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Review Article

Efflux pump inhibitors to address bacterial and fungal resistance

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Introduction

Efflux mechanisms are increasingly recognized as causing resistance to many classes of antibacterial and antifungal agents (1, 2). While certain pumps selectively extrude specific agents, others, referred to as multidrug resistance (MDR) pumps, expel a variety of compounds regardless of their structural class or mechanism of action (3). Bacterial organisms such as *Pseudomonas aeruginosa* are typically refractory to available therapies in the clinic primarily because of their ability to develop high level MDR due to an efflux mechanism. Similarly, the clinical efficacy of the antifungal agent fluconazole is increasingly compromised by the multidrug ATP-binding cassette (ABC) transporters CDR1 and CDR2 (4).

The inhibition of efflux pumps appears to be a logical strategy for improving the clinical efficacy of antibacterial and antifungal agents that are substrates of such pumps (5, 6). Conceptually, this tactic is similar to the successful approach of using a β-lactamase inhibitor to enhance the activity of β-lactams (7). In the following review we highlight several such efforts and demonstrate that the success of various preclinical efflux pump inhibitor (EPI) programs bodes well for possible future clinical applications.

Bacterial efflux pump inhibitors

The field of bacterial drug efflux has witnessed tremendous growth over the past few years, leading to an increasingly fundamental understanding of these pumps and their role in resistance (8-13). Perhaps the most important discovery from much of this work is that the combination of an efflux pump inhibitor and an antibacterial agent would be expected to significantly decrease the frequency of emergence of bacterial strains that are resistant to the antibacterial agent in the clinical setting (14, 15). Based on such studies, there is a growing consensus that strategies focused on the inhibition of these pumps may have a dramatic impact in addressing the problem of bacterial resistance. This can best be reflected in the number of reviews that have been published over the last few years outlining the opportunities that exist through just such an approach (16-18).

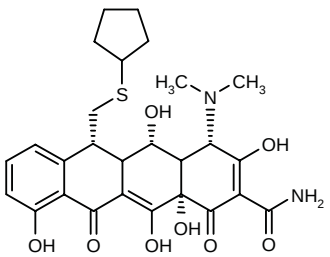
Various industrial and academic institutions have reported inhibitors of efflux pumps in both Gram-positive and Gram-negative bacteria (18). While in some cases these compounds block specific pumps, such as the NorA pump in *Staphylococcus aureus*, others inhibit entire classes of pumps, such as the resistance/nodulation/cell division family (RND) pumps in the Gram-negative bacterium *P. aeruginosa*. In all cases, though, the approaches have resulted in enhancement of the activity of a particular antibacterial agent.

Designing an appropriate strategy for potentiating the activity of a chosen antibacterial agent by inhibiting a particular bacterial efflux pump can be quite complicated (13). Key to this is a comprehensive knowledge of the factors that would lead to a productive effect, including the prevalence of efflux-mediated resistance and the multiplicity of efflux pumps. There must be an appreciation that various antibacterial agents are subject to efflux but at varying degrees, in diverse organisms and by different pumps.

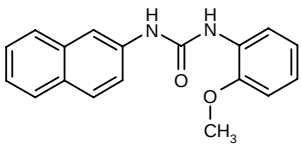
To date, inhibitors of three types of efflux pumps have been identified. They include inhibitors of the major facilitator (MF) tetracycline pumps, the NorA MF pump in Gram-positive bacteria and the RND MDR pumps in Gram-negative organisms.

