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Microbial fermentation-derived inhibitors of efflux-pump-mediated drug resistance

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Abstract

A library of 85 000 microbial fermentation extracts was screened for inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa* and *Candida albicans*. New compounds EA-371α and EA-371δ were isolated and demonstrated to be potent and specific inhibitors of the MexAB–OprM pump in *P. aeruginosa*. Two series of fungal metabolites, enniatins and beauvericins, were found to be ubiquitous and potent inhibitors of ABC transporters. Milbemycins were rediscovered as potent inhibitors of the CDR1 pump in *C. albicans*, and demonstrated to potentiate effectively the antifungal activity of fluconazole and SCH-56592 against a wide variety of *Candida* clinical isolates.

1. Introduction

The development and clinical use of antibiotics has significantly decreased the morbidity and mortality associated with microbial infections. In response to the pressure of antibiotics, microbes have developed multiple drug resistance mechanisms that are threatening the clinical effectiveness of antimicrobial therapy. Drug inactivation and target modification are two well-studied and well-characterized mechanisms of drug resistance [1,2]. More recently, active efflux of antibiotics out of cells by membrane transporter proteins (drug efflux pumps) has been recognized as a major cause of bacterial resistance among diverse classes of antibiotics, including β-lactams, macrolides, tetracyclines, fluoroquinolones and antifungal azoles [3–8]. Among the various resistance mechanisms, active efflux of antibiotics is the most ubiquitous. In addition to acquired resistance, antibiotic efflux by microbes is responsible for much observed intrinsic antibiotic non-susceptibility [9]. Thus, inhibition of microbial efflux pumps is expected to restore simultaneously the usefulness and broaden the antimicrobial spectrum of relevant existing antibiotics.

Drug-resistant efflux pumps in bacteria and fungi are classified based on the energy source they use to export their substrates and sequence homology. ATP hydrolysis is the energy source used by ABC transporters, whereas proton motive force is the energy source of SMR, MFS, MATE, and RND families of transporters [5]. Inhibitors of different classes of pumps are expected to have unrelated chemical structures. The results of screening a natural product extract library for inhibitors of the RND and the ABC families of efflux pumps will be presented in this paper.

2. The natural product extract library

The extract library at Microcide is composed of approximately 85 000 microbial fermentation extracts derived from 3600 strains of actinomycetes and 3500 strains of fungi. In order to harness the widest expression of chemical diversity, each strain was fermented in four complementary media, and each fermentation was harvested at three different elapsed times. To ensure reproducibility, each fermentation was conducted with a seed-stage and a production-stage in 250 ml shake flasks. To maximize the recovery of secondary metabolites, the cell pellet from each fermentation was extracted with wet acetone and the corresponding supernatant was processed on a solid-phase extraction cartridge. The pellet extract and the solid-phase extract derived from each fermentation were combined, creat-

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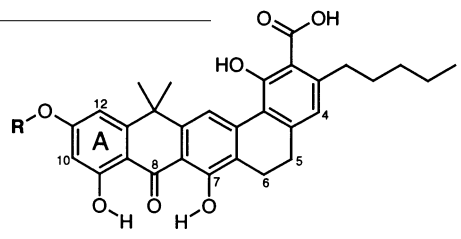
ing 12 extracts from each producing strain for the library. The extracts were formatted in multiple copies of 96-well plates for screening and half of each extract (the equivalent of a 10 ml fermentation) was set aside to create a retention extract bank. The retention extracts were used for confirmation and secondary assays following high throughput screening. In some instances, the retention extracts were also used for chemical dereplication by LC–UV and LC–MS methods.

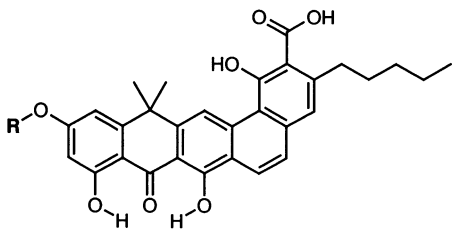
3. Potentiation screens for efflux pump inhibitors

