#### Journal of Medicinal Chemistry

#### Article

Subscriber access provided by University of Newcastle, Australia

NEWCASTLE

#### Identification and Structure-Activity Relationships of Novel Compounds that Potentiate the Activities of Antibiotics in Escherichia coli

Keith M. Haynes, Narges Abdali, Varsha Jhawar, Helen I. Zgurskaya, Jerry M. Parks, Adam T. Green, Jerome Yves Baudry, Valentin V. Rybenkov, Jeremy C. Smith, and John K Walker *J. Med. Chem.*, Just Accepted Manuscript • DOI: 10.1021/acs.jmedchem.7b00453 • Publication Date (Web): 26 Jun 2017 Downloaded from http://pubs.acs.org on June 27, 2017

#### Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.



Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Published by American Chemical Society. Copyright © American Chemical Society. However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

### Identification and Structure-Activity Relationships of Novel Compounds that Potentiate the Activities of Antibiotics in *Escherichia coli*

Keith M. Haynes,<sup>1</sup> Narges Abdali,<sup>2</sup> Varsha Jhawar,<sup>2</sup> Helen I. Zgurskaya,<sup>2</sup> Jerry M. Parks,<sup>3,4</sup> Adam T. Green,<sup>3,4</sup> Jerome Baudry,<sup>3,5</sup> Valentin V. Rybenkov,<sup>2</sup> Jeremy C. Smith<sup>3,5</sup> and John K. Walker<sup>1,6\*</sup>

<sup>1</sup>Department of Pharmacological & Physiological Science, Saint Louis University School of Medicine, St Louis, MO, 63104, USA

 <sup>2</sup>Department of Chemistry and Biochemistry, University of Oklahoma, Norman, OK 73019, USA
 <sup>3</sup>UT/ORNL Center for Molecular Biophysics, Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

<sup>4</sup>Graduate School of Genome Science and Technology, University of Tennessee, Knoxville, TN, 37996, USA

<sup>5</sup>Department of Biochemistry and Cellular and Molecular Biology, University of Tennessee, Knoxville, TN, 37996, USA

<sup>6</sup>Department of Chemistry, Saint Louis University, St. Louis, MO, 63104, USA

\*Corresponding author. Email: walkerjk@slu.edu

**ABSTRACT**: In Gram-negative bacteria, efflux pumps are able to prevent effective cellular concentrations from being achieved for a number of antibiotics. Small molecule adjuvants that act as efflux pump inhibitors (EPIs) have the potential to reinvigorate existing antibiotics that are currently ineffective due to efflux mechanisms. Through a combination of rigorous experimental screening and *in silico* virtual screening we recently identified novel classes of EPIs that interact with the membrane fusion protein AcrA, a critical component of the AcrAB-TolC efflux pump in *E. coli*. Herein, we present initial optimization efforts and structure-activity relationships around one of those previously described hits, NSC 60339 (1). From these efforts we identified two compounds, **SLUPP-225 (17h)** and **SLUPP-417 (17o)**, which demonstrate favorable properties as potential EPIs in *E. coli* cells including the ability to penetrate the outer membrane, improved inhibition of efflux relative to **1** and potentiation of the activity of novobiocin and erythromycin.

#### **INTRODUCTION**

The number of bacterial pathogens exhibiting resistance to at least one or more classes of antibiotics is on the rise and poses an ever-growing health concern. Resistance to almost all major antibacterial drug classes has been observed in both Gram-negative and Gram-positive species. The situation is particularly acute with the significant increase in difficult to treat nosocomial infections resulting from Gram-negative bacteria.<sup>1,2</sup> In Gram-negative bacteria one of the primary obstacles to overcome for any antibacterial agent is the synergistic efforts of the lipopolysaccharide (LPS) rich outer membrane<sup>3</sup> (OM). The OM is effective at limiting permeation of drugs into the bacterial cell and efflux pumps located in the cytoplasmic membrane which extrude compounds that do cross the OM back out of the cell before they can reach their potential site of action.<sup>4,5</sup> Bacterial strains that develop mutations in the OM porins or overexpress efflux pumps tend to exhibit greater drug resistance.<sup>6</sup> As a result, strategies to develop adjuvants that inhibit the action of bacterial efflux pumps (EPI) have the ability to potentiate (i.e., improve antibacterial activity relative to no adjuvant being present) the effectiveness of existing antibacterial agents.<sup>7-9</sup>

In *E. coli* the major efflux pump contributing to resistance is the AcrAB-TolC tripartite pump.<sup>10,11</sup> This class of efflux pumps consists of three separate proteins that assemble to form the active efflux pump complex.<sup>11,12</sup> The AcrB transporter, which belongs to the Resistance Nodulation Cell Division (RND) superfamily of proteins, is responsible for the binding of substrates for efflux. The porin TolC is the channel that spans the OM and allows for passage of substrates from the cell into the extracellular milieu. These two proteins are unable to fully engage on their own but require the membrane fusion protein (MFP) AcrA to bridge the gap between them and form a complete channel that excludes the periplasm.<sup>11-15</sup> The final composition of the active pump complex is believed to be a 3:6:3 ratio of AcrB:AcrA:TolC.<sup>16</sup> The AcrB substrate-binding protein has been studied extensively as a potential therapeutic target for the development of new EPIs.<sup>8,17-19</sup> However, until our recent disclosure,<sup>20</sup> there were no reports, to the best of our knowledge, of specifically targeting the MFP AcrA as a strategy for the development of EPIs.

We were interested in determining whether assembly of the AcrAB-TolC efflux pump system, and thus its function, could be disrupted via binding of a small molecule to AcrA. Toward that end, the Diversity Set V from the DTP program of the NCI/NIH was screened experimentally and virtual docking was used to identify EPIs that potentially work by targeting AcrA. Experimental screening involved initial evaluation of test compounds in an *E. coli* cell line (WT-Pore) engineered to express large pores (~ 2.4 nm) in the OM.<sup>21</sup> This approach allowed us to identify compounds that are potential EPIs and might have been overlooked otherwise due to poor OM penetration. Specifically, compounds were tested in the presence of novobiocin at a concentration (16  $\mu$ g/mL) that is ¼ of its minimum inhibitory concentration (MIC) in that strain. Test compounds showing MICs ~100  $\mu$ M or better were further evaluated to determine if they were acting as EPIs (*vide infra*). The other key step involved using surface plasmon resonance<sup>20, 22</sup> (SPR) to determine whether compounds could bind to AcrA. Compounds that potentiated novobiocin and were also found to bind to AcrA in the SPR experiments were prioritized as potential hits.

From the screening efforts, NSC 60339 (1),<sup>23</sup> (**Figure 1**), a polybasic terephthalic acid derivative studied previously<sup>24,25</sup> as a potential cancer chemotherapeutic agent, stood out as one of the more attractive candidates for medicinal chemistry follow-up. This compound's profile was consistent with an EPI potentially working via binding to AcrA. Testing of **1** in WT-Pore cells, in the

#### Journal of Medicinal Chemistry

presence of novobiocin, resulted in an observed MIC of 25  $\mu$ M while in the absence of novobiocin it had only weak antibacterial activity (MIC = 200  $\mu$ M). In SPR experiments with AcrA protein and 50  $\mu$ M of compound 1 a strong binding signal was observed. Binding of 1 to AcrA was further established through *in vivo* proteolysis experiments with AcrA. It was found that the cleavage products of AcrA differed when exposed to 1 compared to control experiments done in the absence of 1, suggesting 1 binds to AcrA and induces structural changes to AcrA as a result of binding. Furthermore, 1 was shown to dramatically reduce the rate of efflux of a known AcrAB-TolC substrate, bisbenzamide dye H-33342 (HT), in *E. coli* cells.<sup>26</sup> Taken together, these findings suggest 1 is an EPI that functions at least in part through binding of AcrA. Lastly, in terms of synthetic feasibility, 1 is a relatively simple terephthalic acid analog derived from 2chloroterephthalic acid (**2a**) and aniline **3** (**Figure 2**). As a result, we envisioned rapid entry into new analogs to explore critical structure-activity relationships (SAR) around this compound.

Herein we report the synthesis, pharmacological evaluation, and SAR of several new analogs of **1**, some of which demonstrated an equivalent or better profile for potentiation, efflux inhibition and/or cell penetration. These studies are expected to pave the way for identifying more potent efflux pump inhibitors in the future.

#### **RESULTS AND DISCUSSION**

**Design**. To aid in the selection of novel compounds, we used a previously generated model of  $AcrA^{20}$  (**Figure 3A**) to examine relevant possible poses of **1** at the hinge site, which is located at the interface of the  $\alpha$ -helical hairpin and the lipoyl domains. The highest ranked pose of **1** was used to suggest possible modifications that may improve binding affinities. The calculated p $K_a$  values for the dihydroimidazoline groups are > 9, suggesting that **1** is dicationic at pH 7. The two

dihydroimidazolinium groups were calculated to interact with the backbone carbonyls of Ile 65 and Ala 172 (**Figure 3B**). In addition, the orientation of the chloro group, which interacts with the side chain of Val 176, also suggests that modification of this group may alter binding and activity. Our initial set of analogs was designed to test some of these observations. However, we stress that although the binding pose at the hinge site provided guidance for modifying the central and dihydroimidazoline rings, the location of the binding site for **1** has not been determined definitively.

**Chemistry.** General routes for the preparation of new analogs are outlined in **Schemes 1-4**. The simple terephthalic acid derivatives **4a-g** were prepared following one of the two straightforward procedures shown in Scheme 1. Starting with the desired terephthalic acids 2a or **2b** and combining with various anilines using a standard peptide coupling approach mediated by O-(benzotriazol-1-yl)-N,N,N'N',-tetramethyluronium tetrafluoroborate (TBTU) typically led to the desired products in good overall yields. Alternatively, reaction of the appropriate terephthalic acid chlorides with the desired anilines in the presence of an amine base such as N,Ndiisopropylethylamine also gave the corresponding bis-amide analogs **4a-g** in good yield. However, though perhaps not unexpectedly, neither of these protocols could be extended to coupling reactions involving the dihydroimidazoline-substituted aniline, **3**. For example, when trying to resynthesize 1, coupling of acid 2a with aniline 3 using TBTU led exclusively to amide 5. The same outcome was also realized when using the diacid chloride of 2a to couple with 3. None of the desired product from amide formation with the aniline nitrogen was observed (Scheme 1). Interestingly, when amide 5 was profiled in biological testing it was found to potentiate novobiocin in the WT-Pore cells (MPC<sub>4</sub> = 50  $\mu$ M).

#### Journal of Medicinal Chemistry

To prepare analogs **9a-f** from **Table 1**, as well as to resynthesize **1**, we employed either of the two general procedures shown in **Scheme 2**. In one procedure, the starting diacids **2a-f** were converted to the corresponding diacid chloride intermediates of type **6** which could subsequently be converted to the nitrile-containing amides **8**. The resulting nitriles, **8**, were then converted to the dihydroimidazoline moiety with ethylenediamine and sodium sulfide following the procedure of Ji *et al*<sup>27</sup> to give the desired products **9**. The nitriles, **8**, could also be prepared via a standard amide formation protocol using TBTU and either 3-amino (**7a**) or 4-aminobenzonitrile (**7b**). It was also possible to form the desired dihydroimidazoline compounds via a direct coupling of aniline **3** with the diacid chlorides, **6**, in varying yields when the reaction was conducted in glacial acetic acid. The aniline was dissolved in glacial acetic acid first, followed by subsequent addition of the acid chloride to this mixture.<sup>28</sup> In some cases the solubility of the mono-addition product in glacial acetic acid bis-addition product could form. In these cases, the reaction was run in refluxing toluene with a stoichiometric amount of acetic acid.

Analogs **11** and **13**, in which the central phenyl has been replaced with a saturated linker were prepared from trans-1,4-cyclohexanedicarboxylic acid (**10**) and succinic acid (**12**) respectively, as outlined in **Scheme 3**. Formation of the nitrile amides followed by conversion of the nitriles to the dihydroimidazoline group les to analogs **11** and **13**. The 1,3-diamide analog, **16**, was also prepared in a similar fashion starting from isophthalic acid (**15**). The known reversed amide compound **14** was prepared as described previously by Dong et al.<sup>29</sup>

The mono-amide derivatives **17a-q** shown in **Table 3** were prepared as outlined in **Scheme 4**. In general, aniline **3** was coupled directly to a suitable acid chloride using the glacial acetic acid procedure. The acid chloride, **18**, needed to prepare analog **17c**, was made via coupling of

terephthalic acid (2b) with benzyl amine (1.3 eq.) mediated by TBTU. This gave rise to a mixture of the desired mono-benzylamide and the dibenzyl amide. This mixture was easily separated and the resulting acid was converted to 18 with oxalyl chloride and catalytic DMF. The isoindoline intermediate 21 was prepared in 4 total steps from the commercially available ester 19. Exposure of 19 to benzyl bromide in refluxing acetonitrile with cesium carbonate led to efficient introduction of the benzyl group to give 20. Saponification with lithium hydroxide and subsequent acid chloride formation with oxalyl chloride gave the desired acid chloride precursor, 21.