Tet pump inhibitors

Various semisynthetic derivatives of tetracycline have been prepared and tested in an everted membrane vesicle system for their ability to inhibit the tetracycline



13-CPTC (**1**)



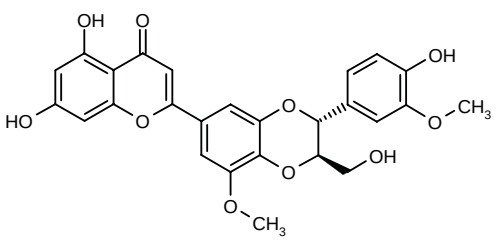
INF-271 (**2**)

specific efflux pump, TetB (19-21). These studies were notable in that they were the first to describe extensive structure-activity relationship (SAR) information regarding a class of pump specific transporter inhibitors. The most potent compound, 13-CPTC (**1**), was a competitive inhibitor of the TetB efflux pump. The combination of **1** with doxycycline against strains of *Escherichia coli* expressing either TetA or TetB resulted in a synergistic decrease in the MIC.

NorA pump inhibitors

Screening of a 9600 compound library led to the identification of various inhibitors of NorA, such as INF-271 (**2**), that potentiate the activity of ciprofloxacin in both NorA-overexpressing and wild-type strains of *S. aureus*. Notably, the compounds were shown to significantly suppress the emergence of ciprofloxacin-resistant *S. aureus* during *in vitro* attempts to select for fluoroquinolone resistance (22).

K. Lewis and colleagues have identified natural product inhibitors of the NorA MDR pump in *S. aureus* (23-25). The compounds, 5'-methoxyhydnocarpin-D (**3**) and the porphyrin pheophorbide *a* demonstrated no antibacterial activity and potentiated the activity of several NorA substrates such as norfloxacin. Based on these initial results, several hydnocarpin-like flavonolignans were synthesized and shown to potentiate berberine, a NorA substrate (26). The isolation of EPIs such as these from natural product sources suggest that pump inhibition may have evolved as a defense mechanism against natural pathogens.



(±) 5'-Methoxyhydnocarpin-D (**3**)

RND pump inhibitors

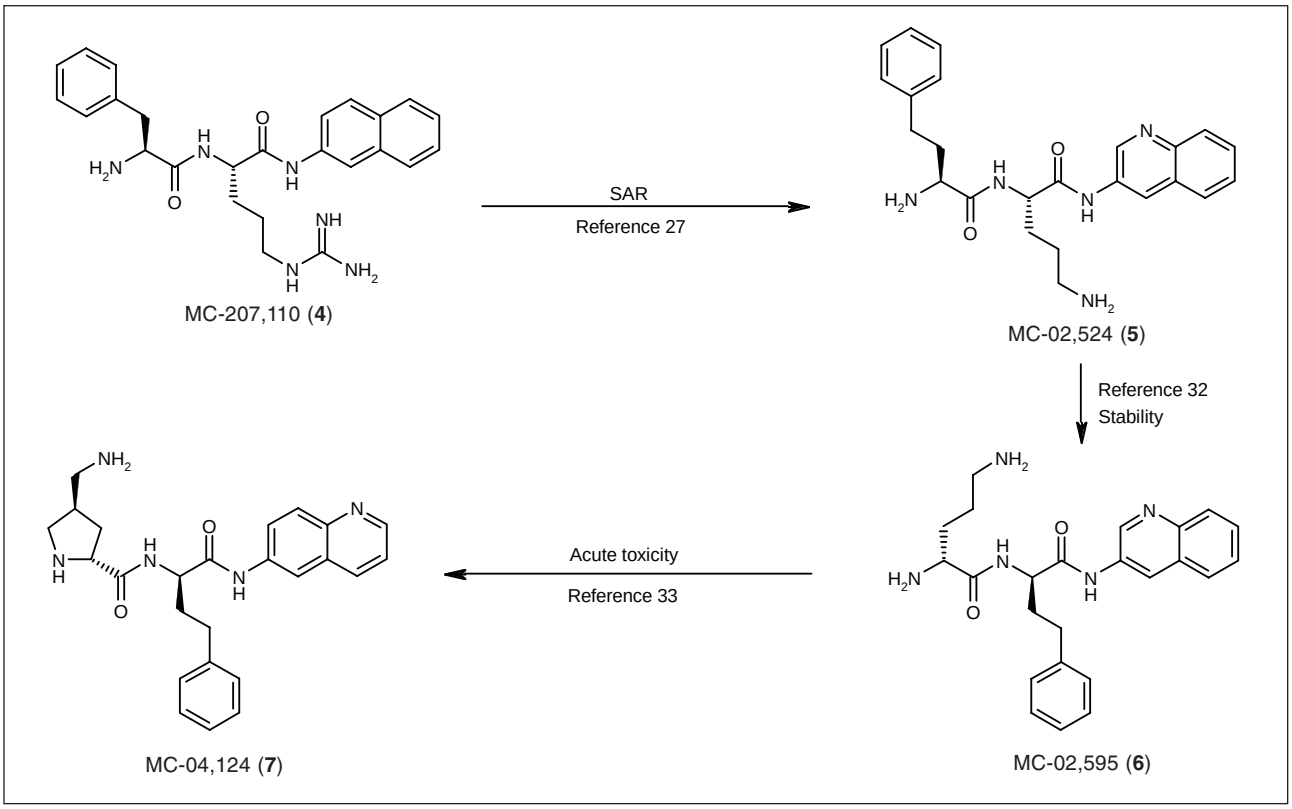
Microcide Pharmaceuticals (now Essential Therapeutics) and Daiichi Pharmaceuticals reported the first known inhibitors of the RND transporters in Gram-negative bacteria in 1999 (27, 28). The discovery program was aimed at identifying EPIs in *P. aeruginosa* that potentiated the activity of the fluoroquinolone levofloxacin. The initial strategy was to identify a broad-spectrum agent that would simultaneously inhibit the RND pumps MexAB-OprM, MexCD-OprJ and MexEF-OprN (a fourth pump, MexXY-OprM, was identified after the program was initiated (29)). A compound identified via high-throughput screening (HTS), MC-207,110 (**4**) (30), served as the lead in an extensive medicinal chemistry program that initially focused on SAR development, an effort that culminated in the identification of MC-02,524 (**5**). To establish selectivity, a collection of randomly chosen compounds from this study was examined for their ability to inhibit P-glycoprotein (Pgp), an efflux pump that is associated with multidrug resistance in mammalian cells (31). None of the compounds tested inhibited Pgp at concentrations where they inhibited the bacterial efflux pumps.