Studies of antibiotic susceptibility patterns of isogenic strains of *Pseudomonas aeruginosa* and *Candida albicans* that overexpress or lack individual efflux pumps allowed us to define the expected maximal potentiation effect of an efflux pump inhibitor [10–12]. The growth of a pump-overexpressing strain is not affected in the presence of subinhibitory concentrations of an antibiotic that is a substrate of the pump, because active efflux maintains a low intracellular concentration. However, when an efflux pump inhibitor is added to the growth media, the growth of the same strain is inhibited because the inactivated pump permits the intracellular antibiotic concentration to rise above the minimal inhibitory concentration. The natural product extract library above was screened in a levofloxacin potentiation assay against *P. aeruginosa*, and a fluconazole potentiation assay against *C. albicans* and *Candida glabrata*. To screen for efflux pump inhibitors, the test samples were assayed against a pump-overexpressing strain grown in the presence (‘+’ condition) and in the absence (‘−’ condition) of a subinhibitory concentration of the antibiotic of interest. A compound that acts as a pure pump inhibitor will cause growth inhibition in the ‘+’ condition but will have no observable effect in the ‘−’ condition. Extracts causing significantly more inhibition in the ‘+’ condition than in the ‘−’ condition are scored as ‘hits’ and the hits were validated in a dose titration format under both the ‘+’ and the ‘−’ conditions. The ideal outcome is growth inhibition, under the ‘+’ condition, over a large concentration range of the extracts and no inhibition, under the ‘−’ condition, over all concentrations.

4. Inhibitors of MexAB and MexEF pumps of *P. aeruginosa*

Much of the acquired antibiotic resistance of *P. aeruginosa* to fluoroquinolones is due to the overexpression of MexAB–OprM, MexCD–OprJ, and MexEF–OprN pumps. MexAB–OprM pump, together with the recently identified MexXY–OprM pump, is also involved in the intrinsic antibiotic resistance of *P. aeruginosa* [13]. These three component trans membrane pumps of the RND family collectively extrude antibiotics (and other chemicals) of practically every structure type. In order to find pump inhibitors that augment the activity of the fluoroquinolone antibiotic, levofloxacin, against resistance *P. aeruginosa*, we screened the extract library in a ‘levofloxacin potentiation’ assay using strains of *P. aeruginosa* overexpressing either the MexAB–OprM or the MexEF–OprN pump. Four new natural products were discovered from the screening of 78 000 extracts, and two of the new compounds, EA-371α (**1**) and EA-371δ (**2**), were produced by a new strain of *Streptomyces* closely related to *Streptomyces vellosus*. The structure and bioactivity of **1** and **2** are presented below.





1, EA-371α: R = SO_3H
3, Benastatin B: R = H

2, EA-371δ: R = SO_3H
4, Benastatin A: R = H

Compounds **1** and **2** were isolated from the fermentation extract of *Streptomyces* MF-EA-371-NS1 by bioassay-guided isolation using the levofloxacin potentiation assay. Benastatin A (**4**) and benastatin B (**3**) [14], inhibitors of glutathione *S*-transferase, were coproduced in the fermentation but were inactive in the levofloxacin potentiation assay. The chemical structures of **1** and **2** were determined by homonuclear and heteronuclear 2D NMR techniques and mass spectrometry. The location of the sulfate group on C-11 was determined based on proton chemical shift differences in ring A between **1** and **3**, and HMBC data in acetone-d_6 showing that C-9 contains a hydroxyl group. As shown in Table 1, EA-371α (**1**) and EA-371δ (**2**) demonstrated specific inhibitory activity against the MexAB–OprM pump. In a whole-cell competition assay with an efflux substrate, direct pump inhibition by EA-371α was demonstrated by intracellular EA-371α

concentration-dependent accumulation of the substrate in cells overexpressing the MexAB–OprM pump, whereas no effect was observed in cells with triple pump deletion (Fig. 1). Substrate accumulation is detected as an increase in fluorescence, since the substrate used for this assay is enzymatically hydrolyzed inside the cells to a fluorescent product. Although EA-371α shows modest cytotoxicity and is not a practical lead candidate for drug discovery, it has proved useful as a molecular tool for further studies of *P. aeruginosa* efflux pumps.

