Inhibition of Antibiotic Efflux in Bacteria by the Novel Multidrug Resistance Inhibitors Biricodar (VX-710) and Timcodar (VX-853)

Steve Mullin,[†] Nagraj Mani, and Trudy H. Grossman*

Vertex Pharmaceuticals, Cambridge, Massachusetts

Received 20 January 2004/Returned for modification 10 April 2004/Accepted 26 June 2004

Inhibitors of mammalian multidrug efflux, such as the plant alkaloid reserpine, are also active in potentiating antibiotic activity by inhibiting bacterial efflux. Based on this precedent, two novel mammalian multiple drug resistance inhibitors, biricodar (VX-710) and timcodar (VX-853), were evaluated for activity in a variety of bacteria. Both VX-710 and VX-853 potentiated the activity of ethidium bromide (EtBr), a model efflux substrate, against three clinically significant gram-positive pathogens: *Staphylococcus aureus*, *Enterococcus faecalis*, and *Streptococcus pneumoniae*. Similar to reserpine, VX-710 and VX-853 directly blocked EtBr efflux in *S. aureus*. Furthermore, these compounds were effective in lowering the MICs of several clinically used antibiotics, including fluoroquinolones, suggesting that VX-710 and VX-853 are representatives of a new class of bacterial efflux inhibitors with the potential for use in combination therapy.

Bacteria can become resistant to antibiotics via three mechanisms: antibiotic inactivation, target modification, and alteration of intracellular antibiotic concentration. The latter mechanism can occur by either decreasing permeability to an antibiotic or increasing the activities of a variety of efflux pumps. While permeability is a significant barrier to antibiotics in gram-negative bacteria due to the presence of an outer membrane, it is an unlikely mechanism of resistance for gram-positive bacteria, since they lack an outer membrane. Efflux of antibiotics is a clinically significant general resistance mechanism for bacteria, often endowing organisms with multiple-drug-resistant (MDR) phenotypes (14). Gram-positive and gram-negative bacteria can possess multiple chromosomal and plasmid-encoded efflux pumps with broad substrate specificities, including both naturally and synthetically produced antibiotics (2, 24, 30). For example, an analysis of the genome sequence of methicillin-resistant *Staphylococcus aureus* N315 indicates that there are >20 open reading frames capable of encoding antibiotic efflux pumps (16; <http://www.membranetransport.org>).

For gram-negative bacteria, pumps belonging to the resistance-nodulation-cell division family play the greatest role in contributing to resistance to many clinically used antibiotics. Examples of this class of efflux pumps include the AcrB pump in *Escherichia coli* and the MexB, MexD, MexF, and MexY pumps in *Pseudomonas aeruginosa* (2, 30). To date, resistance-nodulation-cell division pumps have not been described for gram-positive organisms. For gram-positive bacteria, pumps belonging to the major facilitator superfamily class play the greater role in the efflux of clinically relevant antibiotics, contributing to resistance. Major facilitator superfamily pumps are found in both prokaryotes and eukaryotes, including mammals, and prokaryotic examples of this class of efflux pumps include

the NorA pump of *S. aureus* (28), the PmrA pump of *Streptococcus pneumoniae* (9), and the EmeA pump of *Enterococcus faecalis* (18). Recent reports have described the crystal structures of the *E. coli* AcrB pump (25, 36) and the *Bacillus subtilis* BmrR MDR transcriptional activator (37), both cocomplexed with substrates, and the outer membrane transporter TolC (15). While structural details provide the basis for substrate recognition, the mechanism by which molecules are actually transported to the outside of a cell remains to be elucidated.

Inhibition of efflux is potentially one way to improve the clinical efficacy of an antibiotic, even in the presence of target-based mutations, by increasing intracellular antibiotic concentrations. Because of emerging resistance to all classes of antibiotics, in particular the fluoroquinolones, there has been a significant focus by the pharmaceutical industry on addressing this problem (17). Reserpine, a plant alkaloid, is a known inhibitor of both mammalian and gram-positive bacterial efflux. However, its clinical utility is limited by its neurotoxicity (27). It has activity against the NorA pump of *S. aureus* (22), a known contributor to fluoroquinolone resistance in clinical isolates (12, 23). Homologs of the NorA pump are found in multiple gram-positive bacteria, suggesting that reserpine and other NorA pump inhibitors would inhibit the antibiotic pumps of other clinical pathogens. In this study, the activities in bacteria of two novel mammalian MDR inhibitors, VX-710 and VX-853 (Fig. 1), were evaluated. VX-710 and VX-853 are novel small molecules that inhibit mammalian MDR and confer increased drug sensitivity to cells expressing both P-glycoprotein and MRP-1 (5, 6, 7), with minimal in vivo toxicity (4, 29, 33, 35). Results show that both VX-710 and VX-853 potentiate the activities of multiple antibiotics in several gram-positive pathogens, suggesting that these compounds represent a new class of bacterial efflux inhibitors with potential for use in combination antibiotic therapy.