Studies to address the stability of **5** and related analogs led to MC-02,595 (**6**) (32), while approaches to reduce the acute toxicity of the entire series resulted in the conformationally restricted analog MC-04,124 (**7**) (33). Like other analogs from this series, **7** potentiated the MIC of levofloxacin 8-fold at 10 μg/ml. A thorough evaluation of the physicochemical properties, *in vitro* activity and pharmacokinetic profiles of analogs related to **7** was recently reported (34).

Compounds such as **6** and **7** were critical in providing clear evidence that the potentiation effect observed *in vitro* could be confirmed in an *in vivo* efficacy model, results that also demonstrated that an efflux pump inhibitor approach was a viable strategy in attempting to address the problem of bacterial resistance (35, 36).

Although originally identified as inhibitors of the RND pumps in *P. aeruginosa*, the class of compounds, highlighted by **7,** inhibits RND pumps in other Gram-negative bacteria. For example, the compound potentiates the *in vitro* activity of macrolides such as azithromycin against such Gram-negative bacteria as *E. coli*, *Haemophilus influenzae* and *Klebsiella pneumoniae* (37). These results were extended *in vivo*, where it was demonstrated that efficacy in animal models of infections due to *E. coli* strains with reduced susceptibility due to efflux pumps was enhanced by the combination of **7** and azithromycin (38).