5. Inhibitors of CDR pumps of *C. albicans* and *C. glabrata*

Fluconazole resistance in clinical isolates of *C. albicans* is primarily due to the overexpression of an MFS family pump (BenR) and two pumps of the ABC transporter family (CDR1 and CDR2). Fluconazole resistance in *C. glabrata* has been correlated to the overexpression of a cgCDR pump that has high DNA homology to the CDR1 pump in *C. albicans*. We screened our extract library in a 'fluconazole potentia-

Table 1
Efflux pump inhibitory activity of EA-371α and EA-371δ

Efflux pump inhibitor (EPI)	*P. aeruginosa* strain	EPI MIC (μg/ml)	Levofloxacin MIC (μg/ml)	EPI MPC_4 [a] (μg/ml)	EPI MPC_8 [a] (μg/ml)
EA-371α (**1**)	PAM1020 (wild type)	512	0.25	5	>40
	PAM1032 (MexAB–OprM overexpr)	>512	2	0.625	2.5
	PAM1033 (MexCD–OprJ overexpr)	512	4	>40	>40
	PAM1034 (MexEF–OprN overexpr)	512	4	>40	>40
	PAM1626 (triple pump deletion)	512	0.015	>40	>40
EA-371δ (**2**)	PAM1020 (wild type)	512	0.25	10	>40
	PAM1032 (MexAB–OprM overexpr)	512	2	0.625	1.25
	PAM1033 (MexCD–OprJ overexpr)	512	4	>40	>40
	PAM1034 (MexEF–OprN overexpr)	512	4	>40	>40
	PAM1626 (triple pump deletion)	512	0.015	>40	>40

[a] MPC_4 and MPC_8 are the minimum concentration (μg/ml) of EPI that decreases the MIC of levofloxacin by fourfold and eightfold respectively.

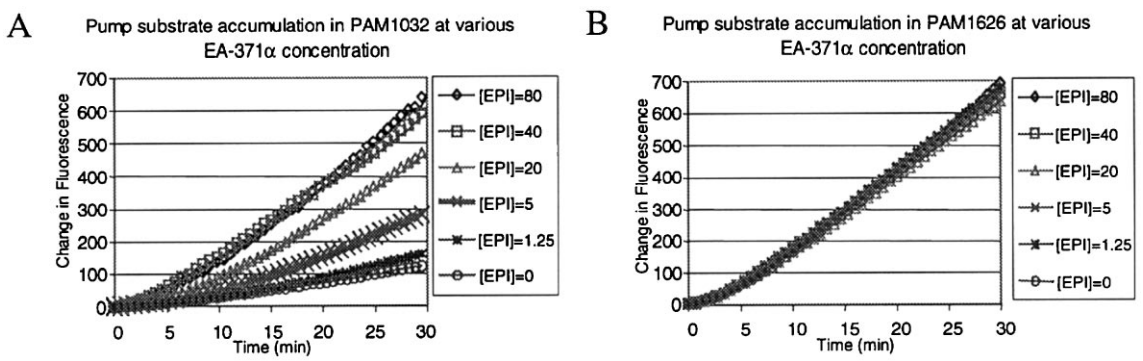


Fig. 1. (A) EA-371α concentration-dependent accumulation of pump substrate in MexAB–OprM overexpressing *P. aeruginosa* strain (PAM1032), and (B) the lack of effect of EA-371α on substrate accumulation by triple-pump-deleted *P. aeruginosa* strain (PAM1626). The pump substrate used in this assay yields a fluorescent compound upon enzymatic hydrolysis inside the bacterial cell. Inhibition of the efflux pump causes the substrate to accumulate inside the cells and results in increased fluorescence after enzymatic hydrolysis. Concentrations of EA-371α at 1, 1.25, 5, 20, 40, and 80 μg/ml were used in this study.

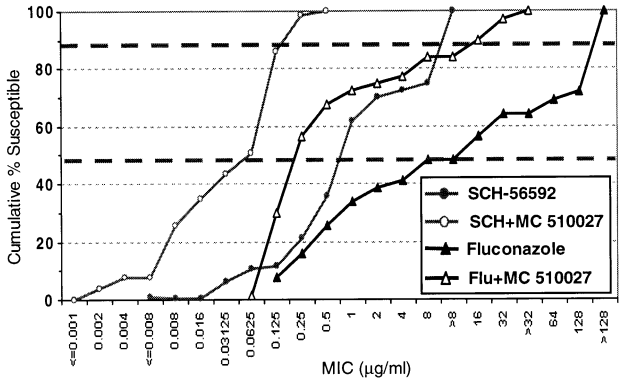


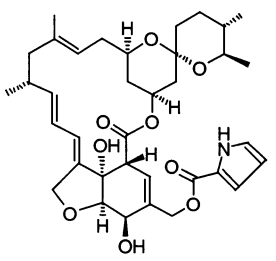
Fig. 2. Cumulative susceptibility of 171 diverse clinical isolates of *C. albicans* to fluconazole and SCH-56592 in the presence and absence of 10 μg/ml of milbemycin α_9.

