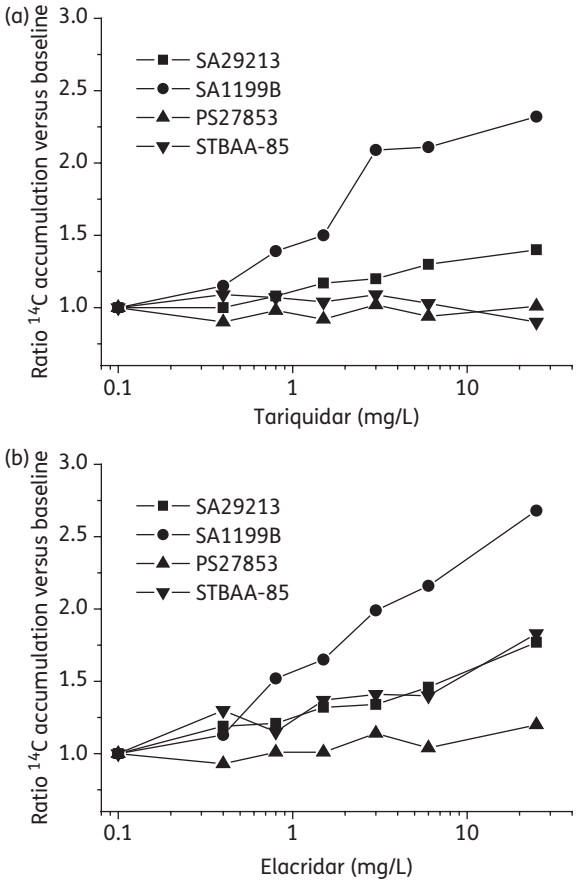

Figure 3. Enhancement of accumulation of [^{14}C]ciprofloxacin into Gram-positive and -negative bacteria by tariquidar (a) or elacridar (b) for SA29213, SA1199B, *P. aeruginosa* 27853 (PS27853) and *S. maltophilia* BAA-85 (STBAA-85).

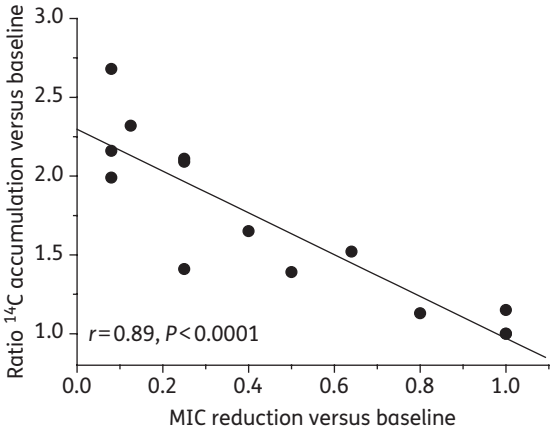

Figure 4. Correlation between change in MIC following the addition of different concentrations of tariquidar and elacridar (pooled data) and accumulation of [^{14}C]ciprofloxacin for SA1199B.

a specific transporter, rather than denial of metabolic energy sources as described for CCCP, which has a much broader spectrum of activity.[14,24] Third-generation MDR inhibitors also have a very low affinity for other enzyme systems, such as CYP3A4, and are therefore less likely to cause undesirable pharmacokinetic interactions.[26] Previously clinically used doses of tariquidar (150 mg) resulted in maximum plasma concentrations of 2–3 mg/L and trough concentrations of 0.3–0.5 mg/L.[27] Even higher doses of tariquidar have been administered to humans in exploratory studies.[28] Considering the dose–response correlations depicted in Figure 1, one could speculate that clinically achievable, well-tolerated concentrations of tariquidar have potent effects on the susceptibility of certain bacterial strains.

Due to the fact that many efflux pumps possess significant structural homology, it may be hoped that one compound will be active against a range of pumps from different bacterial species.[7] However, tariquidar and elacridar are known to be specific, non-competitive inhibitors of P-gp and BCRP, which inhibit ATPase activity, suggesting that the modulating effect is derived from the inhibition of substrate binding, inhibition of ATP hydrolysis or both.[14,29] Interestingly, a dose-dependent reduction was observed for *S. maltophilia* BAA-85, which overexpresses the structurally unrelated multidrug efflux pump SmeDEF, for elacridar but not tariquidar.[20] This finding might be in line with the previous observation that elacridar has broader activity than tariquidar for mammalian ABC transporters in that it inhibits both P-gp and BCRP with comparable potencies whereas tariquidar shows higher selectivity for P-gp versus BCRP.[30] No effect was observed for *P. aeruginosa* wild-type. Since it is known that *P. aeruginosa* wild-type possesses a potent efflux mechanism responsible for intrinsic resistance to some antimicrobial classes, the activity of tariquidar and elacridar clearly will not be transferable to all strains expressing efflux pumps.[31]

As previously described, ciprofloxacin displays concentration-dependent killing (Figure 2a).[32,33] Surprisingly, the addition of tariquidar seems to abrogate this pharmacodynamic characteristic (Figure 2b). Since tariquidar does not have intrinsic antimicrobial activity at the employed concentration, one might speculate that concentration-dependent killing is at least partially mediated by the function of a bacterial efflux pump.