Since the entire series was based on a peptide backbone, peptidomimetics of **6** were also prepared and eval-

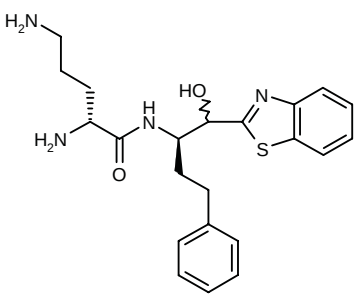


MC-207,110 (**4**)

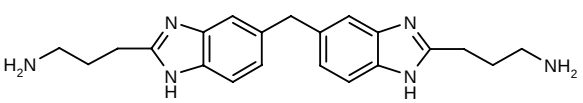
MC-02,524 (**5**)

MC-02,595 (**6**)

MC-04,124 (**7**)



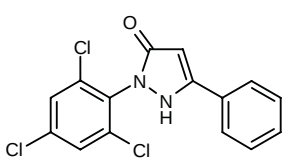
MC-03,749 (**8**)



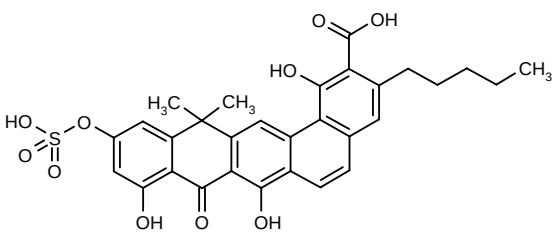
MC-278,537 (**9**)

uated for their ability to potentiate levofloxacin in *P. aeruginosa* (39). Several of these analogs, such as MC-03,749 (**8**), were as active or more active than **6**, demonstrating that a peptide backbone was not essential for activity. As an extension of this work, an entirely new class of broad-spectrum efflux pump inhibitors in *P. aeruginosa*, highlighted by the nonpeptidic benzimidazole MC-278,537 (**9**), has recently been described (40). Taken together, these results suggest that a di-cationic compound with an appropriate lipophilicity range, coupled with an overall topology comparable to that of compounds such as **7-9**, are features that lead to the inhibition of RND pumps.

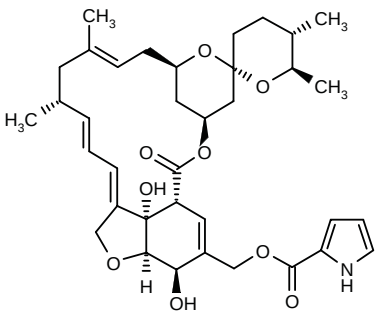
In the HTS that led to the discovery of **1** as an inhibitor of multiple RND efflux pumps, both natural products as well as synthetic compounds were discovered that inhibited each one of three Mex pumps in *P. aeruginosa* (41-43). Pump specific inhibitors, such as the selective MexAB-OprM EPIs MC-02,785 (**10**) and MC-510,050 (**11**), are attractive leads in that β-lactams, for example, are substrates for only MexAB-OprM, making the inhibition of the MexCD-OprJ or MexEF-OprN pumps essentially unnecessary.



MC-02,785 (**10**)



MC-510,050 (**11**)



MC-510,027 (**12**)

Fungal efflux pump inhibitors

Over the last three decades invasive fungal infections, especially in immunocompromised patients, have risen dramatically. A significant complication in current antifungal therapies has been the emergence of drug-resistant pathogens (44-46). Among the recognized mechanisms of resistance, the overexpression of multidrug ABC transporters and multidrug major facilitator superfamily transporters has been associated with azole intrinsic or acquired resistance in *Candida* spp. (47-50) and in *Aspergillus fumigatus* (51), two of the most serious pathogenic fungi. As encouraging results emerge in the case of bacterial EPIs, inhibition of the efflux systems in fungi seems to be an attractive approach for the treatment of infections due to resistant fungi.

