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# Fragment-Based Strategy for Investigating and Suppressing the Efflux of Bioactive Small Molecules

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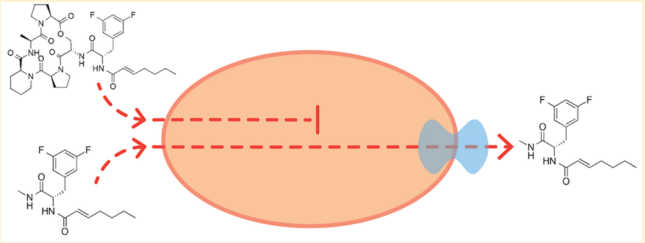
Supporting Information

**ABSTRACT:** Membrane protein-mediated drug efflux is a phenomenon that compromises our ability to treat both infectious diseases and cancer. Accordingly, there is much interest in the development of strategies for suppression of the mechanisms by which therapeutic agents are effluxed. Here, using resistance to the cyclic acyldepsipeptide (ADEP) antibacterial agents as a model, we demonstrate a new counter-efflux strategy wherein a fragment of an actively exported bioactive compound competitively interferes with its efflux and potentiates its activity. A fragment comprising the *N*-heptenoyldifluorophenylalanine side chain of the pharmacologically optimized ADEPs potentiates the antibacterial activity of the ADEPs against actinobacteria to a greater extent than reserpine, a well-known efflux inhibitor. Beyond their validation of a new approach to studying molecular recognition by drug efflux pumps, our findings have important implications for killing *Mycobacterium tuberculosis* with ADEPs and reclaiming the efficacies of therapeutic agents whose activity has been compromised by efflux pumps.



**KEYWORDS:** *efflux, drug resistance, cyclic acyldepsipeptide (ADEP), Streptomyces, Mycobacterium*

Organisms from all kingdoms of life exhibit some measure of resistance to toxic compounds due to the presence and activity of efflux pumps.[1] These membrane-bound proteins are problematic in medicine because they can compromise the efficacies of therapeutic small molecules.[2] Indeed, the treatment of infectious diseases is becoming more challenging because nearly all classes of antimicrobial drugs are known to be acted upon by efflux pumps.[3−11] Moreover, drug resistance phenotypes of pathogenic microorganisms such as *Candida albicans*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis* can be ascribed to genes encoding these proteins.[3,5,9,10,12] Remarkably, some efflux pumps exhibit high specificity for a particular molecule, whereas others act upon molecules of disparate structural classes.[1,6,8,13−17] Those in the latter category underlie a worrisome phenomenon wherein the acquisition of a single genetic locus results in a multidrug resistance phenotype.

Recently, insights into the mechanistic bases of small molecule recognition and export have been gleaned from the few available structures of efflux pumps in complex with their ligands.[18−21] It is anticipated that these structures can guide the design of either efflux inhibitors of various mechanistic classes[3,12,21,23−32] or drugs that are recalcitrant to export.[22] However, structure-based design is not a trivial proposition for many reasons. For example, the identities of efflux pumps underlying a drug resistance phenotype are not always known. Even when the identity of an efflux pump of interest is known, the requisite elucidation of its structure and the means by which it recognizes its ligand are likely to be arduous. A further complication of structure-based design in this context is that the structures of drugs can rarely be altered in ways that preclude efflux without negatively affecting bioactivity.[22,29] To date, a more fruitful approach to the discovery of efflux inhibitors has been high-throughput screens for small molecules that potentiate drug activity against organisms harboring efflux pumps.[3,8,25,27,32] Nevertheless, screening suffers from its reliance on serendipity and has yet to yield a clinically used efflux inhibitor.

Given the challenges of structure-based design and the low "hit" rates of high-throughput screening, we have been exploring an alternative, rational strategy for the development of compounds that perturb drug efflux. Our premise is that efflux is contingent on a molecular recognition event, wherein the pump binds either the entire molecule or a substructure thereof. In the latter scenario, a fragment of an exported molecule containing the moiety recognized by the pump could be acted upon likewise. Accordingly, we predict that such a fragment could potentiate the activity of the full bioactive molecule by competitively interfering with efflux. The key advantage of this approach is that it does not require knowledge of the identity, structure, or mechanism of the efflux pump acting upon the bioactive molecule. Equally important, evidence of fragment-based drug potentiation would reveal insights into how an efflux pump recognizes a bioactive compound, which are notoriously difficult to acquire and could be valuable in designing efflux-resistant drugs.