* Corresponding author. Mailing address: Vertex Pharmaceuticals, 130 Waverly St., Cambridge, MA 02139. Phone: (617) 444-6252. Fax: (617) 444-6210. E-mail: trudy_grossman@vrtx.com.

[†] Present address: ActivBiotics, Lexington, Mass.

MATERIALS AND METHODS

Compounds. VX-710 and VX-853 (Fig. 1) were obtained from in-house stocks. The compounds were dissolved in 100% dimethyl sulfoxide (DMSO) at a con-

FIG. 1. Structures of VX-710 and VX-853.

centration of 100 mg/ml and stored at -20°C . Reserpine was obtained from Sigma Chemical Co. (St. Louis, Mo.), and stocks were stored as described above. All antibiotics were obtained from standard commercial sources, and stocks were prepared at 25.6 mg/ml in 100% DMSO and stored at -20°C .

Bacterial strains. *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, and *S. pneumoniae* ATCC 10015 were obtained from the American Type Culture Collection (Manassas, Va.). *S. aureus* strains SA-1199 (clinical isolate; susceptible), SA-1199B (SA-1199; NorA overproducer, also has an A116E GrlA mutation), SA-8325-4 (NCTC 8325 cured of prophages), and SA-K2068 (SA-8325-4; non-NorA multidrug efflux pump overproducer mutation) were all gifts from Glenn Kaatz (10, 11, 13).

Antibacterial assays. MICs were determined by two or more independent tests using microdilution techniques according to the NCCLS guidelines (26) in cation-adjusted Mueller Hinton broth (caMHB; Fisher Scientific, Pittsburgh, Pa.). Sterile laked horse blood (3%; Quad Five, Ryegate, Mont.) was added to assays for *E. faecalis* and *S. pneumoniae*. A final concentration of 1% DMSO was present in all assays, a concentration which had no antibacterial effect on its own. The MICs for the quality control strains, *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, and *S. pneumoniae* ATCC 10015, were within the NCCLS ranges (26).

MIC₉₀ studies. Susceptibility studies with large panels of resistant clinical isolates to determine the MICs at which 90% of the isolates tested were inhibited (MIC₉₀s) were performed at Focus Technologies, Herndon, Va., according to NCCLS protocols (26). VX-710 and VX-853 were used at 50 $\mu\text{g}/\text{ml}$ in combination with norfloxacin and ciprofloxacin in the presence of 1% DMSO.

Determination of the MECs of EPIs. The MIC of an efflux substrate was determined in the presence of increasing amounts of efflux pump inhibitors (EPIs; 0 to 100 μg of EPI/ml in serial twofold dilutions). The minimal effective concentration (MEC) was determined to be the minimal concentration of EPI that produced the maximal reduction in substrate MIC. No further decrease in substrate MIC was observed at EPI concentrations greater than the MEC.

EtBr efflux. Ethidium bromide (EtBr) efflux assays with *S. aureus* were performed using the method described by Markham et al. (22). Mid-logarithmic-phase *S. aureus* ATCC 29213 cells, grown in caMHB medium, were loaded with 10 μg of EtBr/ml in the presence of 25 μg of reserpine/ml to inhibit efflux during loading. Cells were incubated at 37°C for 20 min and then pelleted by centrifugation. The medium was decanted, and the cell pellet was resuspended in fresh caMHB medium, either with or without an EPI, to an optical density at 600 nm of 0.2. EtBr efflux was determined by continuously monitoring fluorescence at excitation and emission wavelengths of 530 and 600 nm in a black 96-well polystyrene plate with a clear, flat bottom (3904; Costar, Cambridge, Mass.) by using a SpectraMax Gemini spectrofluorimeter (Molecular Devices, Sunnyvale, Calif.). Results are presented as the average from three individual replicate experiments.

RESULTS

Effect of VX-710 and VX-853 on the MICs of EtBr for *S. aureus*, *E. faecalis*, and *S. pneumoniae*. For the initial evaluation of VX-710 and VX-853 in bacteria, EtBr was used as a test substrate. Since EtBr is a nonspecific DNA intercalator, the only known mechanism of resistance to EtBr is via efflux (22).

TABLE 1. Effect of EPIs on the activity of EtBr against *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, and *S. pneumoniae* ATCC 10015

Organism	EPI	MIC of EPI ($\mu\text{g}/\text{ml}$)	MEC of EPI ($\mu\text{g}/\text{ml}$)	MIC of EtBr ($\mu\text{g}/\text{ml}$)		Reduction (<i>n</i> -fold) in EtBr MIC
				Without EPI	With EPI ^a	
<i>S. aureus</i>	Reserpine	>100	6.3	6.3	1.6	4
	VX-710	>100	≥ 100	6.3	0.2	31
	VX-853	>100	1.6	6.3	1.6	4
<i>E. faecalis</i>	Reserpine	>100	≥ 100	6.3	0.8	8
	VX-710	>100	≥ 100	6.3	0.8	8
	VX-853	>100	6.3	6.3	3.1	2
<i>S. pneumoniae</i>	Reserpine	50	25 ^b	2	0.04	50
	VX-710	>100	≥ 100	2	≤ 0.125	≥ 16
	VX-853	>100	12.5	2	1	2

^a EPIs were used at the MEC, except for VX-710, which was used at its solubility limit, 100 $\mu\text{g}/\text{ml}$.