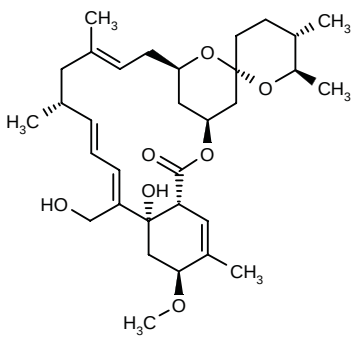
Recently, a group at Microcide Pharmaceuticals (now Essential Therapeutics) reported the isolation and characterization of the first known fungal efflux pump inhibitors (FEPIs) (52, 53). The molecules were identified in a HTS of a library of natural products and synthetic compounds for their ability to potentiate the activity of fluconazole in CDR-pump-overexpressing strains of *Candida albicans* and *Candida glabrata*. Several classes of FEPIs were identified, including broad-spectrum compounds, active against multiple CDR pumps from both *C. albicans* and *C. glabrata*, as well as narrow spectrum agents, which were active only against the CDR1 pump from *C. albicans*. In order to understand the differences in pump selectivity, mutagenesis of a *Saccharomyces cerevisiae* strain expressing the CDR1 pump of *C. albicans* was performed in an attempt to isolate mutants that were resistant to potentiation by various FEPIs. The mutations were sequenced to the *cdr1* gene, thereby providing genetic evidence that the inhibitors interact directly with the efflux pump (54). The experiments also demonstrated that inhibition of the CDR1 pump could be achieved by interaction with various binding sites.

MC-510,027 (**12**), a natural product from the milbemycin family (55), was shown to selectively inhibit *C. albicans* and *C. glabrata* CDR pumps while lacking activity against the major facilitator efflux system. Compound **12** was active against all clinically relevant wild-type *Candida* spp. It potentiated the activity not only of fluconazole but itraconazole, posaconazole and terbinafine as well (56). Its mode of action as an EPI was demonstrated by its ability to increase intrinsic susceptibility to known pump substrates (*e.g.*, fluconazole, terbinafine and rhodamine 6G), but not to agents not subject to efflux (amphotericin B).

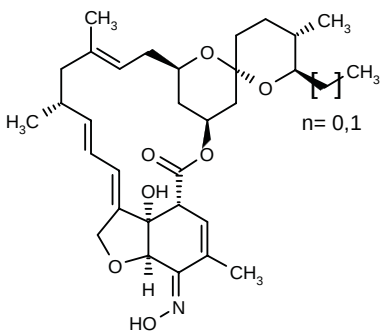
As with the use of bacterial EPIs, coadministration of a FEPI such as MC-510,021 (**13**) with fluconazole reduced the frequency of emergence of resistance to fluconazole in *C. glabrata*. (55)

The *in vivo* efficacy of FEPIs was first demonstrated in the case of a synthetic FEPI, MC-05,172 (**14**, structure not disclosed). In a mouse fungal kidney infection model with a fluconazole-resistant strain of *C. albicans*, compound **14**, at a single dose of 100 mg/kg, was shown to reduce the effective *in vivo* MIC of fluconazole from 128 μg/ml to 5 μg/ml (57). Later, *in vivo* efficacy was also demonstrated in the milbemycin series of FEPIs (58). Further, it was shown that the antifungal effect of fluconazole against either fluconazole-susceptible or fluconazole-resistant *C. albicans* isogenic strains in a mouse model of pyelonephritis was enhanced by coadministration with the milbemycin MC-510,011 (**15**) in a dose-dependent manner.

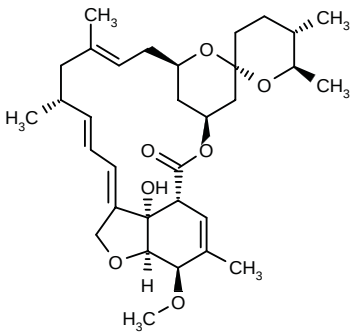
The first medicinal chemistry report on FEPIs appeared in late 2001 (59). In this report, it was anticipated that changing the physical properties of the milbemycins (*i.e.*, decreasing their lipophilicity) might help in