Besides restoring bacterial susceptibility, EPIs may be used to improve the pharmacokinetics of antimicrobial drugs into tissues protected by P-gp. Indeed, elacridar significantly increased cerebral itraconazole concentrations and decreased weight loss during treatment of *Cryptococcus neoformans* meningitis with itraconazole.[34] Likewise, tariquidar has been used to increase the uptake of drugs into the CNS in humans and animals.[27,35] This observation might be prospectively used to create combinations of antibiotics and EPIs, aiming at overcoming both bacterial resistance and penetration barriers in the body. However, the applicability and tolerability of such combinations needs to be investigated in future studies.

An important limitation of the present work is the restriction of investigations to only four strains. While the use of *S. aureus* wild-type and overexpressing strains might be considered well suited for the investigation of suppression of efflux pump activity, the results of investigation of two independent Gram-negative strains can only deliver very preliminary conclusions. However, the use of these two well-defined strains was chosen to support results from NorA-expressing strains rather than to deliver information on the potency of tariquidar to overcome resistance in Gram-negative organisms.

The present study investigated the impact of tariquidar on the activity of bacterial efflux pumps only in MHB, i.e. at a pH of ~7.4.

However, recent literature indicates that many bacteria possess two general efflux pump systems, i.e. proton-motive force-dependent and ABC transporter types, respectively.[36,37] They are activated at low and high pH, respectively; the ones activated at high pH commonly being ABC transporters. Thus, it cannot be excluded that the potency of tariquidar to inhibit efflux pumps might be lower in more acidic environments.

In conclusion, the present study demonstrated that tariquidar has a potent inhibitory effect against bacterial efflux pumps *in vitro*. Although the specificity for selected bacterial efflux pumps might limit clinical applications, the high activity at clinically achievable concentrations might yield this class of drugs promising for future applications in infectious diseases.

Funding

No specific funding was received for this study.

Transparency declarations

None to declare.

References

1 Poole K. Efflux-mediated antimicrobial resistance. *J Antimicrob Chemother* 2005; **56**: 20–51.

2 Normark BH, Normark S. Evolution and spread of antibiotic resistance. *J Intern Med* 2002; **252**: 91–106.

3 Gibbons S, Oluwatuyi M, Kaatz GW. A novel inhibitor of multidrug efflux pumps in *Staphylococcus aureus*. *J Antimicrob Chemother* 2003; **51**: 13–7.

4 Levy SB, Marshall B. Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med* 2004; **10**: S122–9.

5 Huet AA, Raygada JL, Mendiratta K *et al*. Multidrug efflux pump overexpression in *Staphylococcus aureus* after single and multiple in vitro exposures to biocides and dyes. *Microbiology* 2008; **154**: 3144–53.

6 Escribano I, Rodriguez JC, Llorca B *et al*. Importance of the efflux pump systems in the resistance of *Mycobacterium tuberculosis* to fluoroquinolones and linezolid. *Chemotherapy* 2007; **53**: 397–401.

7 Lomovskaya O, Bostian KA. Practical applications and feasibility of efflux pump inhibitors in the clinic—a vision for applied use. *Biochem Pharmacol* 2006; **71**: 910–8.

8 Lomovskaya O, Zgurskaya HI, Totrov M *et al*. Waltzing transporters and 'the dance macabre' between humans and bacteria. *Nat Rev Drug Discov* 2007; **6**: 56–65.

9 Amaral L, Engi H, Viveiros M *et al*. Review. Comparison of multidrug resistant efflux pumps of cancer and bacterial cells with respect to the same inhibitory agents. *In Vivo* 2007; **21**: 237–44.

10 Markham PN, Westhaus E, Klyachko K *et al*. Multiple novel inhibitors of the NorA multidrug transporter of *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1999; **43**: 2404–8.

11 Markham PN. Inhibition of the emergence of ciprofloxacin resistance in *Streptococcus pneumoniae* by the multidrug efflux inhibitor reserpine. *Antimicrob Agents Chemother* 1999; **43**: 988–9.

12 Renau TE, Leger R, Flamme EM *et al*. Inhibitors of efflux pumps in *Pseudomonas aeruginosa* potentiate the activity of the fluoroquinolone antibacterial levofloxacin. *J Med Chem* 1999; **42**: 4928–31.

13 Van Bambeke F, Pages JM, Lee VJ. Inhibitors of bacterial efflux pumps as adjuvants in antibiotic treatments and diagnostic tools for detection of resistance by efflux. *Recent Pat Antiinfect Drug Discov* 2006; **1**: 157–75.

14 Fox E, Bates SE. Tariquidar (XR9576): a P-glycoprotein drug efflux pump inhibitor. *Expert Rev Anticancer Ther* 2007; **7**: 447–59.

15 Malingre MM, Beijnen JH, Rosing H *et al*. Co-administration of GF120918 significantly increases the systemic exposure to oral paclitaxel in cancer patients. *Br J Cancer* 2001; **84**: 42–7.