^b Maximal subinhibitory concentration of reserpine.

Standard MIC assays for measuring the activity of EtBr were performed in the presence and absence of increasing concentrations of EPIs (0 to 100 $\mu\text{g}/\text{ml}$). Neither VX-710 nor VX-853 at 100 $\mu\text{g}/\text{ml}$ had intrinsic activity against *S. aureus*, *E. faecalis*, and *S. pneumoniae*, while reserpine had an MIC of 50 $\mu\text{g}/\text{ml}$ against *S. pneumoniae* (Table 1). The MEC for inhibition of EtBr efflux was defined as the lowest amount of EPI which produces the maximum effect on the MIC of a given antibiotic; no further potentiating activity was observed above the MEC (Table 1). Similar to reserpine, the MIC of EtBr was markedly reduced in the presence of VX-710 and VX-853. This reduction was apparent for all three organisms to different degrees (2- to 31-fold reductions in EtBr MICs) (Table 1). Interestingly, the activity of VX-710 was nonsaturable at ≥ 100 $\mu\text{g}/\text{ml}$ in *S. aureus*, *E. faecalis*, and *S. pneumoniae*; increasing amounts of VX-710 produced a greater reduction in EtBr MICs. This effect was also observed for reserpine in *E. faecalis*. For further experiments with EtBr and other antibiotics, unless otherwise noted, VX-710 was used at 100 $\mu\text{g}/\text{ml}$, its maximum solubility limit. Note that since reserpine possessed an intrinsic activity against *S. pneumoniae* at higher concentrations, it is possible that the 50-fold reduction in the MIC of EtBr in the presence of the maximal subinhibitory concentration of reserpine (25 $\mu\text{g}/\text{ml}$) is due in part to a combination of the activities of reserpine and EtBr.

Inhibition of EtBr efflux in *S. aureus* ATCC 29213 by VX-710 and VX-853. The ability of VX-710 and VX-853 to directly inhibit the efflux of EtBr in *S. aureus* was evaluated by using a fluorescence assay (22). Since EtBr fluoresces only when it is bound to nucleic acid inside cells, a time-dependent decrease in fluorescence of EtBr-loaded bacteria is due to active efflux. All EPIs were used at four times the MEC for EtBr (Table 1), a saturating concentration in *S. aureus* ATCC 29213, except for VX-710, which was used at 100 $\mu\text{g}/\text{ml}$. Results presented in Fig. 2 are the averages from triplicate samples. As shown in Fig. 2, only the control cells without EPIs extruded EtBr, resulting in a significant decrease in fluorescence over the time of the assay. In the presence of each EPI, no loss of fluores-

FIG. 2. Effect of EPIs on EtBr efflux in *S. aureus* ATCC 29213. *S. aureus* cells were loaded with EtBr as described in Materials and Methods. EPIs were used at four times the MEC (25 µg of reserpine/ml, long dashes; 100 µg of VX-710/ml, short dashes; 6.25 µg of VX-853/ml, solid thin line; and no EPI control, solid thick line). Fluorescence was continuously monitored over time at room temperature, and results are an average from three replicate experiments. The standard deviations at the end of the experiment, at 294 seconds, were the following (in fluorescence units): no EPI, ± 4.7 ; reserpine, ± 4.5 ; VX-710, ± 6.9 ; and VX-853, ± 11.3 .

cence was observed, reflecting a blockage of EtBr efflux by reserpine, VX-710, or VX-853.

Potential of antibiotic activity by VX-710 and VX-853 against *S. aureus* ATCC 29213. One potential clinical advantage conferred by bacterial EPIs would be to reduce the MIC of antibiotics, allowing the use of lower doses to treat infections. VX-710 and VX-853 were tested at four times their respective MECs determined for EtBr efflux in combination with a variety of different classes of antibiotics against *S. aureus* ATCC 29213 (Table 2). Results showed that, similar to reserpine, VX-710 and VX-853 lowered the MICs of levofloxacin, ciprofloxacin, norfloxacin, gentamicin, novobiocin, tetracycline, and tetraphenylphosphonium bromide reproducibly by two- to fourfold. No effect was observed on the MICs of gatifloxacin, erythromycin, azithromycin, chloramphenicol, ceftriaxone, and linezolid. The results presented in Table 2 reflect the ability of EPIs to inhibit the normal level of antibiotic efflux expression in *S. aureus* ATCC 29213 in the absence of induc-

tion by prior antibiotic exposure or any mutation conferring overexpression of efflux pumps.