**Cell Assays.** To identify compounds that block efflux and potentiate the antibacterial activity of novobiocin the testing scheme outlined in **Figure 4** was followed for all compounds. Compounds were initially evaluated for their ability to prevent bacterial cell growth in combination with novobiocin (16 µg/mL, 0.25 MIC) in *E. coli* WT-Pore cells. In this way, inhibition of cell growth should be due either to the test compound alone or through working in combination with novobiocin in some manner. In addition, the presence of the large non-specific pore in the outer membrane of WT-Pore cells eliminates the permeability barrier for the compounds and possible non-specific effects on the outer membrane.<sup>21</sup> Compounds were also evaluated for the ability to inhibit bacterial growth both in the wild-type E. coli cells (WT) and in the WT-Pore cells in the absence of novobiocin to determine whether they had any intrinsic antibacterial properties. Compounds having an MIC  $\leq 100 \mu$ M in combination with novobiocin were then evaluated in a checkerboard assay to determine the minimum potentiation concentration (MPC) that results in a fourfold improvement (MPC<sub>4</sub>) in the MIC of novobiocin.<sup>20</sup> Compounds with an MPC<sub>4</sub> Nov  $\leq$  50 µM were examined for their affinity as substrates for the AcrAB-TolC efflux pump and also their ability to permeate the OM barrier. To accomplish this, compounds were tested in two additional cell-based bacterial growth inhibition assays. One assay tested the

#### Journal of Medicinal Chemistry

compound's ability to inhibit growth in *E. coli* cells engineered to lack the AcrAB-TolC efflux pump ( $\Delta$ TolC). The other looked at antibacterial activity in *E. coli* cells in which the AcrAB-TolC ( $\Delta$ TolC-Pore) pump was knocked out and with large pores in the OM. These assays help to define: (i) if potentiation is likely due to blocking the AcrAB-TolC pump or by other mechanisms; (ii) whether the compounds are efflux substrates of the pump; and (iii) how modifications affect the compound's ability to penetrate the OM. Compounds having an MPC<sub>4</sub> Nov  $\leq$  50 µM were also profiled in the SPR and efflux inhibition experiments as described below. Finally, to test the general applicability of this approach the most promising compounds were tested for their ability to potentiate the macrolide antibiotic erythromycin. As an antibiotic, erythromycin works through a different mechanism of action from novobiocin but it is a substrate for the AcrAB-TolC pump and is therefore only weakly effective against *E. coli*.<sup>30</sup> In this way, we can further substantiate the compounds are working generally as EPIs and are not specific just for novobiocin. Similar to experiments with novobiocin, test compounds were administered in the presence of a sub-lethal dose (5 µg/mL, 0.25 MIC) of erythromycin and compounds that potentiated the antibacterial activity of erythromycin were run in the full checkerboard assay format to determine the minimum potentiation concentration.

SPR and HT Assays. In addition to growth inhibition assays, compounds were evaluated for their ability to bind to the purified AcrA protein and to inhibit or slow down the rate of drug efflux in the cell. To determine if new compounds could bind to AcrA, SPR sensorgrams were collected with 50 or 100  $\mu$ M analyte injected over the purified AcrA protein immobilized onto a CM5 sensorchip, using a Biacore instrument. To evaluate the ability of the compound to inhibit drug efflux, the fluorescent HT dye was used. When HT intercalates into membranes or intracellular DNA it gives a fluorescent signal. HT has also been shown to be a substrate for the AcrAB-TolC efflux pump in *E. coli* that is rapidly effluxed out of the cell.<sup>26</sup> As a result, fluorescence from HT accumulation increases very slowly in these cells with active efflux. In the presence of an EPI, however, HT can accumulate in the cell, as indicated by an increase in fluorescence. Therefore, compounds that behave as EPIs, such as **1**, should accelerate the rate of fluorescence change.

**Structure-Activity Relationships**. Compound analogs of **1** were prepared to evaluate the roles of the halo substituent  $(R_1)$  in the central ring and the dihydroimidazoline moiety in the benzamide groups  $(R_2)$  within the hit template (**Table 1**) using the predicted binding pose at the hinge site as a starting point. The docking calculations suggest that the dihydroimidazoline groups could potentially engage in ionic hydrogen bonding interactions with the backbone carbonyl groups of Ile 65, Ala 172, or both. To test this hypothesis, we prepared a small set of analogs (4a-g) in which the dihydroimidazolines were replaced with groups that are capable of either accepting or donating hydrogen bonds. The cyano analogs, 4a and 4b, capable of accepting hydrogen bonds, suffered a significant loss in potentiation. Similarly, the hydroxymethyl compound, 4c, capable of both accepting and donating hydrogen bonds, also failed to show any significant potentiation activity. The same was also true of the negatively charged sulfonic acid derivative, 4d. However, the aminomethyl analog, 4e, which has estimated  $pK_a$  values of ~ 9.0 and 9.6, showed only a minor reduction in potentiation activity (MPC<sub>4</sub> = 50  $\mu$ M) compared to 1 (See Supplemental Material, Figures S1 and Table S1) for additional information and docking poses for these compounds). We also prepared the closely related bioisosteres 4-methyl, 1, 2, 4-triazole, 4f, and imidazole, 4g, which failed to exhibit any significant potentiation of novobiocin. These findings suggest that a basic nitrogen capable of acting as a hydrogen bond donor is required in this position. When the dihydroimidazoline was shifted from the *para* to the *meta* position, **9a**, we observed only a twofold drop (50 µM) in the MPC<sub>4</sub> value. On the basis of the observed MPC<sub>4</sub> values, replacement of

#### **Journal of Medicinal Chemistry**

chlorine with a nitro, **9e**, or methyl group, **9f**, had minimal impact on novobiocin potentiation values (MPC<sub>4</sub> = 25  $\mu$ M). The bromo, **9d**, and des-chloro analog, **9b**, were also similar to **1**, exhibiting only a twofold loss in potentiation. Analog **9c**, where R<sub>1</sub> = F, was the only one to show a significant change in potentiation values with a fourfold loss being observed. Within the symmetrical starting scaffold only minor variations in potentiation values were observed for the majority of analogs containing the dihydroimidazoline group. In contrast, significant losses in potentiation were observed when that group was replaced, strongly highlighting that the dihydroimidazoline is critical for activity but the substituent R<sub>1</sub> does not have a dramatic effect on activity. These findings are generally consistent with the model depicting binding in the hinge site where modifications of the dihydroimidazoline ring would be expected to disrupt favorable interactions and reduce the affinity for AcrA.

We also prepared a small number of analogs with changes to the central phenyl ring (**Table 2**). Preliminary docking experiments suggested that the analog with a saturated trans-cyclohexyl ring, **11**, could bind to AcrA (See Supplemental Material, **Figure S3**). Experimental results, however, showed no potentiation of novobiocin, suggesting that steric hindrance or the lack of aromaticity in the cyclohexyl ring may have a more substantial effect than predicted. Similarly, when the central ring was replaced with a less rigid acyclic core derived from succinic acid, **13**, no potentiation was observed. The reversed amide analog, **14**, which lacks the chlorine substituent in the central ring, potentiated novobiocin better (MPC<sub>4</sub> = 12.5  $\mu$ M) than **1**. Altering the substitution pattern about the central ring, in this case switching from a 1,4-disubstituted amide to a 1,3-disubsituted amide, **16**, also led to significant reduction of potentiation activity relative to **1**.

In light of the result with the *meta*-isomer analog **9a** having similar activity to **1** we speculated that perhaps only one of the dihydroimidazoline groups is critical for activity. Additionally, docking calculations of 1 identified poses in the AcrA site in which only one of the dihydroimidazoline groups was engaged with the protein (See Supplemental Figure S2). With that in mind, we turned our focus to preparing non-symmetrical analogs derived from addition of aniline 3 to various mono-acid chlorides (Scheme 4). We prepared a number of compounds with simple R groups and several of these are shown in **Table 3**. Amides derived from simple substituted benzoyl chlorides such as 17a-17c failed to show any substantial potentiation. The phenylacetamide analog 17d, however, did show modest potentiation (MPC<sub>4</sub> = 100  $\mu$ M), suggesting that was possible to achieve activity with simpler non-symmetrical analogs. The dihydroisoindoline 17e and the phenethyl derivative 17f were also inactive with regard to potentiation. However, the more rigid cinnamoyl derivative, 17g, did show hints of activity but was somewhat obscured by poor solubility. Replacement of hydrogen with chlorine in the phenyl ring of the cinnamoyl group improved solubility and both the *ortho*-analog **17h** and *para*-analog **17l** showed a marked improvement in potentiation, around 25  $\mu$ M, while the *meta*-chloro, **17k**, was only slightly worse than these two. Compounds 17h and 17l are the first simplified analogs with potentiation at least as good as 1. Replacement of the 2-chloro with 2-bromo, 17i, led to a small reduction in potentiation but was still active. A similar result was observed with the 2-trifluoromethyl group, 17*i*. As mentioned above, the 4-chloro analog was active and modifications at this position led to a greater effect on activity. The bromo analog, 17m, showed a twofold loss in activity relative to 17l while the methoxy analog, 17n, suffered a fourfold loss in activity. However, addition of a bulkier alkyl group such as isopropyl, **170**, or *t*-butyl, **17p**, led to analogs with improved potentiation. With the activity shown by the cinnamovl derivatives, we decided to try a simple naphthyl derivative, **17***q*,

#### **Journal of Medicinal Chemistry**

which would be expected to mimic the conformation of the cinnamoyl derivatives. Indeed, 17q proved to be active with an MPC<sub>4</sub> value of 25  $\mu$ M.

**Discussion**. We identified a number of compounds that potentiated the activity of novobiocin in *E. coli* with values equivalent or better than the original compound, **1**. Several of these were profiled in additional cell-based assays to provide a better understanding of their overall properties (**Table 4**). Additionally, we assessed their ability to bind to AcrA, inhibit efflux (as measured by HT uptake) and potentiate erythromycin. From this evaluation, compounds that potentiated novobiocin fell into three broad categories: (i) those that do not appear to act primarily through an EPI mechanism, as evidenced by possessing antibacterial activity, weak or no binding to AcrA and little to no effect on efflux, (ii) those that do appear to act primarily as an EPI, as evidenced by only weak antibacterial activity, binding to AcrA and a reduced rate of efflux, or (iii) those that possess some characteristics of both (i) and (ii).

One interesting compound that did not fall into the above categories was the cyclohexyl derivative **11**, which did not potentiate novobiocin but did possess antibacterial activity with an MIC = 50  $\mu$ M in WT cells but decreased only twofold in WT-pore cells. This compound showed additional twofold reductions in MIC when tested in the  $\Delta$ TolC and  $\Delta$ TolC-Pore cells, further suggesting it is a weak substrate of the efflux pump and readily penetrates the OM. In agreement with these findings, SPR experiments did not show evidence of binding to AcrA, and it only modestly enhanced uptake of HT. The mechanism of action for this antibacterial activity has not been determined. The reversed amide compound, **14**, is an example that falls into the first category described above. It did potentiate novobiocin but is also potent as an antibacterial agent in the WT cells with an MIC = 25  $\mu$ M. It showed good cell penetration, as its MIC was relatively unaffected

in the  $\Delta$ TolC or  $\Delta$ TolC-WT-Pore cell lines. Unlike **11**, it did bind weakly to AcrA (**Figure 5**) but did not improve HT uptake in the cells. These two compounds had interesting antibacterial properties with good penetration of the OM but do not appear to be acting as EPIs. The *meta*-substituted dihydroimidazoline compound, **9a**, a close analog to **1**, profiled in a similar manner to **1**. It showed only weak antibacterial activity by itself with an MIC = 200  $\mu$ M in WT cells. The activity dropped twofold in the WT-Pore cells and was further reduced to 50  $\mu$ M in the  $\Delta$ TolC cells, representing a fourfold change in activity when the efflux pump was deleted. These results suggest that **9a** acts an EPI but is also a substrate of the AcrAB-TolC efflux pump. We were unable to detect binding to AcrA because of non-specific binding of the compound to the chip surface. It did however enhance the rate of HT uptake, but at a rate less than that of **1** (See **Figure 6**).