tion' assay using a strain of *C. albicans* overexpressing the CDR1 pump and a strain of *C. glabrata* overexpressing the cgCDR pump. The overall hit rate of this screen is quite high, and the majority of the hits were produced by fungi. The hit extracts were profiled in a dose titration format in fluconazole potentiation assays against four different pump-overexpressing strains of *Candida*. Extracts with similar profiles were grouped together and representative members of each group were refermented for bioassay-guided isolation. Several enniatins and beauvericins were isolated and fully characterized. LC–UV–MS studies using retention extracts confirmed the presence of enniatins or beauvericins in many of the other hit extracts. The enniatins and the beauvericins both proved to be potent inhibitors of ABC transporters in a substrate accumulation assay similar to that described above. One extract with a unique activity profile was found to contain cytochalasin H and a new compound, both of which showed moderate activity against the BenR pump only. This same extract, however, also contained a small amount of beauvericin, and the unique extract profile can be attributed to the sum of the effects of the three isolated compounds.

The most interesting fungal efflux pump inhibitors discovered in our study are the milbemycins, which are produced by a number of our *Streptomyces* strains. Milbemycins are of commercial interest as insecticides and anthelmintic agents. In our study they are also potent inhibitors of the ABC transporter family of efflux pumps. Over 20 milbemycins were isolated and evaluated against CDR pump-overexpressing laboratory *Candida* strains and clinically isolated fluconazole resistance *Candida* strains. From this effort we were able to glean a structure–activity relationship, and the SAR of the milbemycins as efflux pump inhibitors is quite different from that observed as anthelmintic agents. Milbemycin α_9 (**5**) was found to enhance the activity of azoles and terbinafine against *C. albicans* and *C. glabrata* overexpressing ABC transporters as well as wild-type *C. albicans* and *C. glabrata* [15]. Fig. 2 shows the ability of milbemycin α_9 to decrease the MIC of fluconazole and SCH-56592 against a collection of recent clinical isolates of *C. albicans*. Milbemycin α_9 was also found to enhance the activity of azoles and terbinafine against recent clinical isolates of other *Candida* species, including *Candida guillermondii*, *Candida. lipolytica*, *Candida lusitaniae*, *Candida pseudotropicalis*, and *Candida stellatoidea*, as shown in Table 2.

In conclusion, our microbial fermentation extract library has proved to be a rich source of multiple

Table 2
Milbemycin α_9 (**5**) as an efflux pump inhibitor in combination with SCH-56592 (an antifungal azole currently under development) against *Candida* clinical isolates



Milbemycin α_9 (**5**)

Yeast	Number of strains (n)	MIC of SCH-56592 in the presence of Milbemycin α_9			
		MIC_{50} (μg/ml)		MIC_{90} (μg/ml)	
		W/O**	W**	W/O**	W**
C. albicans	171	1	0.06	>8	0.25
C. glabrata	50	2	0.125	8	0.5
C. krusei	39	0.5	0.06	2	0.06
C. tropicalis	41	>8	0.008	>8	0.016
C. parapsilosis	34	0.125	0.016	0.5	0.03
Other *Candida**	11	0.25	<0.001	>8	0.06

* *C. guillermondii* (n=2); *C. lipolytica* (n=1); *C. lusitaniae* (n=5); *C. pseudotropicalis* (n=2); *C. stellatoidea* (n=1).

** "W/O" is without addition of milbemycin α_9, "W" is with addition of milbemycin α_9 (1 μg/ml for *C. glabrata,* 10 μg/ml for all other *Candida*.)

drug-resistant efflux pump inhibitors. The efflux pump inhibitory activity of two new compounds, EA-371α and EA-371δ, against the *P. aeruginosa* MexAB–OprM pump was clearly demonstrated. Milbemycin α_9, as an efflux pump inhibitor in combination with antifungal azoles, was shown to increase the susceptibility of a wide selection of recent clinical isolates of *Candida* by as much as 16-fold.

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