Hydrophilic fluoroquinolones such as norfloxacin and ciprofloxacin are well-known substrates for the NorA pump and several other less well-characterized pumps in *S. aureus* (10, 13). On the other hand, the more recent hydrophobic fluoroquinolones, such as gatifloxacin and moxifloxacin, are poorer substrates for NorA. Recently, one other pump in *S. aureus* which also recognizes the hydrophobic fluoroquinolones has been described (10). To investigate the potential of VX-710 and VX-853 to block the efflux of several fluoroquinolones in cells with constitutively overexpressed efflux pumps, we used *S. aureus* strains SA-1199B and SA-K2068 (Table 3). All EPIs were used at four times the MEC determined for EtBr efflux in *S. aureus* ATCC 29213, except for VX-710, which was used at 100 µg/ml, its maximal solubility limit. Results showed that for a NorA-overproducing strain (SA-1199B), VX-853 and VX-710 were effective in reducing MICs by 8- and 32-fold for norfloxacin, 2- and 8-fold for ciprofloxacin, 2- and 4-fold for gatifloxacin, and 1- and 2-fold for levofloxacin, respectively. For a non-NorA MDR pump-overproducing strain (SA-K2068), VX-853 and VX-710 were also effective in reducing the norfloxacin MICs by two- and eightfold, the ciprofloxacin MICs by four- and eightfold, the gatifloxacin MICs by two- and fourfold, and the levofloxacin MICs by twofold (both compounds), respectively. These results show that VX-853 and VX-710 are capable of potentiating the antibacterial activities of fluoroquinolones even in the presence of overexpressed efflux proteins, a likely scenario in clinical *S. aureus* isolates. Note that substantial reduction of fluoroquinolone MICs occurred in SA-1199B, which in addition to overexpressing NorA also contains a fluoroquinolone-resistant target-based mutation in *grlA* (A116E) (11).

Effects of VX-710 and VX-853 on the fluoroquinolone susceptibilities of *S. aureus* and *E. faecalis* clinical isolate panels. To be useful in combination therapy, an EPI needs to substan-

TABLE 2. Potentiation of antibiotic activity by EPIs in *S. aureus* ATCC 29213^b

Antibacterial agent	MIC (µg/ml) with no EPI	MIC (µg/ml) in the presence of 4 times the MEC of indicated EPI ^a		
		Reserpine	VX-710	VX-853
EtBr	6.2	1.6	0.2	1.6
Tetracycline	0.25	0.25	0.125	0.25
Novobiocin	0.125	0.063	0.063	0.063
Levofloxacin	0.25	0.125	0.125	0.125
Ciprofloxacin	0.25	0.063	0.063	0.125
Norfloxacin	1	0.5	0.25	0.5
Gentamicin	0.5	0.125	0.125	0.25
Tetraphenylphosphonium bromide	16	8	4	8

^a MEC is the minimal effective concentration determined for EtBr with ATCC 29213 (Table 1). The following concentrations were used: reserpine, 25 µg/ml; VX-710, 100 µg/ml; VX-853, 6.25 µg/ml.

^b Results were reproduced in a minimum of two independent experiments.

TABLE 3. Activity of VX-710 and VX-853 on the MICs of clinically used fluoroquinolones in susceptible and resistant *S. aureus* strains^b

Antibacterial agent	EPI ^a	MIC (μg/ml) for given <i>S. aureus</i> strain				
		ATCC 29213	SA-1199	SA-1199B ^c	SA-8325-4	SA-K2068 ^d
EtBr	None	6.2	6.2	25	3.1	12.5
	Reserpine	0.4	0.4	0.8	0.4	1.6
	VX-710	0.2	0.2	0.4	≤0.1	0.4
	VX-853	0.8	1.6	3.1	0.8	3.1
Norfloxacin	None	0.5	0.5	64	1	8
	Reserpine	0.25	0.125	4	0.25	1
	VX-710	0.125	0.125	2	0.25	1
	VX-853	0.5	0.25	8	0.25	4
Ciprofloxacin	None	0.25	0.25	4	0.25	4
	Reserpine	0.063	0.063	0.5	0.125	0.5
	VX-710	0.063	0.063	0.5	0.125	0.5
	VX-853	0.125	0.125	2	0.125	1
Levofloxacin	None	0.25	0.125	1	0.25	1
	Reserpine	0.125	0.125	0.5	0.25	0.5
	VX-710	0.125	0.125	0.5	0.25	0.5
	VX-853	0.125	0.125	1	0.25	0.5
Gatifloxacin	None	0.063	0.063	0.5	0.125	1
	Reserpine	0.063	0.063	0.125	0.125	0.25
	VX-710	0.063	0.063	0.125	0.125	0.25
	VX-853	0.063	0.063	0.25	0.125	0.5
Gentamicin	None	0.5	0.5	0.5	0.125	0.063
	Reserpine	0.125	0.125	0.125	0.063	0.063
	VX-710	0.125	0.125	0.125	0.063	0.063
	VX-853	0.125	0.125	0.125	0.063	0.063

^a EPIs were used at four-times the MEC determined for EtBr with ATCC 29213 (Table 1), except for VX-710, which was used at 100 μg/ml, its solubility limit.

^b Results were reproduced in a minimum of two independent experiments.