The *ortho*-halo substituted cinnamoyl derivatives, **17h** and **17i**, were also profiled. Neither compound had appreciable antibacterial activity (MICs > 200  $\mu$ M) in the WT or WT-Pore cells. They both showed a twofold decrease in MICs in the  $\Delta$ TolC cells, suggesting they have some affinity as substrates for the efflux pump. Both were found to bind to AcrA (**Figure 5**) with greater affinity than **1** and both yielded a significant enhancement of HT uptake relative to **1** (**Figure 6**). The analog with the chloro substituent moved to the 4- position of the cinnamoyl group, **17l**, behaved similarly to that of **17h** but was slightly more potent as an antibiotic, with an MIC = 200  $\mu$ M in WT cells. When tested in the  $\Delta$ TolC-Pore cells, a fourfold reduction in the MIC was observed, similar to what was observed with **17h** and **17i**. Replacing the halo substituent in **17l** with bulkier alkyl groups promoted some significant changes in the properties of these compounds. For instance, the 4-isopropyl analog **17o** had an MIC = 100  $\mu$ M in WT cells while the larger *t*butyl compound, **17p**, was even more potent with an MIC fourfold lower at 25  $\mu$ M. The target

#### Journal of Medicinal Chemistry

responsible for the greater antibacterial activity of **17p** remains unclear at present. The MICs of these compounds in the  $\Delta$ TolC cells were only twofold lower than in the WT cells, suggesting both **17o** and **17p** are less prone to efflux by the TolC efflux pump (**Table 5**) relative to the other analogs tested. The isopropyl analog **17o** showed one of the strongest binding signals to AcrA we observed in addition to showing a strong enhancement of HT uptake. The *t*-butyl analog **17p** also showed binding to AcrA with a 2.6-fold rate enhancement of the HT uptake rate. Finally, we evaluated the naphthyl compound, **17q**, which showed relatively little activity in the WT cells with only a slight improvement in the WT-Pore cells. However, in the  $\Delta$ TolC-Pore cells it had an MIC = 12.5  $\mu$ M, representing a 16-fold increase in antibacterial activity when the pump was removed. This compound is clearly a strong substrate for the AcrAB-TolC efflux pump. It also displayed strong binding to AcrA but with only a slight enhancement in rate (1.6-fold improvement) for HT uptake in cells.

Due to the cationic nature of these compounds, the possibility exists that they might disrupt the proton motive force that drives the AcrAB-TolC efflux pump. To address this point, we evaluated **1** and the analogs **17h** and **17o** for their effects on the transmembrane potential in *E. coli* cells (**Figure 7a**). From the data, it appears that our compounds had no effect on the membrane potential, providing further evidence they work primarily through binding of AcrA and disrupting the efflux pump assembly. Additionally, because many of these compounds contain a cinnamoyl moiety, which has the potential to act as a Michael acceptor, we performed a basic cytotoxicity assay in HEK293 cells with **17h** and **17o** (**Figure 7b**). The cytotoxicity of the two compounds differed significantly, with IC<sub>50</sub>'s of 10.5  $\mu$ M and >100  $\mu$ M, respectively. From this data, it does not appear that cytotoxicity contributes to the potentiation activity of **17o** but we cannot rule out that there it doesn't make a minor contribution to the potentiation seen with **17h**. Overall, it does

not appear that the cinnamoyl group poses a major liability with these compounds. The difference in observed cytotoxicity  $IC_{50}$ 's is likely due to the nature of the substitution in the cinnamoyl ring.

From a chemical properties standpoint, these new analogs of **1** are characterized primarily by a reduction in molecular weight (MW) (~120-150 Da), increased rigidity, and an increased amphiphilic moment. The latter two properties, along with low globularity and the presence of an unhindered basic nitrogen, were recently shown to be favorable for antibiotic accumulation in Gram-negative bacteria.<sup>31</sup> These compounds fall nicely within the favorable property range for crossing the OM of Gram-negative bacteria (See Supplemental Material, **Table 2S**), which is consistent with our own experimental data. Additionally, with the reduced MW we have the flexibility to optimize these molecules for binding to AcrA, antibacterial targets, or introduce additional modifications to the cinnamoyl group to mitigate potential risk while remaining in the optimal chemical space.

From the data collected a few general SAR trends can be observed. Substitution about the phenyl ring in the cinnamoyl moiety greatly influences the profile of these compounds. Analogs with substituents in the 2-position (i.e. **17h** and **17i**) tend to have lower antibacterial activity, some susceptibility to be substrates for efflux, and also inhibit efflux to a greater extent than other analogs. These compounds also are very effective at crossing the OM as measured by the ratio of activities in WT vs. WT-Pore cells (**Table 5**). Conversely, substitution in the 4-position (i.e. **17l**, **17o** and **17p**) led to compounds with greater antibacterial activity and less susceptibility to efflux, but also were less effective at crossing the OM. The more rigid naphthyl compound **17q** is interesting because it shows the greatest affinity for AcrA in the SPR experiments, but it is also the most susceptible to efflux by the AcrAB-ToIC efflux pump. That it does not show greater

Page 17 of 60

#### **Journal of Medicinal Chemistry**

potentiation is likely attributed to its greater susceptibility to efflux. It is also one of the few analogs that are unable to cross the OM readily, with a ratio similar to the original lead, **1**. Finally, all of the compounds are able to potentiate the activity of a second antibiotic, erythromycin (**See Table 5**). Erythromycin is a macrolide antibiotic that is ineffective against *E. coli* because it is a substrate for AcrAB-TolC. Erythromycin potentiation values for these compounds are consistent with those of novobiocin, further suggesting these compounds are working as EPIs. Taken together, our findings suggest that there are subtle factors that influence whether these compounds act as EPIs, are substrates for efflux or bind to an antibacterial target. In many cases, the compounds exhibit more than one of these characteristics. Of the compounds tested, analogs **17h** and **17o** appear to be the most promising for future development. Both readily cross the OM, bind to AcrA, and inhibit efflux in *E. coli* by potentiating the activity of two separate antibiotics.

**Conclusion**. In this report, we have identified several novel potentiators of novobiocin and erythromycin in *E. coli*. These compounds were designed starting from the recently identified inhibitor **1** with the objective of inhibiting the AcrAB-TolC efflux pump by interacting with the MFP constituent AcrA of the AcrAB-TolC efflux pump. In many cases, the new compounds were shown to bind AcrA and also to inhibit efflux to some extent in assays measuring the uptake of the fluorescent probe HT into cells. Two compounds of particular interest are **17h** and **17o**, both of which show good potentiation of novobiocin and erythromycin, bind to AcrA, and inhibit efflux at a rate significantly higher than the original hit. They differ slightly in that the chloro-substituted analog **17h** has minimal antibacterial activity on its own while **17o** has a good MIC in wild-type cells. Another important aspect of this class of compounds is that they show the potential to cross the outer membrane of Gram-negative bacteria. In summary, we have identified compounds with diverse characteristics that inhibit efflux and potentiate the antibacterial properties of novobiocin

and erythromycin in *E. coli*. Some of these compounds are significantly more potent than our original lead, **1**.

#### **EXPERIMENTAL SECTION**

General Methods. Compounds 1 and 4d (NSC 55156) were obtained from the National Cancer Institute. Novobiocin, erythromycin and Hoechst 33342 were obtained from *Sigma-Aldrich Chemie GMBH* (Steinham, Germany). All reagents and solvents for the synthesis of analogs 4a-g, 5, 9a-f and 17a-g were purchased from *Sigma-Aldrich Chemie GMBH* (Steinham, Germany), *Alfa Aesar GMBH & Co KG* (Karlsruhe, Germany), *Acros Organics* (Geel, Belgium), *TCI America* (Portland, United States) or *Enamine Ltd*. (Monmouth, United States) and used as is without further purification. The purities of the final compounds were characterized by high-performance liquid chromatography (HPLC) using a gradient elution program (Ascentis Express Peptide C18 column, acetonitrile/water 5/95 95/5, 5 min, 0.05% trifluoracetic acid) and UV detection (254 nM). The purities of all compounds final compounds were 95% or greater. 1H-NMR and 13C-NMR were obtained on a Bruker 400 Mhz instrument and all chemical shifts are referenced to residual solvent peaks.

**PAINS**. All of the biologically tested compounds were evaluated for structural attributes consistent with classification as pan-assay interference compounds (PAINS).<sup>32</sup> The results for each compound were negative.

**2-chloro**- $N^1$ , $N^4$ -**bis**(**4-(4,5-dihydro-1***H*-**imidazol-2-yl)phenyl)terephthalamide, (1).** 2chloroterephthalic acid (50 mg, 0.25 mmol) was converted to the corresponding acid chloride via treatment with 2.0 M oxalyl chloride solution (0.312 mL, 0.625 mmol) in dichloromethane. A catalytic quantity of dimethyformamide was added and the reaction was complete in 4 hours on an

#### Journal of Medicinal Chemistry

ice bath. 2-chloroterephthaloyl dichloride was dried to a yellow solid and dissolved in 1.5 mL anhydrous toluene. In a second vial, sodium acetate (150 mg, 1.8 mmol) was dissolved in 2.5 mL glacial acetic acid. To this vial, 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline (88 mg, 0.55 mmol) was added and the mixture sonicated until a homogeneous solution was achieved. The acetic acid solution was added dropwise to the acid chloride, purged with argon, and allowed to react overnight at room temperature. Product precipitated and was collected by filtration. Crude product was dissolved in a minimal amount of water before being made basic with 4.0 M sodium hydroxide solution. Solution was then heated at 50 °C until product had completely precipitated as free-base. Precipitate was collected and triturated with methanol to yield product as a white solid. (18 mg, 15% yield). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.84 (s, 1H), 10.61 (s, 1H), 8.17 (s, 1H), 8.05 (d, *J* = 8.0 Hz, 1H), 7.82 (m, 9H), 3.63 (s, 8H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  164.59, 163.70, 139.21, 137.00, 130.15, 129.15, 128.78, 128.34, 128.29, 128.23, 128.17, 126.81, 119.79, 119.05, 48.21; HRMS: *m/z* calcd. for C<sub>26</sub>H<sub>23</sub>ClN<sub>6</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 487.1651; found [M+H]<sup>+</sup>: 487.1691, [M+2H]: 244.0863.

**2-chloro**- $N^1$ , $N^4$ -**bis(4-cyanophenyl)terephthalamide (4a).** A solution of 2-chloroterephthalic acid (50 mg, 0.25 mmol) in 2.0 mL of dichloromethane was cooled to 0 °C using an ice bath. To this solution was added sequentially a catalytic amount (~ 1-2 drops) of DMF followed by a 2.0 M solution of oxalyl chloride (1.68 mL, 3.3 mmol) in dichloromethane. The resulting mixture was stirred overnight allowing the temperature to gradually warm to rt. The resulting mixture was concentrated down to dryness on a rotoevaporator and then dried under vacuum for 2h. The resulting solid was then dissolved in 3.0 mL of THF and cooled to 0 °C in an ice bath. To this mixture was added triethyl amine (0.087 mL, 1.2 mmol) and the reaction was stirred for 5 min. To this mixture was added 4-aminobenzonitrile (74 mg, 0.62 mmol) as a solution in 1.0 mL of THF.

The resulting mixture was stirred for 1h at 0 °C and then allowed to warm to rt and stir for 2h. The reaction was quenched with saturated solution of sodium bicarbonate, which led to the formation of a white precipitate. The precipitate was isolated by filtration and rinsed several times with deionized water. The resulting solid was collected and dried under vacuum to give a white solid (69 mg, 69%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.96 (s, 2H), 8.18 (d, *J* = 1.6 Hz, 1H), 8.06 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.99 (d, *J* = 8.8 Hz, 2H), 7.91 (d, *J* = 8.8 Hz, 2H), 7.824 (m, 5H); HRMS: *m/z* calcd. for C<sub>22</sub>H<sub>13</sub>ClN<sub>4</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 401.0807; found [M+H]<sup>+</sup>: 401.0780.

**2-chloro**- $N^1$ , $N^4$ -**bis(4-cyanophenyl)terephthalamide (4b).** Compound **4b** was prepared according to the procedure outlined above for example **4a** using 3-aminobenzonitrile. White solid, yield: 85%; <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  10.72 (s, 2H), 8.28 (m, 2H), 8.13 (s, 4H), 8.07 (m, 2H), 7.61 (m, 4H); HRMS: m/z calcd. for C<sub>22</sub>H<sub>14</sub>N<sub>4</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 367.1197; found [M+H]<sup>+</sup>: 367.1199.

**2-chloro**- $N^1$ , $N^4$ -**bis(4-(hydroxymethyl)phenyl)terephthalamide (4c).** To a 25 mL round bottom flask with stir bar, 4-aminobenzyl alcohol (0.500 g, 4.05 mmol) and imidazole (0.303 g, 4.46 mmol) were added. Dichloromethane (10.0 mL) was added followed by the addition of *tert*-butydimethylsilyl chloride (0.610 g, 4.05 mmol). The reaction was allowed to stir overnight at room temperature and stopped with addition of ethyl acetate, which induces precipitation of by-products that can be filtered off before extraction with water. The organic layer was dried with sodium sulfate and concentrated down to a thick oil before being purified on a 25 g silica flash column with 40% ethyl acetate in hexane. 4-(((*tert*-butyldimethylsilyl)oxy)methyl)aniline obtained as colorless oil after drying on high vacuum overnight. (0.944 g, 98% yield). 1H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.98 (s, 1H), 8.83 (s, 1H), 7.71 (t, *J* = 7.2 Hz, 4H), 7.63 (d, *J* = 2.0 Hz, 1H), 7.52

#### **Journal of Medicinal Chemistry**

(dd, J = 8.0, 1.6 Hz, 1H), 7.42 (d, J = 8.0 Hz, 1H), 7.32 (dd, J = 8.4, 4.8 Hz, 4H), 4.73 (d, J = 2.8 Hz, 4H), 0.95 (d, J = 0.8 Hz, 18H), 0.109 (d, J = 1.2 Hz, 12H).