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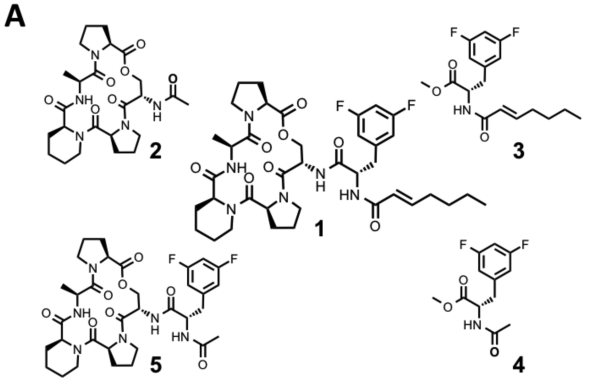
## RESULTS AND DISCUSSION

To assess the viability of the fragment-based strategy for competitively interfering with efflux, we applied it to the discovery of small molecules that suppress efflux of the cyclic acyldepsipeptides (ADEPs), a promising group of antibacterial drug leads.[33−38] These natural products have been extensively optimized via medicinal chemistry efforts;[34−37] one analogue, called ADEP 4, has demonstrable activity in animal models of bacterial infection.[33,35,36] The ADEPs' mechanism of action is distinct from all clinically used antibacterial agents. They bind and dysregulate the catalytic activity of the ClpP peptidase, triggering cell death via the indiscriminate degradation of cellular proteins.[39−41] Although the ADEPs exhibit impressive activity against a wide range of Gram-positive bacterial pathogens such as *S. aureus*, *Streptococcus pneumoniae*, and the *Enterococci*,[34,35,37] they are only weakly active against *M. tuberculosis*, the etiological agent of tuberculosis. Parish and co-workers' observations that ADEP activity against *M. tuberculosis* could be potentiated by reserpine and verapamil (i.e., efflux pump inhibitors) indicated that efflux contributed to the bacterium's resistance.[42] The identities of the efflux pump(s) in *M. tuberculosis* and the means by which they recognize the ADEPs have yet to be reported. Their identification and characterization are likely to be challenging because the bacterium has dozens of putative efflux pump genes.[43−45] The apparent efflux-mediated resistance to the ADEPs and the ease with which ADEP fragments could be synthesized made for a compelling case in which the fragment-based strategy for analyzing and competitively interfering with efflux could be tested.

For initial proof-of-principle experiments, we synthesized a collection of molecules (Figure 1, compounds **2**−**5**) that were substructures of a bioactive ADEP (Figure 1, compound **1**), which is an easily prepared des-methyl analogue of ADEP 4.[33,36] These fragments were evaluated in ADEP potentiation experiments with *S. coelicolor*, wherein the bacterium was grown on solid media supplemented with the ADEP and 2.5-, 5-, 10-, or 20-fold molar excesses of each fragment (Figure 1B). *S. coelicolor* was selected because it is a nonpathogenic relative of *M. tuberculosis* and the ADEP resistance of both streptomycetes and mycobacteria has likewise been linked to efflux pumps.[42,47] *N-E*-2-Heptenoyldifluorophenylalanine methyl ester (**3**) (i.e., the ADEP side-chain moiety) potentiated the activity of ADEP against *S. coelicolor* in a dose-dependent fashion. In contrast, ADEP fragments such as the peptidolactone (**2**), the peptidolactone with a truncated side chain (**5**), or simply the *N*-acetyl difluorophenylalanine methyl ester (**4**) did not display any efficacy as ADEP potentiators. Apparently, the as yet to be unidentified pump(s) recognize the ADEPs primarily by the side chain appended to their peptidolactone.