^c *S. aureus* mutant, NorA overproducer, GrlA A116E.

^d *S. aureus* mutant, non-NorA MDR pump overproducer.

tially reduce the MIC of an antibiotic to within the range of clinical susceptibility. Because many different resistance mechanisms are often present in clinical isolates, including multiple target-based mutations in addition to efflux mutations, the barrier to antibiotic efficacy is much greater. We examined the activities of VX-710 and VX-853, each at 50 μg/ml, in potentiating the activities of two fluoroquinolones, norfloxacin and ciprofloxacin, against panels of clinical isolates of *S. aureus* and *E. faecalis*, including several high-level fluoroquinolone-resistant mutants (Table 4). This relatively high concentration of EPI approached the solubility limit for both compounds and was chosen to maximize the chance of observing an effect in combination with antibiotics against isolates with multiple resistance mechanisms. Oxacillin and vancomycin MICs were also determined in order to identify which isolates were methicillin resistant (*S. aureus*) and vancomycin resistant (*E. faecalis*). Among the *S. aureus* isolates (Table 4), >2-fold decreases in the MICs of ciprofloxacin (10 of 15 isolates) and norfloxacin (13 of 15 isolates) in combination with VX-710 were observed; no significant (>2-fold) decrease in the MIC was seen with VX-853. Interestingly, significant increases in ciprofloxacin (isolates 3 and 5) and norfloxacin (isolates 3, 6, and 9) MICs in combination with VX-853 were observed for several *S. aureus* isolates, while reductions in MICs occurred with VX-710 for the same isolates. The reason for this phenomenon is unclear. Less of an effect was observed with both VX-710 and VX-853 and the *E. faecalis* isolates tested (Table 4); however, a twofold trend in the reduction of MICs of ciprofloxacin and norfloxacin in combination with VX-710 was observed, and a similar trend

was less apparent with VX-853. While VX-710 was generally more active in *S. aureus* than in *E. faecalis*, the activity was still insufficient to restore clinical susceptibility to the highly fluoroquinolone-resistant strains in the panel. VX-710 did significantly impact the fluoroquinolone MICs (4- to >8-fold) for the more susceptible *S. aureus* strains in the panel, suggesting a possible use in combination with fluoroquinolones in treating susceptible isolates.

DISCUSSION

One natural role of efflux pumps in prokaryotic and eukaryotic cells is to remove toxins from the interior of the cell. This protective function enables bacterial cells to survive in hostile environments, including the presence of antibiotics during the treatment of infections. The up-regulation of efflux systems through physiological induction and spontaneous mutation can significantly lower the intracellular concentration of many antibiotics, causing an impact on clinical efficacy. For this reason, many academic and pharmaceutical programs have focused on identifying inhibitors of gram-negative and gram-positive efflux systems that could potentially be used in combination with antibiotics to improve efficacy and suppress resistance (1, 2, 3, 8, 10, 21, 31, 34). In vitro, EPIs have been shown to reduce spontaneous resistance frequencies to antibiotics in *P. aeruginosa* (19), *S. pneumoniae* (20), and *S. aureus* (21, 22). In an animal model of *P. aeruginosa* infection, Renau et al. (32) showed that levofloxacin plus an EPI was more efficacious than levofloxacin alone, demonstrating the potential for combination therapy in vivo.

While there is limited structural homology between bacterial and mammalian efflux systems, there is significant substrate overlap (2, 27). Because of this overlap, it is not surprising that many mammalian MDR inhibitors, such as reserpine, verapamil, and GG918, also affect bacterial efflux (1, 8, 27). In this study, we have shown that two novel mammalian MDR inhibitors were active in potentiating the activity of EtBr against *S. aureus*, *E. faecalis*, and *S. pneumoniae* (Table 1). Since EtBr is a substrate of many different bacterial pumps, the number of pumps these EPIs are acting on in each organism is unknown. In the case of other antibiotics, it is also not clear whether the potentiating activities of VX-710 and VX-853 are due to the inhibition of one or more pumps (Table 2). For *S. aureus*, however, we have demonstrated that both VX-710 and VX-853 can partially reverse fluoroquinolone resistance conferred by the overexpression of at least two different pumps (Table 3). Furthermore, it is interesting that the activity of VX-710 on the MIC of EtBr was nonsaturable for *S. aureus*, *E. faecalis*, and *S. pneumoniae*, which might suggest the targeting of multiple efflux pumps.