To a reaction vial with magnetic stir bar and Teflon cap, 2-chloroterephthalic acid (100 mg, 0.50 mmol) and TBTU (320 mg, 1.00 mmol) were added and the vial was purged with argon. 5.0 ml DMF was added through the cap followed by diisopropylethylamine (0.44 mL, 2.50 mmol). The reaction was allowed to stir for 15 minutes at room temperature before the addition of 4-(((tertbutyldimethylsilyl)oxy)methyl)aniline (296 mg, 1.25 mmol). The reaction was run overnight before being quenched with water and extracted into ethyl acetate. The organic layer was treated with sodium sulfate and dried to give an oil. The product was run through a 25 g silica flash column with 2% methanol in dichloromethane and dried to a white solid (256 mg, 80% yield). Cleavage of the TBDMS group was achieved by dissolving the compound in THF (5.0 ml), followed by addition of 0.1 M tetrabutylammonium fluoride (0.98 mL) and stirring overnight. The reaction mixture was dried to a solid and run through a 24 g silica flash column with 2% methanol in dichloromethane. The product was dried to a white solid (37 mg, 22% yield). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  10.58 (s, 1H), 10.40 (s, 1H), 8.14 (d, J = 1.6 Hz, 1H), 8.03 (dd, J = 8.0, 1.6 Hz, 1H), 7.74 (m, 3H), 7.67 (d, J = 8.8 Hz, 2H), 7.31 (dd, J = 8.8, 4 Hz, 4H) 5.15 (dt, J = 5.6, 1.6 Hz, 2H), 4.48 (dd, J = 5.6, 3.2 Hz, 4H); <sup>13</sup>C NMR (400 MHz, DMSO- $d_6$ )  $\delta$  164.22, 163.26, 139.38, 138.27, 138.21, 137.33, 137.08, 130.11, 129.01, 128.59, 126.96, 126.81, 126.61, 120.27, 119.41, 62.59, 62.56; HRMS: m/z calcd. for C<sub>22</sub>H<sub>19</sub>ClN<sub>2</sub>O<sub>4</sub> [M+H]<sup>+</sup>: 411.1113; found [M+H]<sup>+</sup>: 411.1090.

 $N^1$ , $N^4$ -bis(4-(aminomethyl)phenyl)terephthalamide (4e). To a reaction vial with magnetic stir bar and teflon cap, terephthaloyl chloride (40 mg, 0.20 mmol) was added along with 2.0 mL anhydrous dichloromethane. 4-((*N*-Boc)aminomethyl)aniline (93 mg, 0.42 mmol) was added along with triethylamine (0.06 mL, 0.42 mmol). The vial was purged with argon and allowed to react overnight at room temperature. Methanol was added to the reaction mixture and it was filtered to collect the Boc-protected product as a white solid. The protecting group was removed with the addition of TFA and stirred for 4 hours before drying to a solid. The crude product was sonicated in 4M NaOH and filtered to collect the product as a white solid (21 mg, 28 % yield); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.50 (s, 2H), 8.11 (s, 8H), 7.83 (d, *J* = 8.4 Hz, 4H), 7.45 (d, *J* = 8.4 Hz, 4H), 4.02 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  164.88, 139.19, 137.32, 129.46, 129.36, 127.85, 120.53, 42.00; HRMS: *m/z* calcd. for C<sub>22</sub>H<sub>22</sub>N<sub>4</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 375.1823; found [M+H]<sup>+</sup>: 375.1822, [M+2H]: 171.0680.

# **2-chloro**- $N^{1}$ , $N^{4}$ -**bis**(4-(4-methyl-4*H*-1,2,4-triazol-3-yl)phenyl)terephthalamide (4f). Compound 4f was prepared from 2-chloroterephthalic acid and 4-(4-methyl-4*H*-1,2,4-triazol-3-yl)aniline as described above for the synthesis of compound 4a. White solid, yield: 57%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) $\delta$ 10.89 (s, 1H), 10.66 (s, 1H), 8.56 (s, 2H), 8.20 (d, *J* = 0.8 Hz, 1H), 8.08 (dd, *J* = 7.6, 1.6 Hz, 1H), 7.99 (d, *J* = 8.8 Hz, 2H), 7.91 (d, *J* = 8.8 Hz, 2H), 7.83 (d, *J* = 7.6 Hz, 1H), 7.78 (m, 4H), 3.76 (d, *J* = 3.6 Hz, 6H); HRMS: *m/z* calcd. for C<sub>26</sub>H<sub>21</sub>ClN<sub>8</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 513.1556; found [M+H]<sup>+</sup>: 513.1557, [M+2H]: 257.0821.

 $N^{1}$ , $N^{4}$ -bis(4-(1*H*-imidazol-2-yl)phenyl)-2-chloroterephthalamide (4g). Compound 4g was prepared from 2-chloroterephthalic acid and 4-(1*H*-imidazol-2-yl)aniline as described above for the synthesis of compound 4a. White solid, yield: 24%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  12.48 (s, 2H), 10.75 (s, 1H), 10.54 (s, 1H), 8.18 (d, *J* = 1.6 Hz, 1H), 8.06 (dd, *J* = 7.6, 1.6 Hz, 1H), 7.93 (m, 4H), 7.88 (d, *J* = 9.2 Hz, 2H), 7.80 (m, 3H), 7.13 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$ 164.38, 163.45, 139.32, 138.64, 138.58, 137.09, 130.18, 129.14, 128.70, 126.72, 126.41, 125.45, 125.30, 120.50, 119.72;

(2-chloro-1,4-phenylene)bis((2-(4-aminophenyl)-4,5-dihydro-1*H*-imidazol-1-yl)methanone) (5). 2-chloroterephthalic acid (102 mg, 0.51 mmol) was added to a reaction vial with a stir bar. TBTU was added (327 mg, 1.02 mmol) before the addition of 10 mL anhydrous DMF. Diisopropylethylamine (0.45 mL, 2.55 mmol) was added and the reaction stirred for 10 minutes before the addition of 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline (3) (210 mg, 1.29 mmol). The vial was purged with argon and allowed to react overnight at room temperature before extracting the product into dichloromethane from water. The organic layer was dried with sodium sulfate and dried to a solid on a rotavap. It was then purified on an ISCO 12 g silica column (4% methanol in DCM). The product was obtained as a yellow solid. (51 mg, 21% yield). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  7.43 (s, 3H), 7.15 (s, 4H), 6.39 (s, 4H), 5.47 (m, 4H), 3.81 (s, 8H).

 $N^{1}$ , $N^{4}$ -bis(3-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)terephthalamide (9a). Compound 9a was prepared from bis-nitrile intermediate  $N^{1}$ , $N^{4}$ -bis(3-(4,5-dihydro-1*H*-imidazol-2yl)phenyl)terephthalamide, **4b**, as described below for the preparation of compound **9b**. White solid; yield: 30%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.85 (s, 2H), 10.60 (s, 4H), 8.54 (m, 2H), 8.17 (s, 4H), 7.98 (m, 2H), 7.68 (m, 4H), 4.03 (s, 8H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  165.32, 165.07, 139.65, 137.01, 129.73, 128.01, 126.58, 124.07, 122.76, 120.36, 44.43; HRMS: *m/z* calcd. for C<sub>26</sub>H<sub>24</sub>N<sub>6</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 453.2041; found [M+H]<sup>+</sup>: 453.2043, [M+2H]: 227.1057.

 $N^1$ ,  $N^4$ -bis(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)terephthalamide dihydrochloride (9b). To a reaction vial with magnetic stir bar and Teflon cap, terephthaloyl dichloride (70 mg, 0.34 mmol) and 4-aminobenzonitrile (100 mg, 0.86 mmol) were added and vial was purged with argon. 3.0 ml THF was added along with trimethylamine (0.12 mL, 0.86 mmol). The reaction was allowed to stir at room temperature overnight. The reaction was quenched with the addition of saturated sodium bicarbonate solution and filtered to collect crude product, which was then purified with trituration in methanol to collect  $N^{I}$ , $N^{4}$ -bis(4-cyanophenyl)terephthalamide intermediate as a white solid. (107 mg, 86% yield). 1H NMR (400 MHz, DMSO- $d_{6}$ )  $\delta$  10.92 (s, 2H), 8.21 (s, 4H), 8.02 (d, J = 9.2 Hz, 4H), 7.85 (d, J = 8.8 Hz, 4H).

 $N^{J}$ , $N^{d}$ -bis(4-cyanophenyl)terephthalamide (40 mg, 0.11 mmol) was added to a 2 dram vial with stir bar. Ethylenediamine (0.768 mL, 11.5 mmol) was added along with 1.0 ml dimethylacetamide before the addition of sodium hydrosulfide hydrate (88 mg, 1.57 mmol). The solution was capped and carefully heated to 115°C. The reaction was set on a timer and allowed to react 12 hours with constant stirring. Once cooled, the reaction was quenched with water to precipitate product and filtered to collect product as the free base, which was not soluble in dimethylsulfoxide. It was then converted to the HCl salt by adding 8 eq. HCl as a 4.0 M solution in dioxane and heating at 90 °C for one hour. The product was collected as a white solid (19 mg, 39% yield) and converted to the triflate salt with trifluoroacetic acid (1 eq.) <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.95 (s, 2H), 10.47 (s, 4H), 8.17 (s, 4H), 8.10 (d, *J* = 9.2 Hz, 4H), 8.01 (d, *J* = 9.2 Hz, 4H), 4.00 (s, 8H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  165.47, 164.42, 144.55, 137.24, 129.53, 128.07, 120.05, 116.80, 44.32; HRMS: *m/z* calcd. for C<sub>26</sub>H<sub>24</sub>N<sub>6</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 453.2041; found [M+H]<sup>+</sup>: 453.2038, [M+2H]: 227.1061.

 $N^1$ ,  $N^4$ -bis(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)-2-fluoroterephthalamide (9c). Compound 9c was prepared from 2-fluoroterephthalic acid and 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline as outlined above for compound 1. White solid, converted to TFA Salt, yield: 18%; <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.15 (s, 1H), 10.99 (s, 1H), 10.52 (s, 1H), 8.11 (d, J = 9.2 Hz, 2H), 8.02 (m, 8H), 7.91 (t, J = 8.0 Hz, 1H), 4.01 (s, 8H); HRMS: m/z calcd. for C<sub>26</sub>H<sub>23</sub>FN<sub>6</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 471.1947; found [M+H]<sup>+</sup>: 471.1945, [M+2H]: 236.1010.  $N^{1}$ , $N^{4}$ -bis(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)-2-bromoterephthalamide (9d). Compound 9d was prepared from 2-bromoterephthalic acid and 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline as outlined above for compound 1. Tan solid, yield: 17%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.79 (s, 1H), 10.58 (s, 1H), 8.31 (d, *J* = 1.6 Hz, 1H) 8.08 (dd, *J* = 4.8, 2.0 Hz, 1H), 7.84 (d, *J* = 4.4 Hz, 4H), 7.81 (s, 2H), 7.76 (m, 3H), 3.61 (s, 8H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  165.43, 163.49, 163.22, 163.17, 141.45, 140.69, 140.58, 136.98, 131.73, 128.94, 127.94, 127.77, 127.23, 125.68, 125.18, 119.71, 119.05, 118.95, 49.36; HRMS: *m/z* calcd. for C<sub>26</sub>H<sub>23</sub>BrN<sub>6</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 531.1146; found [M+H]<sup>+</sup>: 531.1120.

 $N^{1}$ , $N^{4}$ -bis(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)-2-nitroterephthalamide (9e). Compound 9e was prepared from 2-nitroterephthalic acid and 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline as outlined above for compound 1. White solid, yield: 9%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.95 (s, 1H), 10.77 (s, 1H), 8.73 (d, *J* = 1.6 Hz, 1H), 8.45 (dd, *J* = 4.8, 1.6 Hz, 1H), 8.00 (d, *J* = 8.0 Hz, 1H), 7.84 (m, 6H), 7.72 (d, *J* = 8.8 Hz, 2H), 3.61 (s, 8H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$ 163.72, 163.22, 163.18, 162.88, 146.28, 140.47, 140.46, 136.81, 134.80, 133.26, 129.81, 128.01,127.83, 126.04, 125.92, 123.58, 119.83, 119.01, 49.52; HRMS: *m/z* calcd. for C<sub>26</sub>H<sub>23</sub>N<sub>7</sub>O<sub>4</sub> [M+H]<sup>+</sup>: 498.1892; found [M+H]<sup>+</sup>: 498.1896, [M+2H]: 249.5984.

