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A Forward Chemical Screen Identifies Antibiotic Adjuvants in *Escherichia coli*

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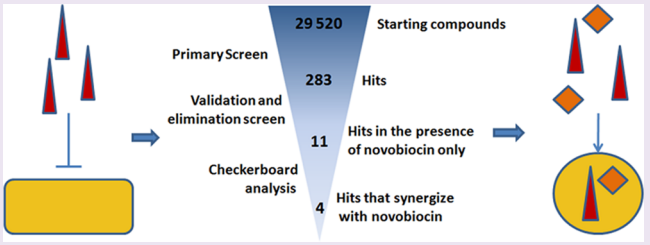
Supporting Information

ABSTRACT: Multi-drug-resistant infections caused by Gram-negative pathogens are rapidly increasing, highlighting the need for new chemotherapies. Unlike Gram-positive bacteria, where many different chemical classes of antibiotics show efficacy, Gram-negatives are intrinsically insensitive to many antimicrobials including the macrolides, rifamycins, and aminocoumarins, despite intracellular targets that are susceptible to these drugs. The basis for this insensitivity is the presence of the impermeant outer membrane of Gram-negative bacteria in addition to the expression of pumps and porins that reduce intracellular concentrations of many molecules. Compounds that sensitize Gram-negative cells to "Gram-positive antibiotics", antibiotic adjuvants, offer an orthogonal approach to addressing the crisis of multi-drug-resistant Gram-negative pathogens. We performed a forward chemical genetic screen of 30,000 small molecules designed to identify such antibiotic adjuvants of the aminocoumarin antibiotic novobiocin in *Escherichia coli*. Four compounds from this screen were shown to be synergistic with novobiocin including inhibitors of the bacterial cytoskeleton protein MreB, cell wall biosynthesis enzymes, and DNA synthesis. All of these molecules were associated with altered cell shape and small molecule permeability, suggesting a unifying mechanism for these antibiotic adjuvants. The potential exists to expand this approach as a means to develop novel combination therapies for the treatment of infections caused by Gram-negative pathogens.



The development of antimicrobial agents in the 20th century resulted in a dramatic decline in the burden of disease and death from infectious disease. The widespread use, and misuse, of these drugs however has led to the selection of bacteria that are resistant to many commonly used antibiotics. Antibiotic resistance can be classified in two broad categories: acquired and intrinsic.[1] Acquired resistance can occur as a result of mutations that arise in chromosomal genes or through the acquisition of new genes. These mutations or new genetic elements convey a selective advantage in the presence of antibiotics. This advantage is passed on to progeny, resulting in the emergence of an antibiotic-resistant strain. On the other hand, intrinsic resistance exists independent of antibiotic selection in that all members of the species or group of microbes have equal survival opportunity.

An example of intrinsic antibiotic resistance is offered in the physiology of Gram-negative bacteria. These show remarkable antibiotic resistance due to the permeability barrier presented by the lipopolysaccharide (LPS)-containing outer membrane (OM) in addition to a network of efflux pumps and selective porins that hinder the uptake and retention of many antibiotics, thereby decreasing drug effectiveness.[2−4] Coupled with this intrinsic resistance is an increased incidence of acquired resistance in Gram-negative bacteria to current first line drugs such as the β-lactams and fluoroquinolones.[5−7] The result is the emergence of extremely drug-resistant Gram-negative pathogens that increasingly defy available therapy. There is no question that novel antibiotics and/or antibiotic alternatives are needed to keep pace with the rapidly increasing levels of acquired resistance mechanisms.

The emergence and clinical relevance of multi-drug-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) in the 1980s and 1990s focused much of new antimicrobial discovery toward Gram-positive pathogens. These efforts yielded three new classes of approved antibiotic drugs over the past decade: the oxazolidinones (linezolid),[8,9] the lipodepsipeptides (daptomycin),[10] and the pleuromutilins (retapamulin).[11] These compounds though have little or no effect on Gram-negative pathogens owing to the challenge of intrinsic resistance, a property that also excludes use of many antibiotic drug classes including the glycopeptides, macrolides, aminocoumarins, and rifamycins. At the same time multi-drug-resistant (MDR) Gram-negative pathogens, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and Enterobacteriaceae, which combine both intrinsic and acquired drug resistance, are increasingly prevalent.[12,13] These are proving much more challenging for the drug discovery community to address, resulting in fewer leads for new Gram-negative-targeted antibiotics in clinical trials and an increasingly desperate situation in the clinic.