MC-510,021 (**13**)
MPC_8 = 2 μg/ml
TC_{50} = 1.9 μg/ml



MC-510,011 (**15**)
$MPC_8 \leq 0.0625$ μg/ml
TC_{50} = 2.4 μg/ml

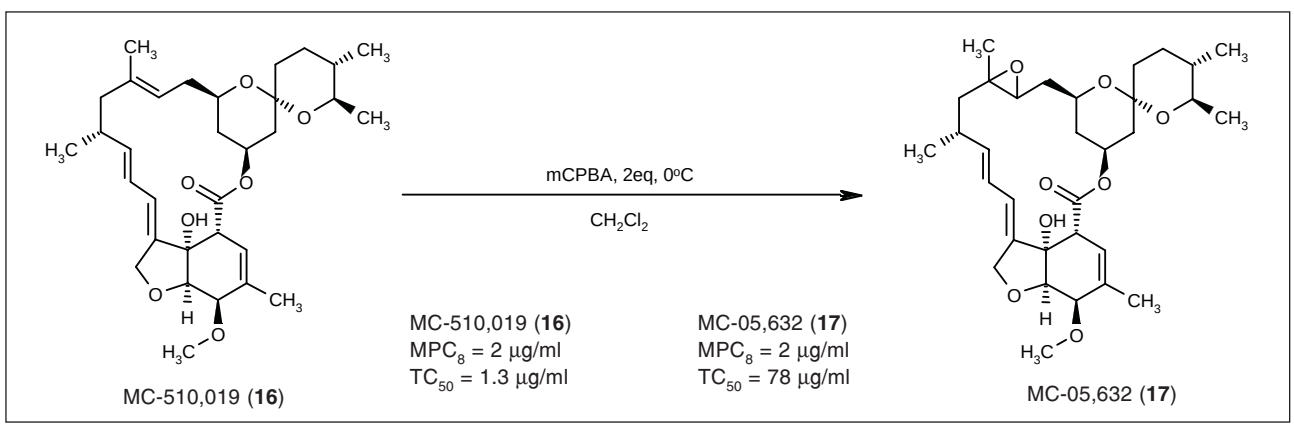


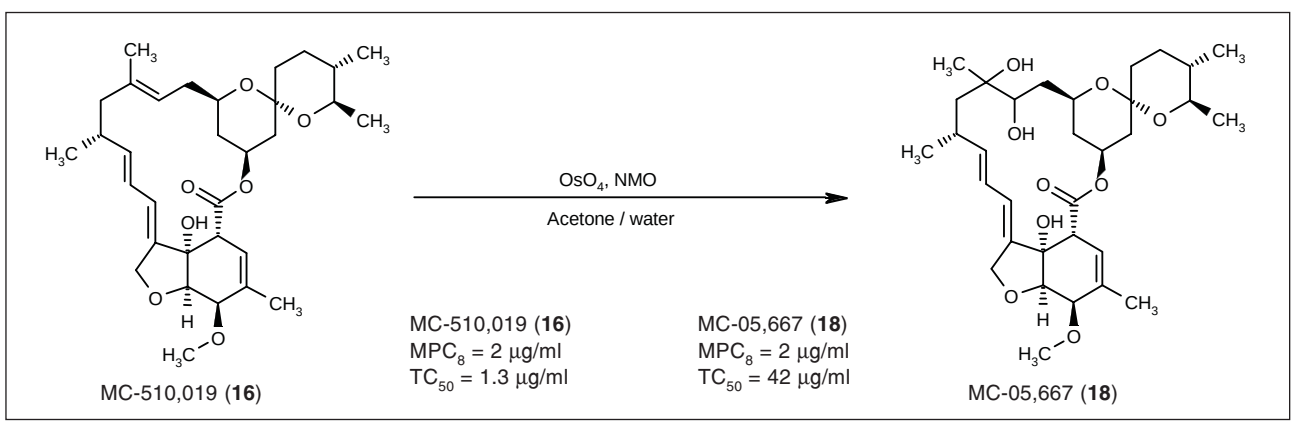
MC-510,019 (**16**)
MPC_8 = 2 μg/ml
TC_{50} = 1.3 μg/ml