The in vitro data reported here suggest that VX-710 and VX-853 are representatives of a novel class of bacterial EPIs that should be explored for their clinical potential. We observed that VX-710 was consistently more active than VX-853. However, in combination with ciprofloxacin and norfloxacin, VX-710 still fell short of restoring fluoroquinolone susceptibility to highly resistant clinical isolates (Table 4). These results do not rule out the possibility that other derivatives of this EPI class may be more effective in completely blocking fluoroquinolone efflux or that VX-710 may be more effective in combi-

TABLE 4. Effect of VX-710 and VX-853 on fluoroquinolone susceptibility of *S. aureus* and *E. faecalis* clinical isolates

Isolate	MIC (μg/ml) of:							
	Ciprofloxacin with:			Norfloxacin with:			Oxacillin with no EPI ^a	Vancomycin with no EPI ^a
	No EPI	VX-710 ^b	VX-853	No EPI	VX-710	VX-853		
<i>S. aureus</i>								
1	2	0.5	1	16	2	8	16	
2	0.5	0.12	0.5	1	≤0.25	1	>64	
3	0.12	≤0.06	1	1	≤0.25	4	32	
4	>64	64	64	256	128	128	>64	
5	0.12	≤0.06	1	1	≤0.25	2	2	
6	16	8	32	32	8	128	16	
7	0.25	≤0.06	0.5	2	≤0.25	2	2	
8	0.5	0.25	1	4	1	4	4	
9	0.5	≤0.06	1	1	≤0.25	4	2	
10	16	8	16	64	16	64	>64	
11	16	8	32	64	32	64	>64	
12	0.5	≤0.06	0.25	1	≤0.25	1	2	
13	>64	32	64	256	64	128	>64	
14	>64	32	64	256	64	128	>64	
15	>64	32	>64	256	64	256	>64	
<i>E. faecalis</i>								
1	1	0.5	1	4	2	2		1
2	1	0.5	1	4	1	2		4
3	64	32	64	128	64	128		>256
4	64	32	64	128	64	128		1
5	32	32	64	64	64	64		2
6	1	0.5	1	2	1	8		2
7	1	0.5	1	2	1	2		1
8	64	32	32	128	64	128		128
9	1	1	1	4	2	2		1
10	32	16	64	64	32	64		2
11	32	16	32	128	64	64		16
12	0.5	0.5	1	2	1	2		4
13	1	0.5	0.5	2	1	2		2
14	0.5	0.25	0.5	4	0.5	2		1
15	2	1	2	4	2	4		4
16	1	1	1	4	2	4		0.5
17	1	0.5	1	4	2	2		4
18	64	32	64	128	64	128		>256
19	1	1	1	4	2	4		2
20	1	0.5	1	4	2	2		2
21	0.5	0.5	1	2	1	4		1
22	64	32	64	128	64	128		1
23	64	32	32	64	32	64		2
24	32	16	32	>256	32	64		1
25	>64	32	64	128	64	128		1
26	64	32	64	>256	64	128		>256

^a The MIC indicates the methicillin- or oxacillin-resistant or -susceptible phenotype. Oxacillin breakpoint, ≥ 4 $\mu\text{g/ml}$; vancomycin breakpoint, ≥ 32 $\mu\text{g/ml}$.

^b EPIs were used at 50 $\mu\text{g/ml}$.

nation with other quinolone antibiotics. It is also possible that some highly resistant strains have acquired too many target-based mutations in topoisomerase IV and/or DNA gyrase, making it difficult, even with an ideal EPI, to achieve sufficient intracellular antibiotic concentrations to overcome the reduction in target binding affinity.

One advantage of this new class of EPIs is that VX-710 has already been evaluated for safety in the clinic and in combination with chemotherapeutic agents for cancer therapies. In these studies, only mild adverse events were observed with VX-710 (4, 29, 33, 35) compared to other EPIs (31). Furthermore, in bacteria, VX-710 and VX-853 have no intrinsic antibacterial activity, thereby demonstrating the specificity of their pharmacological effect. While VX-710 and VX-853 have been

shown to be active in gram-positive organisms, preliminary data with gram-negative organisms suggest that more bacterially permeable derivatives of this compound class could extend their usefulness to inhibiting gram-negative bacterial efflux mechanism(s) (data not shown).

It has been speculated that bacterial antibiotic EPIs with mammalian MDR activity may manifest toxicities in the clinic (17); proof of this remains to be demonstrated. In fact, a well-tolerated dually active bacterial and mammalian EPI may have some favorable pharmacological effects, such as the following: (i) promoting gastrointestinal absorption of antibiotic (raising plasma levels); (ii) improving permeation of the blood-brain barrier for central nervous system infections; (iii) increasing mammalian intracellular antibiotic concentrations for

the eradication of invasive pathogens; and (iv) enabling the use of lower concentrations of antibiotics to minimize their undesirable side effects. Such effects could significantly improve antibiotic efficacy by raising physiological levels of an antibiotic and act synergistically by reducing bacterial efflux. The potential clinical utility of this new class of bacterial efflux inhibitors as potentiators of antibiotic activity warrants further investigation.

ACKNOWLEDGMENTS

We thank Bill Markland, Eric Olson, and John Thomson for critically reviewing the manuscript. We also thank Ann Kwong, Matt Harding, Bob Kauffman, John Alam, and Michael Briggs for helpful discussions, suggestions, and support. We are grateful to James Karlowsky and Elena Karginova of Focus Technologies for their help in designing and performing the MIC₉₀ studies.