16 Kuppens IE, Witteveen EO, Jewell RC *et al*. A phase I, randomized, open-label, parallel-cohort, dose-finding study of elacridar (GF120918) and oral topotecan in cancer patients. *Clin Cancer Res* 2007; **13**: 3276–85.

17 Pusztai L, Wagner P, Ibrahim N *et al*. Phase II study of tariquidar, a selective P-glycoprotein inhibitor, in patients with chemotherapy-resistant, advanced breast carcinoma. *Cancer* 2005; **104**: 682–91.

18 Raguz S, Yague E. Resistance to chemotherapy: new treatments and novel insights into an old problem. *Br J Cancer* 2008; **99**: 387–91.

19 Alonso A, Martinez JL. Multiple antibiotic resistance in *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 1997; **41**: 1140–2.

20 Alonso A, Martinez JL. Cloning and characterization of SmeDEF, a novel multidrug efflux pump from *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 2000; **44**: 3079–86.

21 Mahamoud A, Chevalier J, Alibert-Franco S *et al*. Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. *J Antimicrob Chemother* 2007; **59**: 1223–9.

22 Muller M, Brunner M, Hollenstein U *et al*. Penetration of ciprofloxacin into the interstitial space of inflamed foot lesions in non-insulin-dependent diabetes mellitus patients. *Antimicrob Agents Chemother* 1999; **43**: 2056–8.

23 de Lange EC, Marchand S, van den Berg D *et al*. In vitro and in vivo investigations on fluoroquinolones: effects of the P-glycoprotein efflux transporter on brain distribution of sparfloxacin. *Eur J Pharm Sci* 2000; **12**: 85–93.

24 Kaatz GW, Moudgal VV, Seo SM. Identification and characterization of a novel efflux-related multidrug resistance phenotype in *Staphylococcus aureus*. *J Antimicrob Chemother* 2002; **50**: 833–8.

25 Abraham J, Edgerly M, Wilson R *et al*. A phase I study of the P-glycoprotein antagonist tariquidar in combination with vinorelbine. *Clin Cancer Res* 2009; **15**: 3574–82.

26 Wong HL, Bendayan R, Rauth AM *et al*. Simultaneous delivery of doxorubicin and GG918 (elacridar) by new polymer–lipid hybrid nanoparticles (PLN) for enhanced treatment of multidrug-resistant breast cancer. *J Control Release* 2006; **116**: 275–84.

27 Wagner CC, Bauer M, Karch R *et al*. A pilot study to assess the efficacy of tariquidar to inhibit P-glycoprotein at the human blood–brain barrier with (*R*)-^{11}C-verapamil and PET. *J Nucl Med* 2009; **50**: 1954–61.

28 Kreisl WC, Liow JS, Kimura N *et al*. P-glycoprotein function at the blood–brain barrier in humans can be quantified with the substrate radiotracer ^{11}C-*N*-desmethyl-loperamide. *J Nucl Med* 2010; **51**: 559–66.

29 Kuppens IE, Breedveld P, Beijnen JH *et al*. Modulation of oral drug bioavailability: from preclinical mechanism to therapeutic application. *Cancer Invest* 2005; **23**: 443–64.

30 Kuhnle M, Egger M, Muller C *et al*. Potent and selective inhibitors of breast cancer resistance protein (ABCG2) derived from the P-glycoprotein (ABCB1) modulator tariquidar. *J Med Chem* 2009; **52**: 1190–7.

31 Kiser TH, Obritsch MD, Jung R *et al*. Efflux pump contribution to multidrug resistance in clinical isolates of *Pseudomonas aeruginosa*. *Pharmacotherapy* 2010; **30**: 632–8.

32 Gould IM, Milne K, Jason C. Concentration-dependent bacterial killing, adaptive resistance and post-antibiotic effect of ciprofloxacin alone and in combination with gentamicin. *Drugs Exp Clin Res* 1990; **16**: 621–8.

33 Joyanes P, Pascual A, Gimenez MJ *et al*. Differences between two new quinolones (gemifloxacin and trovafloxacin) and ciprofloxacin in their concentration-dependent killing of *Streptococcus pneumoniae*. *Chemotherapy* 2001; **47**: 409–14.

34 Imbert F, Jardin M, Fernandez C *et al*. Effect of efflux inhibition on brain uptake of itraconazole in mice infected with *Cryptococcus neoformans*. *Drug Metab Dispos* 2003; **31**: 319–25.

35 Bankstahl JP, Kuntner C, Abrahim A *et al*. Tariquidar-induced P-glycoprotein inhibition at the rat blood–brain barrier studied with (*R*)-^{11}C-verapamil and PET. *J Nucl Med* 2008; **49**: 1328–35.

36 Martins A, Spengler G, Martins M *et al*. Physiological characterisation of the efflux pump system of antibiotic-susceptible and multidrug-resistant *Enterobacter aerogenes*. *Int J Antimicrob Agents* 2010; **36**: 313–8.

37 Martins A, Spengler G, Rodrigues L *et al*. pH modulation of efflux pump activity of multi-drug resistant *Escherichia coli*: protection during its passage and eventual colonization of the colon. *PLoS One* 2009; **4**: e6656.