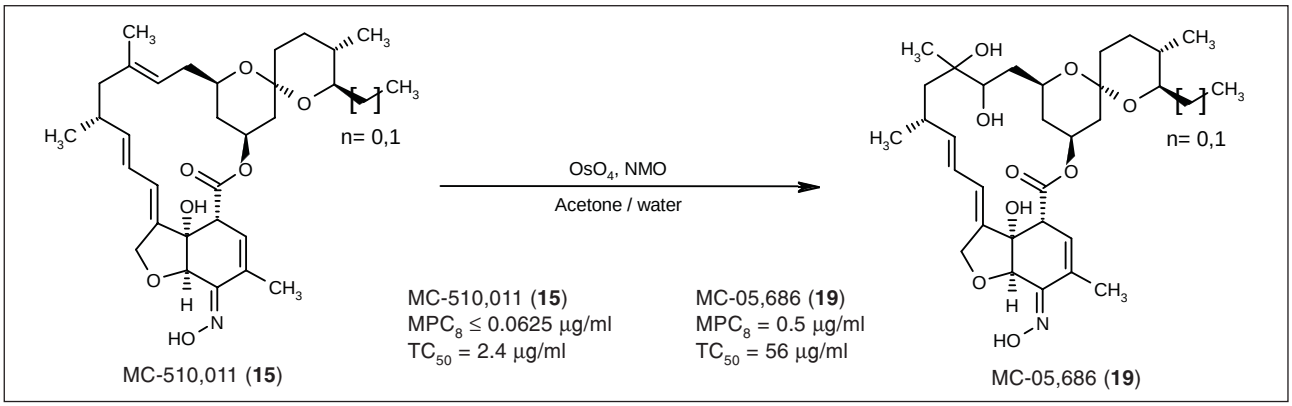
differentiating FEPI activity from intrinsic cytotoxicity, thereby increasing the therapeutic window of the milbemycins. This was a concern due to high cytotoxicity of the milbemycins. It was also anticipated that the poor water solubility of this series might be improved as well. The authors decreased the global lipophilicity of the milbemycins by introducing either a positive or a negative charge, or by introducing heteroatoms into the molecule. The chemical modifications were performed on three milbemycins: **13**, **15** and **16**. Against a strain of *C. albicans* overexpressing CDR pumps, the concentrations of FEPI required to reduce the *in vitro* MIC of fluconazole 8-fold (MPC_8) were 0.0625, 2 and 2 μg/ml for **15**, **13** and **16**, respectively, whereas the TC_{50} measures of cytotoxicity of the compounds in the K-562 human chronic myelogenous leukemia cell line were 2.4, 1.9 and 1.3 μg/ml, respectively. The authors were successful in differentiating FEPI activity from cytotoxicity when they introduced heteroatoms in the milbemycin scaffold. For example, epoxidation of the Δ-14 olefin of **16** gave MC-05,632 (**17**), a semisynthetic milbemycin equipotent to its parent milbemycin but 60-fold less cytotoxic. Similarly, the dihydroxylation of the same double bond gave MC-05,667 (**18**), an equipotent FEPI where the cytotoxicity was reduced 33-fold. Dihydroxylation of the Δ-14 olefin of **15** led to the formation of MC-05,686 (**19**), a compound 23 times less cytotoxic than **15** but where the FEPI activity was somewhat reduced. Nevertheless, **19** was still a very potent FEPI with a concentration required to reduce the MIC of fluconazole 8-fold *in vitro* equal to 0.5 μg/ml. As anticipated, the water solubility of **19** (150 μg/ml at pH 7) was 150-fold greater than that of **15**.

Summary

Several series of prototype EPIs and FEPIs have emerged that have provided the critical proof-of-principle potentiation experiments both *in vitro* and *in vivo* to validate the inhibitor approach. As more compounds are







identified and disclosed over the next several years it should become increasingly clear whether the inhibition of these pumps in bacteria and fungi can address an unmet clinical need in infectious disease.

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