 $N^{1}$ , $N^{4}$ -bis(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)-2-methylterephthalamide (9f). Compound 9f was prepared from 2-methyterephthalic acid and 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline as outlined above for compound 9b. White solid, yield: 44%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$ 11.00 (s, 1H), 10.88 (s, 1H), 8.10 (d, *J* = 9.2, 2H), 8.02 (m, 7H), 7.96 (d, *J* = 8.4, 1H), 7.69 (d, *J* = 8.0 Hz, 1H), 3.99 (s, 8H), 2.49 (s, 3H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  167.37, 165.05, 140.92, 140.88, 139.73, 135.61, 135.57, 129.79, 127.82, 127.71, 127.39, 125.14, 119.59, 118.98, 49.35, 19.36; HRMS: *m/z* calcd. for C<sub>27</sub>H<sub>26</sub>N<sub>6</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 467.2197; found [M+H]<sup>+</sup>: 467.2183.

(1*r*,4*r*)-*N*<sup>1</sup>,*N*<sup>4</sup>-bis(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)cyclohexane-1,4-dicarboxamide (11). Compound 11 was prepared from the bis-nitrile, (1r,4r)-*N*<sup>1</sup>,*N*<sup>4</sup>-bis(4cyanophenyl)cyclohexane-1,4-dicarboxamide, following the procedure described above for compound 9b. White solid, yield: 23%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.52 (s, 2H), 7.93 (d, *J* = 9.6 Hz, 4H), 7.87 (d, *J* = 8.8 Hz, 4H), 3.98 (s, 8H), 3.04 (s, 2H), 1.96 (d, *J* = 6.8 Hz, 4H), 1.49 (t, *J* = 10.0 Hz, 4H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  174.93, 164.38, 144.96, 129.66, 118.73, 115.88, 44.29, 44.14, 28.07; HRMS: *m/z* calcd. for C<sub>26</sub>H<sub>30</sub>N<sub>6</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 459.2510; found [M+H]<sup>+</sup>: 459.2512, [M+2H]: 230.1294.

 $N^{1}$ ,  $N^{4}$ -bis(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)succinamide (13). Succinic acid (35 mg, 0.3 mmol) was converted to the corresponding acid chloride via treatment with 2.0 M oxalyl chloride solution in dichloromethane. A catalytic quantity of DMF was added and the reaction was complete in 4 hours on an ice bath. The solvent was removed, leaving succinyl chloride as a yellow solid. 4-(4,5-dihydro-1H-imidazol-2-yl)aniline (97 mg, 0.6 mmol) was dissolved in 1.5 mL glacial acetic acid before being added to the acid chloride. The solution was sonicated to mix before being allowed to react overnight at room temperature. The solvent was removed and crude product was purified on a 50 g C18 reversed-phase column (acetonitrile/water). White solid, 2 TFA salt, yield: 9%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.57 (s, 2H), 10.34 (s, 4H), 7.89 (d, *J* = 9.2 Hz, 4H), 7.82 (d, *J* = 8.8 Hz, 4H), 3.98 (s, 8H), 2.75 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  171.37, 164.37, 144.76, 129.69, 118.56, 115.83, 44.26, 30.95; HRMS: *m/z* calcd. for C<sub>22</sub>H<sub>24</sub>N<sub>6</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 405.2041; found [M+H]<sup>+</sup>: 405.2041, [M+2H]: 203.1056.

 $N^1$ , $N^3$ -bis(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)isophthalamide (16). Compound 16 was prepared from the bis-nitrile,  $N^1$ , $N^3$ -bis(4-cyanophenyl)isophthalamide, following the procedure described above for compound 9a. White solid, 2 HCl salt, yield: 45%; <sup>1</sup>H NMR (400 MHz,

#### **Journal of Medicinal Chemistry**

DMSO- $d_6$ )  $\delta$  11.20 (s, 2H). 10.44 (s, 4H), 8.84 (s, 1H), 8.21 (m, 6H), 8.01 (d, J = 8.8 Hz, 4H), 7.76 (t, J = 7.6 Hz, 1H), 4.00 (s, 8H); <sup>13</sup>C NMR (400 MHz, DMSO- $d_6$ )  $\delta$  165.48, 164.29, 144.79, 134.14, 131.66, 129.49, 119.81, 116.59, 44.22; HRMS: m/z calcd. for C<sub>26</sub>H<sub>24</sub>N<sub>6</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 453.2041; found [M+H]<sup>+</sup>: 453.2037, [M+2H]: 227.1056.

#### 4-chloro-N-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl)benzamide (17a). 4-chlorobenzoic

acid (47 mg, 0.3 mmol) was converted to the corresponding acid chloride via treatment with 2.0 M oxalyl chloride solution (0.18 mL, 0.36 mmol) in dichloromethane. A catalytic quantity of DMF was added and the reaction was complete in 4 hours on an ice bath. The solvent was removed, leaving 4-chlorobenzoyl chloride as a yellow solid. 4-(4,5-dihydro-1H-imidazol-2-yl)aniline (48 mg, 0.3 mmol) was dissolved in 1.5 mL glacial acetic acid before being added to the acid chloride. The solution was sonicated to mix before being allowed to react overnight at room temperature. The solvent was removed and crude product was purified on a 50 g C18 reversed-phase column (acetonitrile/water). The product was obtained as a TFA salt, (45 mg, 36% yield).<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.76 (s, 1H), 10.36 (s, 2H), 8.04 (d, *J* = 8.8 Hz, 2H), 8.00 (d, *J* = 8.4 Hz, 2H), 7.94 (d, *J* = 9.2 Hz, 2H), 7.66 (d, *J* = 8.8 Hz, 2H), 4.00 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  165.11, 164.39, 144.57, 136.95, 129.81, 129.47, 128.59, 119.92, 116.63, 44.28; HRMS: *m*/*z* calcd. for C<sub>16</sub>H<sub>14</sub>ClN<sub>3</sub>O [M+H]<sup>+</sup>: 300.0905; found [M+H]<sup>+</sup>: 300.0904.

Methyl 4-((4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)carbamoyl)benzoate (17b). Compound 17b was prepared from 4-(methoxycarbonyl)benzoic acid and 4-(4,5-dihydro-1*H*-imidazol-2yl)aniline (3) following the procedure described above for compound 17a. Tan solid, yield: 32%; <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  10.63 (s, 1H), 8.10 (dd, J = 12.8, 8.8 Hz, 4H), 7.85 (dd, J =16.5, 9.2 Hz, 4H), 3.90 (s, 3H), 3.65 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO- $d_6$ )  $\delta$  165.65, 165.02, 163.47, 141.62, 138.75, 132.23, 129.23, 128.19, 128.15, 119.70, 52.47; HRMS: m/z calcd. for C<sub>18</sub>H<sub>17</sub>N<sub>3</sub>O<sub>3</sub> [M+H]<sup>+</sup>: 324.1350; found [M+H]<sup>+</sup>: 324.1352.

 $N^{1}$ -benzyl- $N^{4}$ -(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)terephthalamide (17c). Compound 17c was prepared from acid chloride 18, described below, and 4-(4,5-dihydro-1*H*-imidazol-2yl)aniline (3) following the procedure described above for compound 17a. White solid, yield 25%; <sup>1</sup>H NMR (400 MHz, DMSO- $d_{6}$ )  $\delta$  10.53 (s, 1H), 9.24 (s, 1H), 8.05 (s, 4H), 7.84 (s, 4H), 7.31 (m, 5H), 4.52 (s, 2H), 3.60 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO- $d_{6}$ )  $\delta$  165.48, 165.04, 163.15, 140.66, 139.50, 139.48, 137.05, 136.95, 128.33, 127.78, 127.61, 127.32, 127.27, 126.82, 126.06, 119.59, 42.72; HRMS: *m/z* calcd. for C<sub>24</sub>H<sub>22</sub>N<sub>4</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 399.1823; found [M+H]<sup>+</sup>: 399.1828.

**2-(4-chlorophenyl)**-*N*-(**4-(4,5-dihydro-1***H*-imidazol-2-yl)phenyl)acetamide (17d). Compound 17d was prepared from 2-(4-chlorophenyl)acetic acid and 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17a**. Tan solid, TFA salt, yield: 11%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 10.70 (s, 1H), 10.32 (s, 2H), 7.88 (d, J = 9.2 Hz, 2H) 7.83 (d, *J* = 8.8 Hz, 2H), 7.40 (d, *J* = 8.4 Hz, 2H), 7.35 (d, *J* = 8.8 Hz, 2H), 3.98 (s, 4H), 3.73 (s, 2H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 169.77, 164.34, 144.61, 134.41, 131.45, 131.17, 129.68, 128.28, 118.81, 116.18, 44.27, 42.41; HRMS: *m/z* calcd. for C<sub>17</sub>H<sub>16</sub>ClN<sub>3</sub>O [M+H]<sup>+</sup>: 314.1062; found [M+H]<sup>+</sup>: 314.1059.

## **2-benzyl-***N***-(4-(4,5-dihydro-1***H***-imidazol-2-yl)phenyl)-1-oxoisoindoline-5-carboxamide (17e).** Ester **20** (75 mg, 0.48 mmol) was dissolved in 5 mL of a 9-1 mixture of THF-H<sub>2</sub>O and stirred vigorously. To this mixture was added LiOH (25 mg, 1 mmol) and the reaction was stirred at rt overnight. The reaction was judged complete and the solvent was removed to near dryness by passing a gentle stream of air over the vial. The resulting residue was neutralized to ~ pH 5-6 with

#### **Journal of Medicinal Chemistry**

1N HCl and the product was extracted with dichloromethane (4X). The organics, were combined, dried and concentrated in vacuo. The resulting acid was converted to the acid chloride without further purification.

The acid (55 mg, 0.23 mmol) was added to a vial fitted with a Teflon screwcap and slurried in 1.5 mL of dichloromethane. The reaction was then cooled to 0 °C and a catalytic amount of DMF (0.01 mL) was added followed by addition of 2.0 M solution of oxalyl chloride (0.125 mL, 0.5 mmol) in dichloromethane. The resulting solution was stirred at 0 °C for 10 min then allowed to warm to rt and stir for 1h to give a dark yellow homogeneous solution. The solvent was then removed under vacuum to give a yellow-brown residue and further dried under vacuum for 2h. In a separate vial, 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline (3) (37 mg, 0.23 mmol) was dissolved in 1 mL of glacial acetic acid. The resulting homogeneous mixture was stirred for 5 min at rt and then added all at once to the acid chloride residue from above. A thick yellow precipitate forms and the reaction was allowed to stir overnight. The resulting reaction mixture was filtered through a fritted funnel with washing from diethyl ether and then dried. The solid is then slurried in water and a saturated sodium bicarbonate solution was added until the pH  $\sim$  10. The solution was then filtered and washed sequentially with water and diethyl ether and further dried under vacuum to give a tan solid (15 mg, 16%). This was converted to the TFA salt as described above, <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  10.90 (s, 1H), 10.41 (s, 2H), 8.13 (s, 1H), 8.09 (d, J = 7.6 Hz, 1H), 8.05 (d, J = 9.2Hz, 2H), 7.95 (d, J = 8.8 Hz, 2H), 7.91 (d, J = 8.0 Hz, 1H), 7.37 (m, 2H), 7.30 (m, 3H), 4.78 (s, 2H), 4.48 (s, 2H), 4.00 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 166.53, 165.88, 164.42, 141.92, 137.28, 137.13, 129.53, 128.75, 127.75, 127.68, 127.45, 123.32, 123.05, 119.95, 116.72, 49.40, 45.53, 44.30; HRMS: m/z calcd. for C<sub>25</sub>H<sub>22</sub>N<sub>4</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 411.1823; found [M+H]<sup>+</sup>: 411.1801.

*N*-(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)-3-phenylpropanamide (17f). Compound 17f was prepared from 3-phenylpropanoic acid and 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17a**. White solid, TFA salt, yield: 28%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.43 (s, 1H), 10.31 (s, 2H), 7.87 (d, *J* = 9.2 Hz, 2H) 7.81 (d, *J* = 8.8 Hz, 2H), 7.27 (m, 4H), 7.10 (t, *J* = 6.8 Hz, 1H), 3.98 (s, 4H), 2.92 (t, *J* = 7.6 Hz, 2H), 2.70 (t, *J* = 8.0 Hz, 2H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  171.46, 164.38, 144.71, 140.96, 129.68, 128.37, 128.27, 126.04, 118.67, 115.93, 44.27, 38.00, 30.53; HRMS: *m/z* calcd. for C<sub>18</sub>H<sub>19</sub>N<sub>3</sub>O [M+H]<sup>+</sup>: 294.1608; found [M+H]<sup>+</sup>: 294.1607.