Through a positional scanning analysis of compound **3**, we set out to define the structural requirements for its ADEP potentiation and by extension those for ADEP recognition by the pump(s). First, we assessed the importance of the 2-heptenoyl group of compound **3** by preparing and evaluating analogues having other acyl moieties, including those that were saturated, polyunsaturated, and/or cyclic (**6**−**10**). In the ADEP potentiation assays with *S. coelicolor*, we found that none of the compounds were superior to the parent compound **3** (Figure 2). We were intrigued to find that a fragment with the polyunsaturated acyl moiety of a naturally occurring ADEP exhibited no potentiation activity (compound **10**, Figure 2).[36] From the perspective of evolution, one might anticipate that an efflux pump conferring ADEP resistance would be biased to recognize and act on a fragment of a natural product rather than a synthetic compound. However, an alternative explanation for the weak ADEP potentiation of compound **10** could be its lability via inter- or intramolecular reactions.[36] In the next stage of the structural analysis, we compared the ADEP potentiation activity of compound **3** to that of analogues with other amino acid residues (**11** and **12**). The compound with a phenylalanine (**11**) in place of difluorophenylalanine was an active potentiator, but was less effective than **3** (Figure 2).




| Frag. : ADEP | ADEP MIC [μg/mL] | | | |
|---|---|---|---|---|
| | 2.5 : 1 | 5 : 1 | 10 : 1 | 20 : 1 |
| **2** | 16 | 16 | 16 | 16 |
| **3** | 13 | 13 | 10 | 9 |
| **4** | 16 | 16 | 16 | 16 |
| **5** | 16 | 16 | 16 | 16 |

**Figure 1.** ADEP potentiation by fragments: (A) structures of a bioactive ADEP and synthetic fragments thereof; (B) dose-dependent effects of fragments on the minimal inhibitory concentration (MIC) of the ADEP against *S. coelicolor* grown on Difco nutrient agar.[46] The ADEP MIC of *S. coelicolor* is 16 μg/mL.

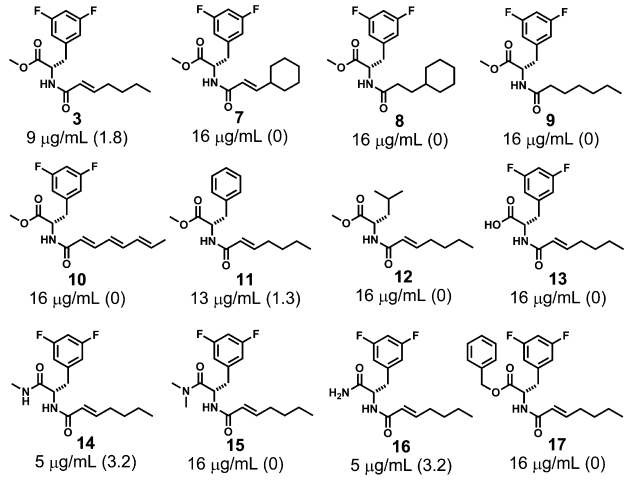



**Figure 2.** Assessment of analogues of compound **3**, a potentiator of ADEP activity. Concentrations indicate the ADEP MIC when it is combined with analogues at a 20:1 molar excess. The degree of potentiation (i.e., the fold-change in MIC relative to ADEP alone) is shown in parentheses.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Again, these results are interesting in the context of efflux pump evolution because the ADEP natural products have phenylalanine at the analogous position. In contrast, substitution of the difluorophenylalanine with leucine (**12**) completely abolished potentiation activity. Finally, the significance of the methyl ester of compound **3** for ADEP potentiation was assessed via the syntheses and testing of analogues with other groups at their carboxy termini (**13**−**17**, Figure 2). Only the compounds with a primary or secondary amide (**14**, **16**) were superior to compound **3** as ADEP potentiators, which is interesting because the ADEPs have an amide bond at the analogous position. Compound **14** was the most effective of the potentiators at fragment to ADEP ratios lower than 20:1 and was also a better potentiator than reserpine when the compounds were used at the same molar ratios (3.2- vs 1.8-fold). Interestingly, despite strong congruence between the structure−activity relationships of the fragments for potentiation and those of the ADEPs for antibacterial activity,[36] the potentiating fragments do not inhibit growth of *S. coelicolor* at concentrations as high as 200 μg/mL in assay conditions described in Figures 1 and 2 (surface-grown cultures on solid media). Furthermore, at the concentration at which ADEP potentiation was observed, compound **14** had only a marginal effect on the growth rate of the bacterium in liquid media (see Supporting Information, Figure S1).