REFERENCES

- Aeschlimann, J. R., L. D. Dresser, G. W. Kaatz, and M. J. Rybak. 1999. Effects of NorA inhibitors on in vitro antibacterial activities and postantibiotic effects of levofloxacin, ciprofloxacin, and norfloxacin in genetically related strains of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **43**:335–340.
- Bambeke, F. V., E. Balzi, and P. M. Tulkens. 2000. Antibiotic efflux pumps. *Biochem. Pharmacol.* **60**:457–470.
- Barrett, J. F. 2001. MC-207110 Daiichi Seiyaku/Microcide Pharmaceuticals. *Curr. Opin. Investig. Drugs* **2**:212–215.
- Bramwell, V. H. C., D. Morris, D. S. Ernst, I. Hings, M. Blackstein, P. M. Venner, E. I. Ette, M. W. Harding, A. Waxman, and G. D. Demetri. 2002. Safety and efficacy of the multidrug-resistance inhibitor biricodar (VX-710) with concurrent doxorubicin in patients with anthracycline-resistant advanced soft tissue sarcoma. *Clin. Cancer Res.* **8**:383–393.
- Germann, U. A., D. Shlyakhter, V. S. Mason, P. J. Ford, and M. W. Harding. 1996. VX-853: a novel bispecific chemosensitizer which reverses P-glycoprotein- and MRP-mediated multidrug resistance, abstr. 2283. *Proc. 87th Annu. Meet.*, vol. 37. American Association for Cancer Research, Washington, D.C.
- Germann, U. A., P. J. Ford, D. Shlyakhter, V. S. Mason, and M. W. Harding. 1997. Chemosensitization and drug accumulation effects of VX-710, verapamil, cyclosporin A, MS-209 and GF120918 in multidrug resistant HL60/ADR cells expressing the multidrug resistance-associated protein MRP. *Anticancer Drugs* **8**:141–155.
- Germann, U. A., D. Shlyakhter, V. S. Mason, R. E. Zelle, J. P. Duffy, V. Galullo, D. M. Armistead, J. O. Saunders, J. Boger, and M. W. Harding. 1997. Cellular and biochemical characterization of VX-710 as a chemosensitizer: reversal of P-glycoprotein-mediated multidrug resistance in vitro. *Anticancer Drugs* **8**:125–140.
- Gibbons, S., M. Oluwatuyi, and G. W. Kaatz. 2003. A novel inhibitor of multidrug efflux pumps in *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **51**:13–17.
- Gill, M. J., N. P. Brenwald, and R. Wise. 1999. Identification of an efflux pump gene, *pmrA*, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **43**:187–189.
- Kaatz, G. W., V. V. Moudgal, and S. M. Seo. 2002. Identification and characterization of a novel efflux-related multidrug resistance phenotype in *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **50**:833–838.
- Kaatz, G. W., V. V. Moudgal, S. M. Seo, and J. E. Kristiansen. 2003. Phenothiazines and thioxanthenes inhibit multidrug efflux pump activity in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **47**:719–726.
- Kaatz, G. W., and S. M. Seo. 1995. Inducible NorA-mediated multidrug resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **39**:2650–2655.
- Kaatz, G. W., S. M. Seo, and C. A. Ruble. 1993. Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **37**:1086–1094.
- Kohler, T., J. C. Pechere, and P. L. Plesiat. 1999. Bacterial antibiotic efflux systems of medical importance. *Cell. Mol. Life Sci.* **56**:771–778.
- Koronakis, V., A. Sharf, E. Koronakis, B. Luisi, and C. Hughes. 2000. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405**:914–919.
- Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, K. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu. 2001. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* **357**:1225–1240.
- Lawrence, L. E., and J. F. Barrett. 2000. Inhibition of bacterial efflux: needs, opportunities, and strategies. *Curr. Opin. Antimicrob. Invest. Drugs* **2**:145–153.
- Lee, E. W., J. Chen, M. N. Huda, T. Kuroda, T. Mizushima, and T. Tsuchiya. 2003. Functional cloning and expression of *emeA*, and characterization of EmeA, a multidrug efflux pump from *Enterococcus faecalis*. *Biol. Pharm. Bull.* **26**:266–270.
- Lomovskaya, O., M. S. Warren, A. Lee, J. Galazzo, R. Fronko, M. Lee, J. Blais, D. Cho, S. Chamberland, T. Renau, R. Leger, S. Hecker, W. Watkins, K. Hoshino, H. Ishida, and V. J. Lee. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. *Antimicrob. Agents Chemother.* **45**:105–116.
- Markham, P. N. 1999. Inhibition of the emergence of ciprofloxacin resistance in *Streptococcus pneumoniae* by the multidrug efflux inhibitor reserpine. *Antimicrob. Agents Chemother.* **43**:988–989.