*N*-(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)cinnamamide (17g). Compound 17g was prepared from cinnamic acid and 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17a**. Tan solid, TFA salt, yield: 20%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  13.48 (s, 1H) 10.90 (s, 1H), 8.81 (d, *J* = 2.4 Hz, 1H), 8.71 (s, broad, 2H), 8.67 (s, 1H), 8.36 (d, *J* = 2.0 Hz, 1H), 8.09 (m, 4H), 7.86 (d, *J* = 8.8 Hz, 1H), 7.78 (dd, *J* = 8.8, 2.0 Hz, 1H), 6.61 (s, 1H), 2.60 (s, 3H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  164.41, 164.31, 144.82, 141.54, 134.46, 130.18, 129.73, 129.11, 127.96, 121.56, 118.96, 116.19, 44.29; HRMS: *m/z* calcd. for C<sub>18</sub>H<sub>17</sub>N<sub>3</sub>O [M+H]<sup>+</sup>: 292.1452; found [M+H]<sup>+</sup>: 292.1449.

#### (E)-3-(2-chlorophenyl)-N-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl)acrylamide (17h).

Compound **17h** was prepared from (*E*)-3-(2-chlorophenyl)acrylic acid and 4-(4,5-dihydro-1*H*imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17a**. White Solid, yield 16%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.55 (s, 1H), 7.90 (d, *J* = 15.6 Hz, 1H), 7.79 (m, 5H), 7.57 (m, 1H), 7.45 (m, 2H), 6.92 (d, 15.6 Hz, 1H), 3.62 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO*d*<sub>6</sub>)  $\delta$  163.27, 163.23, 141.05, 135.71, 133.50, 132.43, 131.34, 130.11, 128.01, 127.90, 127.74,

 125.16, 124.96, 118.62, 49.09; HRMS: *m/z* calcd. for C<sub>18</sub>H<sub>16</sub>ClN<sub>3</sub>O [M+H]<sup>+</sup>: 326.1062; found [M+H]<sup>+</sup>: 326.1060.

#### (E)-3-(2-bromophenyl)-N-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl)acrylamide (17i).

Compound **17i** was prepared from (*E*)-3-(2-bromophenyl)acrylic acid and 4-(4,5-dihydro-1*H*imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17a**. White solid, TFA salt, yield: 34%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.85 (s, 1H), 10.36 (s, 2H), 7.92 (m, 5H), 7.78 (dd, *J* = 7.6, 1.6 Hz, 1H), 7.75 (dd, *J* = 8.0, 1.2 Hz, 1H), 7.51 (t, *J* = 7.2 Hz, 1H), 7.38 (dt, *J* = 8.0, 1.6 Hz, 1H), 6.89 (d, *J* = 15.6 Hz, 1H), 4.00 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  164.39, 163.74, 144.61, 139.21, 133.97, 133.42, 131.82, 129.78, 128.52, 127.90, 124.74, 124.59, 119.11, 116.44, 44.32; HRMS: *m/z* calcd. for C<sub>18</sub>H<sub>16</sub>BrN<sub>3</sub>O [M+H]<sup>+</sup>: 370.0557; found [M+H]<sup>+</sup>: 370.0559.

#### (E)-N-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl)-3-(2-(trifluoromethyl)phenyl)acrylamide

(17j). Compound 17j was prepared from (*E*)-3-(2-(trifluoromethyl)phenyl)acrylic acid and 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17a**. White solid, TFA salt, yield: 16%; <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$ ; <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$ ; <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  10.93 (s, 1H), 10.39 (s, 2H), 7.87 (m, 8H), 7.66 (t, *J* = 7.6 Hz, 1H), 6.95 (d, *J* = 15.2 Hz, 1H), 4.00 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO- $d_6$ )  $\delta$  164.40, 163.45, 144.44, 135.98, 133.26, 132.86, 130.19, 129.74, 127.97, 127.19, 126.90, 126.12, 119.16, 116.53, 44.3;

#### (E)-3-(3-chlorophenyl)-N-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl)acrylamide (17k).

Compound **17k** was prepared from (*E*)-3-(3-chlorophenyl) acrylic acid and 4-(4,5-dihydro-1*H*imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17k**. White solid, TFA salt, yield: 16%; <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  10.74 (s, 1H), 10.34 (s, 2H), 7.93 (s, 4H), 7.74 (s, 1H), 7.64 (m, 2H), 7.50 (m, 2H), 6.91 (d, J = 15.6 Hz, 1H), 4.00 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO- $d_6$ )  $\delta$  164.39, 163.97, 144.65, 139.81, 136.76, 133.77, 130.89, 129.72, 127.62, 126.33, 123.32, 118.98, 116.29, 44.28; HRMS: m/z calcd. for C<sub>18</sub>H<sub>16</sub>ClN<sub>3</sub>O [M+H]<sup>+</sup>: 326.1062; found [M+H]<sup>+</sup>: 326.1036.

#### (E)-3-(4-chlorophenyl)-N-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl)acrylamide (17l).

Compound **171** was prepared from (*E*)-3-(4-chlorophenyl) acrylic acid and 4-(4,5-dihydro-1*H*imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17a**. White solid, TFA salt, yield: 35%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.74 (s, 1H), 10.33 (s, 2H), 7.93 (s, 2H), 7.66 (m, 3H), 7.54 (d, *J* = 8.4 Hz, 2H), 6.86 (d, 16.4 Hz, 1H), 3.99 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  164.38, 164.09, 144.71, 140.09, 134.58, 133.42, 129.71, 129.61, 129.12, 122.38, 118.97, 116.23, 44.31; HRMS: *m/z* calcd. for C<sub>18</sub>H<sub>16</sub>ClN<sub>3</sub>O [M+H]<sup>+</sup>: 326.1062; found [M+H]<sup>+</sup>: 326.1053.

#### (E)-3-(4-bromophenyl)-N-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl)acrylamide (17m).

Compound **17m** was prepared from (*E*)-3-(4-bromophenyl) acrylic acid and 4-(4,5-dihydro-1*H*imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17a**. Yellow solid, TFA salt, yield: 22%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.44 (s, 1H), 10.31 (s, 2H), 7.87 (d, *J* = 9.2 Hz, 2H), 7.80 (d, 9.2 Hz, 2H), 7.47 (d, *J* = 8.4 Hz, 2H), 7.21 (d, *J* = 8.0 Hz, 2H), 3.98 (s, 4H), 2.90 (t, *J* = 7.6 Hz, 2H), 2.70 (t, *J* = 7.6 Hz, 2H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  171.22, 164.34, 144.64, 140.44, 131.19, 130.62, 129.68, 119.07, 118.67, 115.94, 44.26, 37.64, 29.81; HRMS: *m/z* calcd. for C<sub>18</sub>H<sub>18</sub>BrN<sub>3</sub>O [M+H]<sup>+</sup>: 372.0713; found [M+H]<sup>+</sup>: 372.0706.

#### (*E*)-*N*-(4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl)-3-(4-methoxyphenyl)acrylamide (17n). Compound 17n was prepared from (*E*)-3-(4-methoxyphenyl) acrylic acid and 4-(4,5-dihydro-1*H*-

#### **Journal of Medicinal Chemistry**

imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17a**. Tan solid, yield 6%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 10.63 (s, 1H), 10.32 (s, 2H), 7.92 (d, *J* = 2.8 Hz, 4H), 7.61 (m, 3H), 7.03 (d, *J* = 8.4 Hz, 2H), 6.71 (d, *J* = 15.6 Hz, 1H), 3.99 (s, 4H), 3.81 (s, 3H).

#### (E)-N-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl)-3-(4-isopropylphenyl)acrylamide (170).

Compound **170** was prepared from (*E*)-3-(4-isopropylphenyl) acrylic acid and 4-(4,5-dihydro-1*H*imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17a**. White solid, TFA salt, yield 8%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.70 (s, 1H), 10.33 (s, 2H), 7.93 (d, *J* = 2.8 Hz, 4H), 7.61 (m, 3H), 7.34 (d, *J* = 8.8 Hz, 2H), 6.81 (d, *J* = 15.6 Hz, 1H), 3.99 (s, 4H), 2.93 (m, 1H), 1.22 (d, *J* = 6.8 Hz, 6H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  164.46, 164.40, 150.91, 144.89, 141.55, 132.12, 129.74, 128.09, 127.09, 120.53, 118.93, 116.11, 44.29, 33.40, 23.68; HRMS: *m/z* calcd. for C<sub>21</sub>H<sub>23</sub>N<sub>3</sub>O [M+H]<sup>+</sup>: 334.1921; found [M+H]<sup>+</sup>: 334.1906.

#### (E)-3-(4-(tert-butyl)phenyl)-N-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl)acrylamide (17p).

Compound **17p** was prepared from (*E*)-3-(4-*tert*-butyllphenyl) acrylic acid and 4-(4,5-dihydro-1*H*imidazol-2-yl)aniline (**3**) following the procedure described above for compound **17a**. White solid, TFA salt, yield 7%; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.70 (s, 1H), 10.33 (s, 2H), 7.93 (d, *J* = 2.8 Hz, 4H), 7.61 (m, 3H), 7.49 (d, *J* = 8.8 Hz, 2H), 6.82 (d, *J* = 15.6 Hz, 1H), 3.99 (s, 4H), 1.30 (s, 9H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  164.43, 164.37, 153.04, 144.87, 141.36, 131.71, 129.70, 127.78, 125.87, 120.63, 118.89, 116.07, 44.26, 34.63, 30.92; HRMS: *m/z* calcd. for C<sub>22</sub>H<sub>25</sub>N<sub>3</sub>O [M+H]<sup>+</sup>: 348.2078; found [M+H]<sup>+</sup>: 348.2049.

#### 6-bromo-N-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl)-2-naphthamide (17q). 6-bromo-2-

naphthoic acid (63 mg, 0.25 mmol) was converted to the corresponding acid chloride via treatment with 2.0 M oxalyl chloride solution (0.15 mL, 0.3 mmol) in dichloromethane. A catalytic quantity

of DMF was added and the reaction was complete in 4 hours on an ice bath. The solvent was removed, leaving 6-bromo-2-naphthoyl chloride as a yellow solid. 4-(4,5-dihydro-1*H*-imidazol-2-yl)aniline (40 mg, 0.25 mmol) was dissolved in 1.5 mL glacial acetic acid before being added to the acid chloride. The solution was sonicated to mix before being allowed to react overnight at room temperature. The solvent was removed and crude product was purified on a 50 g C18 reversed-phase column (acetonitrile/water). The product was obtained as a TFA salt (15 mg, 12% yield). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.93 (s, 1H), 10.37 (s, 2H), 8.62 (s, 1H), 8.35 (d, *J* = 2.0 Hz, 1H), 8.08 (m, 5H), 7.96 (d, *J* = 8.8 Hz, 2H), 7.78 (dd, *J* = 8.8, 2.0 Hz, 1H), 4.01 (s, 4H); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  166.09, 164.44, 144.79, 135.58, 132.22, 131.23, 130.59, 130.11, 129.74, 129.56, 128.46, 127.51, 125.61, 121.58, 119.92, 116.60, 44.32; HRMS: *m/z* calcd. for C<sub>20</sub>H<sub>16</sub>BrN<sub>3</sub>O [M+H]<sup>+</sup>: 394.0557; found [M+H]<sup>+</sup>: 394.0553.

**4-(benzylcarbamoyl)benzoyl chloride (18).** To a solution of terephthalic acid (300 mg, 1.8 mmol) in 5 mL of dichloromethane and 1.0 mL of DMF was added sequentially diisopropylethylamine (0.95 mL, 5.4 mmol) and benzylamine (0.11 mL, 1.08 mmol). This mixture was stirred together at rt for 30 min followed by addition of TBTU (415 mg, 1.26 mmol). The mixture was stirred at rt overnight. By LC-MS the desired mono-addition product is the major one. The reaction was stopped by addition of water resulting in a small of a white precipitate to form. This was filtered off and determined to be the bis-amide product. The resulting biphasic mixture was separated and the aqueous layer was extracted with dichloromethane (4X). The organics were combined, dried and concentrated in vacuo to give a thick oil. Addition of a few drops of water to this oil resulted in a thick precipitate forming that was filtered and washed successively with water and diethyl ether (2X) to give a resulting white solid of 95% purity which

#### **Journal of Medicinal Chemistry**

was carried on in the next step. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  9.21 (t, J = 6 Hz, 1H), 8.03 – 7.85 (m, 4H), 7.41 – 7.32 (m, 4H), 7.24 – 7.20 (m, 1H), 4.49 (d, J = 6 Hz, 2H).

The resulting acid (65 mg, 0.25 mmol) from above was added to a reaction vial with a stir bar and fitted with a teflon cap and dissolved in 1.5 mL of dichloromethane. The reaction was then cooled to 0 °C and a catalytic amount of DMF was added to the reaction. To this solution was added a 2.0 M solution of oxalyl chloride (0.14 mL, 0.28 mmol) in dichloromethane. The resulting mixture was stirred for 10min at 0 °C and then allowed to warm to rt. After stirring for 1h at rt the reaction was concentrated to dryness under vacuum. The resulting residue was then used as is in the next step.