As part of our efforts to define the mechanism by which compound **14** potentiates ADEP activity against *S. coelicolor*, we sought to characterize ADEP efflux in the bacterium. Although the ADEP susceptibility of wild-type *S. coelicolor* can be affected by the efflux inhibitor reserpine (see Supporting Information, Table S4), the identity of efflux pump(s) that confer resistance to the ADEPs is not known. We attempted to resolve this matter using a published report about ADEP resistance in *Streptomyces lividans*.[47] Curiously, we found that disruption of a genetic locus in *S. coelicolor* (SCO4359-60) encoding orthologues of an ABC transporter that confers ADEP resistance in *S. lividans* did not affect the bacterium's ADEP susceptibility. Nevertheless, we cloned and homologously expressed *S. coelicolor* genes encoding ABC transporters with high sequence similarities to the ADEP-resistance determinant from *S. lividans*. The resulting strains were systematically tested for ADEP susceptibility. We found that overexpression of one genetic locus (SCO1719-20) markedly increased ADEP resistance. The ADEP MIC of this strain was 36 μg/mL, whereas that of the wild-type strain was 16 μg/mL. Interestingly, we consider the locus to be a cryptic ADEP resistance determinant because it is not transcriptionally active in wild-type *S. coelicolor* and its disruption does not change the bacterium's ADEP susceptibility (see Supporting Information, Figure S2). Our finding that disruption of a divergently transcribed gene encoding a transcription factor increases the ADEP resistance of *S. coelicolor* to the same extent that does overexpression of SCO1719−20 suggests that the cryptic nature of the resistance locus is a consequence of transcriptional repression (see Supporting Information, Table S5). In any case, we assessed the capacity of compound **14** and reserpine to potentiate ADEP activity against the *S. coelicolor* strain expressing the ABC transporter. We were gratified to find that both compounds were potentiators of ADEP activity; the former was more potent than the latter (Figure 3). Importantly, the coadministration of only 6.25 μg/mL of compound **14** reduced the ADEP MIC of the *S. coelicolor* strain overexpressing the efflux pump by 3-fold, whereas a concentration

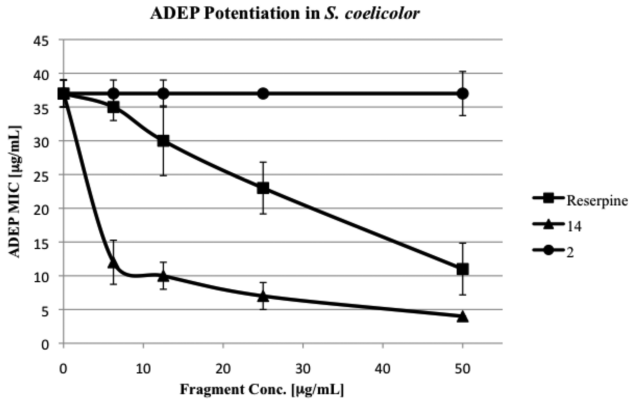


**Figure 3.** Potentiation of ADEP activity against a *S. coelicolor* strain that overexpresses genes (SCO1719-20) encoding an ABC family efflux pump that confers ADEP resistance. This strain has an ADEP MIC that is 2.3-fold greater than that of the wild-type strain.

of 43 μg/mL of it was required to reduce the ADEP MIC of the wild-type strain by the same degree (Figures 2 and 3). These observations confirm that efflux is an ADEP resistance mechanism, indicate that the ABC transporter encoded by SCO1719-20 confers more ADEP resistance than the as yet to be unidentified ADEP efflux pump(s) in *S. coelicolor*, and are consistent with our proposal that a fragment can competitively interfere with ADEP efflux. The fact that compound **14** is a strong ADEP potentiator when its molar ratio with ADEP is only 1:4.5 (i.e., at 6.25 μg/mL) indicates that the fragment is preferentially acted upon by the ABC transporter. Further evidence of the selectivity of the transporter can be gleaned from the observation that the potency and degree of ADEP potentiation by compound **14** are greater than those of reserpine (Figure 3).