- Markham, P. N., and A. A. Neyfakh. 1996. Inhibition of the multidrug transporter NorA prevents emergence of norfloxacin resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **40**:2673–2674.
- Markham, P. N., E. Westhaus, K. Klyachko, M. E. Johnson, and A. A. Neyfakh. 1999. Multiple novel inhibitors of the NorA multidrug transporter of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **43**:2404–2408.
- Muñoz-Bellido, J. L., M. A. Alonso Manzanarez, J. A. Martínez Andrés, M. N. Gutiérrez Zufiaurre, G. Ortiz, M. Segovia Hernández, and J. A. García-Rodríguez. 1999. Efflux pump-mediated quinolone resistance in *Staphylococcus aureus* strains wild type for *gyrA*, *gyrB*, *grrA*, and *norA*. *Antimicrob. Agents Chemother.* **43**:354–356.
- Munoz-Bellido, J. L. M., G. Y. Guirao, N. G. Zufiaurre, and A. A. Manzanarez. 2002. Efflux-mediated antibiotic resistance in Gram-positive bacteria. *Rev. Med. Microbiol.* **13**:1–13.
- Murakami, S., R. Nakashima, E. Yamashita, and A. Yamaguchi. 2002. Crystal structure of bacterial multidrug efflux transporter AcrB. *Nature* **419**:587–593.
- National Committee for Clinical Laboratory Standards. 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 5th ed. Approved standard M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
- Neyfakh, A. A., V. E. Bidnenko, and L. B. Chen. 1991. Efflux-mediated multidrug resistance in *Bacillus subtilis*: similarities and dissimilarities with the mammalian system. *Proc. Natl. Acad. Sci. USA* **88**:4781–4785.
- Neyfakh, A. A., C. M. Borsch, and G. W. Kaatz. 1993. Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter. *Antimicrob. Agents Chemother.* **37**:128–129.
- Peck, R. A., J. Hewett, M. W. Harding, Y. M. Wang, P. R. Chaturvedi, A. Bhatnagar, J. Ziessman, F. Atkins, and M. J. Hawkins. 2001. Phase 1 and pharmacokinetic study of the novel MDR1 and MRP1 inhibitor biricodar administered alone and in combination with doxorubicin. *J. Clin. Oncol.* **19**:3130–3141.
- Putman, M., H. W. van Veen, and W. N. Konings. 2000. Molecular properties of bacterial multidrug transporters. *Microbiol. Mol. Biol. Rev.* **64**:672–693.
- Renau, T. E., R. Leger, L. Filonova, E. M. Flamme, M. Wang, R. Yen, D. Madsen, D. Griffith, S. Chamberland, M. Dudley, V. Lee, O. Lomovskaya, W. Watkins, T. Ohta, K. Nakayama, and Y. Ishida. 2003. Conformationally-restricted analogues of efflux pump inhibitors that potentiate the activity of levofloxacin in *Pseudomonas aeruginosa*. *Bioorg. Med. Chem. Lett.* **13**:2755–2758.
- Renau, T. E., R. Leger, E. Flamme, J. Sangalang, M. W. She, R. Yen, C. L. Gannon, D. Griffith, S. Chamberland, O. Lomovskaya, S. J. Hecker, V. J. Lee, T. Ohta, and K. Nakayama. 1999. Inhibitors of efflux pumps in *Pseudomonas aeruginosa* potentiate the activity of the fluoroquinolone levofloxacin. *J. Med. Chem.* **42**:4928–4931.
- Rowinsky, E. K., L. Smith, Y. M. Wang, P. Chaturvedi, M. Villalona, E. Campbell, C. Aylesworth, S. G. Eckhardt, L. Hammond, M. Kraynak, R. Drengler, J. Stephenson, M. W. Harding, and D. von Hoff. 1998. Phase I and pharmacokinetic study of paclitaxel in combination with biricodar, a novel agent that reverses multidrug resistance conferred by overexpression of both MDR1 and MRP. *J. Clin. Oncol.* **16**:2964–2976.
- Thorarensen, A., A. L. Presley-Bodanar, K. R. Marotti, T. P. Boyle, C. L. Heckaman, M. J. Bohanon, P. K. Tomich, G. E. Zurenko, M. T. Sweeney, and B. H. Yagi. 2001. 3-Arylpiperidines as potentiators of existing antibacterial agents. *Bioorg. Med. Chem. Lett.* **11**:1903–1906.
- Toppmeyer, D., A. D. Seidman, M. Pollak, C. Russell, K. Tkaczuk, S. Verma, B. Overmeyer, V. Garg, E. Ette, M. W. Harding, and G. D. Demetri. 2002. Safety and efficacy of the multidrug resistance inhibitor Incel (biricodar; VX-710) in combination with paclitaxel for advanced breast cancer refractory to paclitaxel. *Clin. Cancer Res.* **8**:670–678.
- Yu, E. W., G. McDermott, H. I. Zgurskaya, H. Nikaido, and D. E. Koshland, Jr. 2003. Structural basis of multiple drug-binding capacity of the AcrB multidrug efflux pump. *Science* **300**:976–980.
- Zhelezanova, E. E., P. N. Markham, A. A. Neyfakh, and R. G. Brennan. 1999. Structural basis of multidrug recognition by BmrR, a transcription activator of a multidrug transporter. *Cell* **96**:353–362.