**Methyl 2-benzyl-1-oxoisoindoline-5-carboxylate (20).** To a screwtop vial fitted with a teflon cap was added methyl 1-oxoisoindoline-5-carboxylate (100 mg, 0.52 mmol). To this was added cesium carbonate (252 mg, 0.78 mmol) and the resulting mixture was slurried in 4 mL of acetonitrile. Benzyl bromide (0.08 mL, 0.68 mmol) was added to the reaction, which was subsequently heated to 75 °C and allowed to stir at this temperature overnight. Additional cesium carbonate (100 mg, 0.3 mmol) and benzyl bromide (0.03 mL, 0.25 mmol) and the reaction and heating again overnight led to complete consumption of starting material. The reaction was allowed to cool to rt and the solvent was removed by passing a gentle stream of air over the top of the vial. To the resulting residue was added water and dichloromethane. The layers were separated and the aqueous layer was extracted with dichloromethane (4X). The organics were combined, dried over magnesium sulfate and concentrated *in vacuo*. The crude product was purified by flash chromatography using a 40g Teledyne Isco Redisep column. Elution with Hexanes-EtOAc (5:1  $\rightarrow$ 2:1) gave 70 mg (48%) of the desired product as a white solid. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>0</sub>)  $\delta$ 

8.16 (d, *J* = 8.0 Hz, 1H), 8.07 (s, 1H), 7.95 (d, *J* = 8.0 Hz, 1H), 7.40-7.30 (M, 5H), 4.83 (s, 2H), 4.32 (s, 2H), 3.96 (s, 3H).

**Molecular Modeling.** Docked poses of **1** and selected analogs were obtained using a procedure described previously<sup>20</sup> and summarized here. Marvin Calculator Plugins<sup>33</sup> were used to assign the most likely protonation and tautomerization state for each compound. A full-length model of monomeric AcrA was generated with an X-ray structure of truncated AcrA lacking 21 residues at the N-terminus and the entire membrane-proximal domain (PDB entry 2F1M).<sup>13</sup> An X-ray structure of CusB from *E. coli*<sup>34</sup> was used as the template for the MP domain. After a brief molecular dynamics (MD) simulation of the model in water, the resulting MD snapshots were clustered to generate a diverse ensemble of conformations. Ensemble docking<sup>35</sup> was then performed on each protein conformation with VinaMPI<sup>36</sup> to generate and rank ligand binding poses. Selected 2D and 3D molecular descriptors were calculated with the program MOE.<sup>37</sup>

**Biology.** *E. coli* WT-Pore and ΔTolC-Pore strains are derivatives of BW 25113 and GD102.<sup>21</sup> Plasmid pEZ11 expressing a soluble AcrA<sup>His</sup> variant under the IPTG-inducible T7-promoter was used for purification of AcrA.<sup>38</sup> MICs were analyzed using a two-fold broth dilution method.<sup>39</sup> For the checkerboard assay, an antibiotic and a test compound were serially diluted into 96-well plates as described previously.<sup>40</sup> SPR experiments were carried out with the purified AcrA immobilized onto a CM5 chip (Biacore).<sup>20,22</sup> The SPR assay was validated using both negative and positive controls, as described previously.<sup>20</sup> The HT uptake assay was performed in a temperaturecontrolled micro-plate reader (Tecan Spark 10M) equipped with a sample injector, in fluorescence

#### **Journal of Medicinal Chemistry**

mode. Data were fitted to extract initial rates, as described previously.<sup>20</sup> The effect of compounds on the transmembrane potential was analyzed using DiSC3(5), as described previously.<sup>41</sup> The ionophore valinomycin was used as a positive control. This ionophore, which is usually inactive against Gram-negative bacteria, depolarizes the inner membrane of *E. coli* WT-Pore cells, because it can reach into the periplasm through the large non-specific pore present in the outer membrane of these cells.

To measure transmembrane potential, exponentially grown and induced WT-Pore cells were collected, washed and resuspended to the final OD600 of 0.2 in a buffered glucose solution supplemented with either valinomycin or indicated EPIs in final concentration 10  $\mu$ M. 1% DMSO was added as a negative control. Cells were added to the same buffer solution containing 1  $\mu$ M of the voltage-sensitive probe DiSc3(5). Upon addition of cells the DiSc3(5) fluorescence quenches due to the presence of transmembrane potential.

To test for cytotoxicity, the Promega Cell Titer Glo Luminescent Cell Viability Assay was used according to manufacturer's protocol. HEK293 cells (ATCC CRL-1573) were plated in 96-well white plates with clear, tissue culture-treated flat bottoms (Corning 3903) at 20,000 cells per well in 75ul of DMEM media (Gibco) with 5% FBS (Gemini Biosystems) and L-Glutamine (Corning) added. Cells were incubated at 37°C overnight in the presence of 5% CO<sub>2</sub>. The next day, compounds of interest were serially diluted and added to the cells to make a total volume of 100ul per well, with DMSO used as a negative control. Cells were again incubated overnight in 37°C with 5% CO<sub>2</sub>. The following day, ATP standards were added to corresponding wells, luminescent substrate was added to all wells and incubated for 10 minutes at room temperature. Luminescence

was measured on a Biotek Neo plate reader according to manufacturer's protocol. Data was analyzed using Graphpad Prism Software using standard LD<sub>50</sub> non-linear regression analysis.

#### ASSOCIATED CONTENT

#### Supporting information available:

Figures depicting additional docking poses for **1**, **4a-e** and **11**, molecular descriptor analysis of all new compounds, detailed characterization of compounds **1**, **17h**, **17o** and **17q**, characterization of intermediates **9a**, **11**, and **16**, and LC-MS traces for all reported compounds (PDF).

Molecular formula strings (CSV)

#### **AUTHOR INFORMATION**

Corresponding Author

\*E-mail: walkerjk@slu.edu. Phone: 1-314-977-6427,

#### ORCID

John K. Walker: 0000-0002-8683-0026

Helen Zgurskaya: 0000-0001-8929-4727

Valentin Rybenkov: 0000-0002-5300-4369

Jeremy C. Smith: 0000-0002-2978-3227

Jerome Baudry: 0000-0002-3587-0589

Jerry M. Parks: 0000-0002-3103-9333

#### Notes

The Authors declare no competing financial interest.

#### ACKNOWLEDGEMENTS

The University of Saint Louis School of Medicine and the National Institutes of Health AI052293 (H.I.Z) supported this work. We thank the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI, USA for providing us with plated and vialed samples. We gratefully acknowledge Dr. Stacy Arnett and the Center for World Health & Medicine for performing high-resolution mass spectrometry experiments. We acknowledge Dr. Kristine Griffett for performing cytotoxicity measurements on compounds **17h** and **17o**. We also acknowledge Miss Ashley Anderson and the NMR facility in the Saint Louis University Department of Chemistry for acquiring <sup>1</sup>H and <sup>13</sup>C NMR spectra. We thank Mr. David Wolloscheck at the University of Oklahoma for help with fitting the kinetic data and Sarah J. Cooper for calculating molecular descriptors.

#### **ABBREVIATIONS USED**

DCM, dichloromethane; DMAC, dimethyl acetamide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EPI, efflux pump inhibitor; EtOAc, ethyl acetate; HOAc, glacial acetic acid; MPC, minimum potentiation concentration; MW, molecular weight; NCI, National Cancer Institute; NIH, National Institutes of Health; OM, outer membrane; SPR, surface plasmon resonance; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N'N'*,-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; WT, wild-type.

#### REFERENCES

Page, M. GP; Heim, J. Prospects for the next anti-*Pseudomonas* drug. *Curr. Opin. Pharmacol.* 2009, 9, 558-565.

(2) Dorotkiewicz-Jach, A.; Augustyniak, D.; Olszak, T.; Drulis-Kawa, Z. Modern therapeutic approaches against *Pseudomonas aeruginosa*. *Curr. Med.Chem.* **2015**, *22*, 1642-1664.

(3) Delcour, A. Outer membrane permeability and antibiotic resistance. *Biochim. Biophys. Acta 1794.* **2009**, 808-816.

(4) Poole, K.; Srikumar, R. Multidrug Efflux in Pseudomonas aeruginosa: Components, mechanisms and Clinical Significance. *Curr. Top. Med. Chem.* **2001**, *1*, 59-71.

(5) Nikaido, H.; Pages, J.-M. Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiol. Rev.* **2012**, *36*, 340-363.

(6) Mazzariol, A.; Zulianai, J.; Cornaglia, G.; Rossolini, G. M.; Fontana, R. AcrAB efflux system:expression and contribution to fluoroquinoline resistance in Klebsiella spp. *Antimicrob. Agents Chemother.* 2002, *46*, 3984-3986.

(7) Nakashima, R.; Sakurai, K.; Yamasakai, S.; Hayashi, K.; Nagata, C.; Hoshino, K.; Onodera,

Y.; Nishino, K.; Yamaguchi, A.; Structural basis for the inhibition of bacterial multidrug exporters. *Nature* **2013**, *500*, 102-106.

(8) Opperman, T. J.; Nguyen, S.T. Recent advances toward a molecular mechanism of efflux pump inhibition. *Front. Microbiol.* **2015**, *6*, 1-16.

(9) Jamshidi, S.; Sutton, J. M.; Rahman, K. M. An overview of bacterial efflux pumps and computational approaches to study efflux pump inhibitors. *Future Med. Chem.* **2016**, *2*, 195-215.

(10) Nikaido, H.; Zgurskaya, H. I. AcrAB and related multidrug efflux pumps of Escherichia coli.*J. Mol. Microbial Biotechnol*, **2001**, *3*, 215-218.

(11) Krishnamoorthy, G.; Tikhonova, E.B.; Dhamdhere, G.; Zgurskaya, H.I. On the role of TolC in multidrug efflux: the function and assembly of AcrAB-TolC tolerate significant depletion of intracellular TolC protein. *Mol. Microbiol.* **2013**, *87*, 982-997.

(12) Du, D.; Wang, Z.; James, J. R.; Voss, J. E.; Klimont, E.; Ohene-Agyei, T.; Venter, H.; Chiu,

W.; Luisi, B. F. Structure of the AcrAB-TolC multidrug efflux pump. Nature, 2014, 509, 512-515.

(13) Mikolosko, J.; Bobyk, K.; Zgurskaya, H. I.; Ghosh, P. Conformational flexibility in the multidrug efflux system protein AcrA. *Structure* **2006**, *14*, 577-587.

(14) Jeong, H.; Kim, J. S.; Song, S.; Shigemetsu, H.; Yokoyama, T.; Hyun, J.; Na, N. C.
Pseudoatomic structure of the tripartite multidrug efflux pump AcrAB-TolC reveals the intermeshing cogwheel-like interaction between AcrA and TolC. *Structure* 2016, *24*, 272-276.
(15) Vargiu, A.V.; Nikaido, H. Multidrug binding properties of the AcrB efflux pump characterized by molecular dynamics simulations. *Proc. Natl. Acad. Sci. U.S.A.* 2013, *109*, 20637-

20642.

(16) Lu, W.; Zhong, M.; Chai, Q.; Wang, Z.; Yu, L.; Wei, Y. Functional relevance of AcrB trimerization in pump assembly and substrate binding. *PLoS ONE* **2014**, *9*, e89143.

P.; Vargiu, A. V.; Baylay, A. J.; Smith, H. E.; Brandon, Y.; Galloway, D.; Piddock, L. J. AcrB drug-binding pocket substitution confers clinically relevant resistance and altered substrate specificity. *Proc. Natl. Acad. Sci. U.S.A.* **2015**, *112*, 3511-3516.

(17) Blair, J. M.; Bavro, V. N.; Ricci, V.; Modi, N.; Cacciotto, P.; Kleinekathfer, U.; Ruggerone,

(18) Bohnert, J. A.; Schuster, S.; Kern, W. V.; Karcz, T.; Olejarz, A.; Kaczor, A.; Handzlik, J.; Kiec-Kononowicz, K. Novel piperazine arylideneimidazolones inhibit the AcrAB-TolC pump in Escherichia coli and simultaneously act as fluorescent probes in a combined real-time influx and efflux assay. *Antimicrob Agents Chemother*. **2016**, *60*, 1974-1983.

(19) Sjuts, H.; Vargiu, A. V.; Kwasny, S. M.; Nguyen, S. T.; Kim, H.-S.; Ding, X.; Ornik, A. R.;

Ruggerone, P.; Bowlin, T. L.; Nikaido, H.; Pos, K. M.; Opperman, T. J. Molecular basis for the inhibition of AcrB multidrug efflux pump by novel and powerful pyranopyridine derivatives.

Proc. Natl. Acad. Sci. U.S.A. 2016, 113, 3509-3514.