The efficacy of compound **14** as a potentiator of ADEP activity against *S. coelicolor* motivated us to test it in the same fashion against phylogenetically related *Mycobacteria* that are reported to exhibit ADEP resistance via efflux.[41] Initially, we assessed the dose dependence of ADEP potentiation using *Mycobacterium smegmatis* MC2155, a nonpathogenic surrogate for *M. tuberculosis* (Figure 4A). Compound **14** was a 4-fold potentiator of ADEP activity at 50 μg/mL on solid growth media. On the basis of the success of the experiments with *M. smegmatis*, we investigated the capacity of compound **14** to potentiate ADEP activity against a virulent strain of *M. tuberculosis* (Figure 4B). Remarkably, the highest degree of ADEP potentiation by compound **14** (i.e., 5-fold) was greater in *M. tuberculosis* than those observed in both *M. smegmatis* and wild-type *S. coelicolor*. It should also be noted that reserpine was a 1.25-fold potentiator of ADEP activity at the same concentration (50 μg/mL). Although we do note that the apparent degree of ADEP potentiation against *M. tuberculosis* by reserpine was lower than that reported by Parrish and coworkers (2.5-fold),[35] the design principle that yielded compound **14** and its superior efficacy as a potentiator of ADEP activity are truly noteworthy and bode well for the use of ADEPs in treating tuberculosis.

The capacity of compound **14** to potentiate ADEP activity against resistant actinobacteria is consistent with our proposal that a fragment can competitively interfere with efflux of the cognate bioactive compound. Indeed, we found that compound **14** potentiated ADEP activity at much lower concentrations in a *S. coelicolor* strain overexpressing an efflux pump than in the


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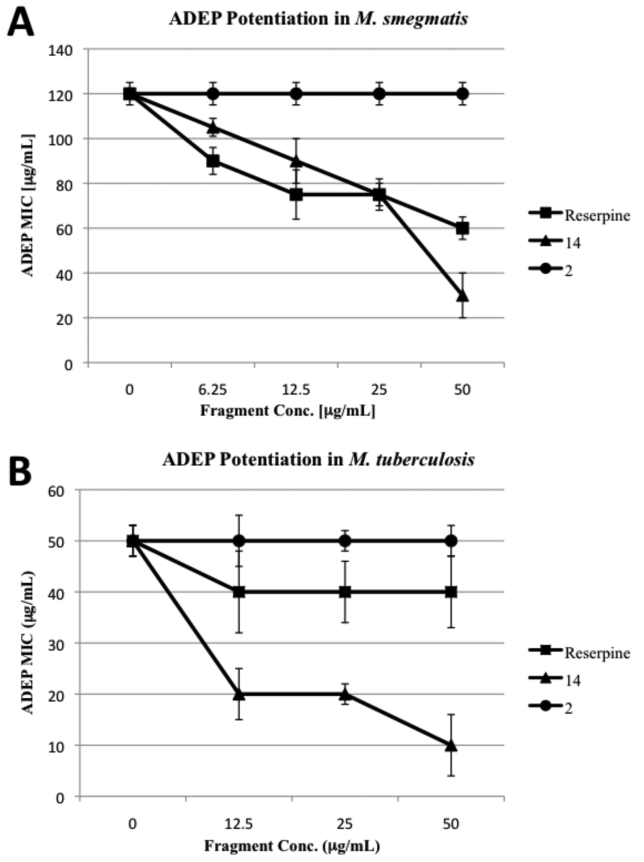


**Figure 4.** Potentiation of ADEP activity against mycobacteria: (A) potentiation of ADEP activity against *M. smegmatis* MC2155 in solid growth media; (B) potentiation of ADEP activity against *M. tuberculosis* H37Rv in liquid growth media. Compound **2** was used as a negative control.