(20) Abdali, N.; Parks, J. M.; Haynes, K. M.; Chaney, J. L.; Green, A. T.; Wolloscheck, D.;

Walker, J. K.; Rybenkov, V. V.; Baudry, J.; Smith, J. C.; Zgurskaya, H. I. Reviving antibiotics:

efflux pump inhibitors that interact with AcrA, a membrane fusion protein of the AcrAB-TolC multidrug efflux pump. *ACS Infect. Dis.* **2017**, *3*, 89-98.

(21) Krishnamoorthy, G.; Wolloscheck, D.; Weeks, J. W.; Croft, C.; Rybenkov, V. V.; Zgurskaya,

H. I. Breaking the permeability barrier of Escherichia coli by controlled hyperporination of the outer membrane. *Antimicrob. Agents Chemother.* **2016**, *60*, 7372-7381.

(22) Tikhonova, E. B.; Dastidar, V.; Rybenkov, V. V.; Zgurskaya, H. I. Kinetic control of TolC recruitment by multidrug efflux complexes. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 16416-16421.

(23) Wander, A.G. Polybasic compounds and processes for their production. GB patent 1007334, 1965.

(24) Pine, M. J.; Harzewski, E.; Wissler, F. C. Action of Phthalanilide drugs on *Escherichia coli*. *Cancer Res.* 1963, *23*, 932-937

(25) Yesair, D. W.; Kohner, F. A.; Rogers, W. I.; Baronowsky, P. E.; Kensler, C. J. Relationship of the phthalanilide-lipid complexes to uptake and retention of 2-chloro-4',4''-di(2-imidazolin-2-

yl)terephthalanilide (NSC 60339) by sensitive and resistant p388 leukemia cells. *Cancer Res.* **1966**, *26*, 202-207.

(26) Coldham, N. G.; Webber, M.; Woodward, M. J.; Piddock, L. J. V. A 96-well plate fluorescence assay for assessment of cellular permeability and active efflux in *Salmonella enterica* serovar Typhimurium and *Escherichia coli. J. Antimicrob. Chemother.* 2010, *65*, 1655-1663.
(27) Sun, M.; Wei, H.-T.; Li, D.; Zheng, Y.-G.; Cai, J.; Ji, M. Mild and efficient one-pot synthesis

of 2-imidazolines from nitriles using sodium hydrosulfide as catalyst. *Syn. Comm.* **2008**, *38*, 3151-3158.

(28) Hagmann, W. K. Substituted aminoquinolines as modulators of chemokine receptor activity.WO 98/27815, 1998.

(29) Dong, Y.; Wang, X.; Cal, M.; Kaiser, M.; Vennerstrom, J. L. Activity of diimidazoline amides against African trypanosomiasis. *Bioorg. Med. Chem. Lett.* **2014**, 24, 944-948.

(30) Chollet, R.; Chevalier, J.; Bryskier, A.; Pages, J.-M.; *Antimicrob. Agents Chemother*. The AcrAB-TolC pump is involved in macrolide resistance but not in telithromycin efflux in *Enterobacter aerogenes* and *Escherichia coli*. **2004**, 48, 3621.

(31) Richter, M. F.; Drown, B. S.; Riley, A. P.; Garcia, A.; Shirai, T.; Svec, R. L.; Hergenrother, P.
J. Predictive compound accumulation rules yield a broad-spectrum antibiotic. Nature 2017, *545*, 299-304.

(32) Baell, J. B.; Holloway, G. A New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem.*2010, *53*, 2719-2740.

(33) Calculator Plugins were used for structure property prediction and calculation, Marvin 17.9.0,2017, ChemAxon (<u>http://www.chemaxon.com</u>).

(34) Su, C.-C.; Long, F.; Zimmerman, M. T.; Rajashankar, K. R.; Jernigan, R. L.; Yu, E. W. Crystal structure of the CusBA heavy-metal efflux complex of *Escherichia coli*. Nature **2011**, *470*, 558-563.

(35) Ellingson, S. R.; Miao, Y.; Baudry, J.; Simth, J. C. Multi-conformer ensemble docking to difficult protein targets. *J. Phys. Chem. B* **2015**, *119*, 1026-1034.

(36) Ellingson, S. R.; Smith, J. C.; Baudry, J. VinaMPI: facilitating multiple receptor high-throughput virtual docking on high-performance computers. *J. Comput. Chem.* **2013**, *34*, 2212-2221.

(37) *Molecular Operating Environment* (MOE), 2015: Chemical Computing Group ULC, 1010 Sherbrooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7

(38) Zgurskaya, H. I.; Nikaido, H. AcrA is a highly asymmetric protein capable of spanning the periplasm. *J. Mol. Biol.* **1999**, *285*, 409-420.

(39) Tikhonova, E. B.; Wang, Q., Zgurskaya, H. I. Chimeric analysis of the multicomponent multidrug efflux transporters from Gram-negative bacteria. *J. Bacteriol*, 2002, *184*, 6499-6507.
(40) Lomovskaya, O.; Warren, M. S.; Lee, A.; Galazzo, J.; Fronko, R.; Lee, M.; Blais, J.; Cho, D.; Chamberland, S.; Renau, T.; leger, R.; Hecker, S.; Watkins, W.; Hoshino, K.; Ishida, H.; Lee, V. J. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. *J. Antimicrob. Chemother.* 2001, *45*, 105-116.

(41) Farha, M. A.; Verschoor, C. P.; Bowdish, D.; Brown, E. D. Collapsing the Proton Motive
Force to Identify Synergistic Combinations against *Staphylococcus Aureus*. Chem. Biol. 2013, *20*, 1168-1178.





Figure 1. NSC 60339 (1)



Figure 2. Retrosynthesis of 1.



**Figure 3**. (**A**) Homology model of AcrA with the hinge site labeled. (**B**) Highest-ranking docking pose for **1** at the hinge site. Colors: Carbon atoms in AcrA and **1** in cyan and gold, respectively; nitrogen in blue; oxygen in red; chlorine in green.





Figure 4. Testing scheme used to identify compounds acting as efflux pump inhibitors.



Figure 5. SPR analysis of test compounds (50 µM) binding to immobilized AcrA.



**Figure 6**. Kinetic analyses of intracellular uptake of HT alone and in the presence of EPIs. (**A**) Real-time changes in HT fluorescence as a function of time. Exponentially grown and induced WT-Pore cells were collected, washed and resuspended in a buffered glucose solution. HT alone was added to cells (arrows) at increasing concentrations and fluorescence was monitored over a period of time. (**B**) and (**C**) the same as in (**A**) but HT was pre-mixed either with 12.5  $\mu$ M of compound **170** or 12.5  $\mu$ M compound **17h**. (**D**) Data were collected as above with various analogs and the kinetic curves were fitted into a two-exponential model to extract the initial rates of uptake. The rates are plotted as a function of HT concentration in the solution (N=4, error bars are SD).



**Figure 7**. (**A**) Effect of EPIs on the transmembrane potential in live *E. coli* cells. Valinomycin, but not EPIs or DMSO, affected the transmembrane potential of WT-Pore cells, as seen from the time-dependent increase in DiSc3(5) fluorescence. (**B**) Evaluation of HEK293 cell viability in presence of test compounds **17h** and **17o** (IC<sub>50</sub> values = 10.5 and <100  $\mu$ M respectively, N = 4).



<sup>a</sup>Reagents and conditions: (a)  $R_2NH_2$ , TBTU, diisopropylethyl amine, DMF, rt; (b) 2.0 M (COCl)<sub>2</sub>-CH<sub>2</sub>Cl<sub>2</sub>, cat. DMF, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C to rt; (c) evaporate to dryness, dissolve in THF, add Et<sub>3</sub>N then add  $R_2NH_2$ , 0 °C; (d) Aniline **3**, TBTU, *N*,*N*-diisopropylethylamine, DMF, rt.

**ACS Paragon Plus Environment** 



#### Scheme 2. General synthetic procedures dihydroimidazoline-containing compounds 9a-f<sup>a</sup>

<sup>a</sup>Reagents and conditions: (a) 2.0 M (COCl)<sub>2</sub>-CH<sub>2</sub>Cl<sub>2</sub>, cat. DMF, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C to rt; (b) Aniline **3**, HOAc, rt, 1h; (c) evaporate to dryness, dissolve in THF, add Et<sub>3</sub>N then add 3-aminobenzonitrile (**7a**) or 4-aminobenzonitrile (**7b**), 0 °C; (d) **7a** or **7b**, TBTU, diisopropylethyl amine, DMF, rt; (e) H<sub>2</sub>NCH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>, NaSH, DMAC, 115 °C.



Scheme 3. Synthesis of analogs 11, 13 and 16<sup>a</sup>



<sup>a</sup>Reagents and conditions: (a) **7a**, TBTU, *N*,*N*-diisopropylethylamine, DMF, rt; (b) H<sub>2</sub>NCH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>, NaSH, DMAC, 115 °C; (c) 2.0 M (COCl)<sub>2</sub>-CH<sub>2</sub>Cl<sub>2</sub>, cat. DMF, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C to rt; (d) Aniline **3**, HOAc, rt, 1h.





<sup>a</sup>Reagents and conditions: (a) RC(O)Cl, **3**, HOAc; (b) benzyl amine (1.3 eq.), TBTU, *N*,*N*,diisopropylethylamine, DMF, rt; (c) 2.0 M (COCl)<sub>2</sub>-CH<sub>2</sub>Cl<sub>2</sub>, cat. DMF, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C to rt; (d) BnBr, Cs<sub>2</sub>CO<sub>3</sub>, CH3CN, 75 °C, 2d; (e) LiOH, 9: THF:H<sub>2</sub>O.





<sup>a</sup>Concentration of test compound at which novobiocin activity is potentiated fourfold (N=2).







<sup>a</sup>Concentration of test compound at which novobiocin activity is potentiated fourfold (N = 2).



#### Table 3. Potentiation for analogs of 1 containing only one dihydroimidazoline group.

<sup>a</sup> Concentration of test compound where Novobiocin is potentiated 4-fold

<sup>b</sup> Value refers to MPC<sub>2</sub>, 2-fold potentiation

<sup>a</sup>Concentration of test compound at which novobiocin is potentiated fourfold (N=2). <sup>b</sup>Value refers

to MPC<sub>2</sub>, twofold potentiation.

Cmpd #	MIC(µM) wt <sup>a</sup>	MIC(μM) Pore <sup>b</sup>	MIC (μM) ΔToIC <sup>c</sup>	MIC (μM) Pore-∆TolC <sup>d</sup>	AcrA binding <sup>e</sup>	Efflux <sup>f</sup>
1	>200	100	25	12.5	+	+
9a	200	100	50	25	+	+
11	50	25	12.5	6.25	-	+
14	50	50	25	12.5	+	-
17h	>200	400	200	100	+	+
17i	>400	>400	200	100	+	+
171	200	100	50	25	ND <sup>g,h</sup>	+
170	100	50	25	25	++	+
17 <b>p</b>	25	25	12.5	12.5	+	+
17q	> 400	200	25	12.5	++	+

Table 4. SAR data for analogs showing better potentiation than 1.

<sup>a</sup>Wild type BW 25113 strain of *E. coli*. <sup>b</sup>Engineered strain of *E. coli* with 2.4 nM pores in the OM. <sup>c</sup> *E. coli* strain with the AcrAB-TolC efflux pump deleted. <sup>d</sup>Represents a strain of *E. coli* engineered with 2.4 nM pores in the OM and the AcrAB-TolC efflux pump deleted. <sup>e</sup>Evaluation of binding to immobilized AcrA with test compound (25  $\mu$ M). <sup>f</sup>Evaluation of the rate of cell uptake by HT in the presence of test compound (25  $\mu$ M). <sup>g</sup>ND = not determined. <sup>h</sup>Compound stuck to the SPR control surface (N=2),

#	<b>OM</b> <sup>a</sup>	Eff <sup>b</sup>	HTc	NOV Pot <sup>d</sup>	Ery Pot <sup>e</sup>
1	4	8	3.2	16	8
9a	2	4	1.7	4	4
17h	1	4	9.7	32	8
17i	1	8	11.6	32	32
171	2	4	2.5	8	4
170	2	2	6.3	8	8
17p	1	2	2.6	2	2
17q	4	16	16	16	16

 Table 5. Analysis of SAR data comparing ratios between different cell types.

<sup>a</sup>Evaluation of OM permeability, Ratio of WT MIC/WT-Pore MIC. <sup>b</sup>Evaluation of compound susceptibility to efflux, Ratio of WT-Pore MIC/Pore- $\Delta$ TolC MIC. <sup>c</sup>Rate HT uptake in presence of 25  $\mu$ M test compound/ HT uptake alone. <sup>d</sup>WT-Pore/WT-Pore + novobiocin (16  $\mu$ g/mL) MIC. <sup>e</sup>WT-Pore MIC/WT-Pore + erythromycin (5  $\mu$ g/mL) MIC.