wild-type strain (Figures 2 and 3). Nevertheless, we carried out additional experiments to assess the possibility of other mechanisms of potentiation. For example, we confirmed that compound **14** did not present any antibacterial activity in and above the concentration ranges at which it potentiated ADEP activity. To exclude the possibility that compound **14** was a general and nonspecific adjuvant of antibiotics, we assessed its ability to potentiate the activities of chloramphenicol and spiramycin against *S. coelicolor*. These experiments were chosen because *S. coelicolor* is known to have specific efflux pumps that confer resistance to these antibiotics.[47,48] Because compound **14** did not potentiate the antibacterial activities of chloramphenicol or spiramycin against *S. coelicolor* (see Supporting Information, Table S6), we conclude that it is not a universal adjuvant of antibacterial agents or a ligand of multiple efflux pumps like reserpine or verapamil. The superior ADEP potentiation activity of compound **14** relative to that of reserpine (Figures 3 and 4) suggests either that the ADEP efflux pumps weakly bind reserpine or that compound **14** may act by competitive interference of efflux and perhaps another mechanism. In fact, we did find that a close analogue of compound **14** was a weak activator of the *M. tuberculosis* ClpP in vitro, but exhibited some synergy in combination with an ADEP in ClpP activation assays.[51] We are currently working to delineate the relative contributions of synergistic binding to ClpP and competitive interference with ADEP efflux in the capacity of compound **14** to potentiate ADEP activity against *M. tuberculosis*.

In conclusion, we present a fundamentally new strategy for suppressing efflux of a bioactive compound. It is distinct from other counter-efflux approaches such as the use of small molecules to occlude a pump's outer-membrane channel, to perturb pump assembly, or to inhibit the energy-consuming mechanism that mediates efflux.[8,9,11,12,21,23−27,29−32,50] The use of fragments to competitively interfere with efflux of therapeutic agents does not require any knowledge of the pump's identity, structure, or its mechanism of action. In fact, we show how it can be used to reveal new insights into the structural basis by which efflux occurs. Although it is not a panacea for multidrug efflux, this approach could be generalized to any small molecule therapeutics that are acted upon by efflux pumps. The results reported herein have implications for infectious disease treatment, but the approach could also be applied to cancer therapy, wherein the efficacies of drugs are compromised by P-glycoprotein and other multidrug-resistant pumps.[2b] Despite the fact that the structural complexity of many bioactive molecules presents synthetic challenges for further demonstrations of this fragment-based strategy, such efforts are now underway in these laboratories.

## METHODS

**Chemical Syntheses of ADEP Fragments.** Experimental procedures are described in the Supporting Information. All reagents were purchased from Sigma-Aldrich or VWR. Column chromatography was performed using 60 Å (230−400 mesh ASTM) silica gel. NMR analyses were performed on a Bruker Advance Ultrashield spectrometer (400 or 600 MHz).

**Antibacterial Assays.** Assays with *S. coelicolor* M145 were performed on Difco nutrient agar medium supplemented with the indicated concentrations of compound. Germinated spores ($10^6$) were added to each well, and growth was assessed after incubation at 30 °C for 48 h. The lowest drug concentration that resulted in visual clearing of the wells was considered to be the MIC. All experiments were performed six times.

Assays with *M. smegmatis* MC2155 were performed on Difco nutrient agar medium supplemented with 0.2% glycerol and the indicated concentrations of compound. After the clumped cells had been allowed to settle, liquid cultures with an OD of 0.6 were plated onto the DNA plates supplemented with compounds, and growth was assessed after incubation at 30 °C for 72 h. The lowest drug concentration that resulted in visible clearing of the wells was considered to be the MIC. All experiments were performed in triplicate.

To determine the MICs of compounds for *M. tuberculosis* H37Rv, OD600-based assays were used. Bacteria were grown to mid log phase and plated in 96-well plates at OD600 = 0.025 in the presence of small molecule inhibitors for 7 days, and growth was assessed by reading OD600. The MIC value was determined as the lowest concentration that inhibited growth by >90% relative to the DMSO control. All experiments were performed in triplicate.

## ASSOCIATED CONTENT

### Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/id500009f.

All experimental details and spectral characterizations (PDF)

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### Notes

The authors declare no competing financial interest.

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