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The growing clinical need has spurred numerous strategies that are currently being explored as a means to combat Gram-negative bacterial infection. These include the development of new antibiotics such as the third generation semisynthetic tetracycline tigecycline,[14] and the redeployment of old antibiotics, in particular the lipo-cationic peptide colistin that was previously abandoned due to severe toxicity.[15,16] Furthermore, recent chemical genetic screens revealed that deletion of a number of otherwise nonessential genes (genes that can be deleted in the laboratory with no effect on cell growth) sensitize Gram-negative bacteria to a number of antibiotics including those that show effectiveness only against Gram-positives.[17] These so-called chemical synthetic lethal interactions offer a diverse and deep reservoir of targets for antibiotic adjuvants, agents that increase the efficacy of antibiotics when given in combination with drugs.[18] Antibiotic adjuvants differ from traditional antibiotic combinations in that the antibiotic is paired with a molecule that does not necessarily have intrinsic antibiotic activity itself.

Such combination therapies represent an untapped potential to repurpose "old" antibiotics with limited or diminished clinical utility due to intrinsic or acquired resistance. For example, in recent studies we showed that loperamide, a common anti-diarrheal drug (Imodium), is able to potentiate the activity of the tetracycline antibiotic minocycline in *P. aeruginosa* and other Gram-negative bacteria, including drug-resistant isolates,[19] and that sertraline (the antidepressant Zoloft) synergizes with fluconazole to kill fungal pathogens.[20]

In this study we systematically explored combinations of the aminocoumarin novobiocin, an antibiotic that has negligible anti-Gram-negative activity, with a library of 30,000 small molecules against *Escherichia coli* BW25113 in order to identify pairs that synergize to increase antimicrobial activity. We identified four non-obvious synergistic combinations that overcome the intrinsic resistance of Gram-negative bacteria to novobiocin; each of these compounds alters cell shape, suggesting a unifying mechanism of antibiotic potentiation.

RESULTS

Screen of Compounds That Potentiate Novobiocin in *E. coli* BW25113. The goal of this study was to identify small molecules that potentiate and synergize with the aminocoumarin antibiotic novobiocin to achieve antibiotic activity against *E. coli*, an organism that is intrinsically resistant to this antibiotic (Figure 1).

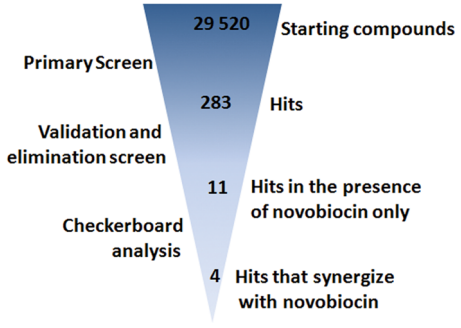

Figure 1. Strategy for identifying compounds that synergize with novobiocin to inhibit growth of *E. coli* BW25113.

A high-throughput screen of approximately 30,000 small molecules was first employed to identify compounds that could potentiate the activity of novobiocin in *E. coli* BW25113. The MIC of novobiocin against *E. coli* BW25113 in M9 minimal media was determined to be 1024 μg/mL, and the primary screen was performed at a concentration of 1/4 MIC (256 μg/mL) to select for robust potentiators of the antibiotic. We selected 1/4 MIC on the basis of our experience in compound combination screens as a workable compromise between setting a concentration too low, and thus missing possible lead compounds, and too high where the hit rate is increased, but chemical genetic links between compounds can become too distant for meaningful analysis. We defined hits as molecules that were able to inhibit bacterial growth to 60% or less than the growth of the controls resulting in a short-list of 283 compounds (0.9% hit rate) (Figure 2).

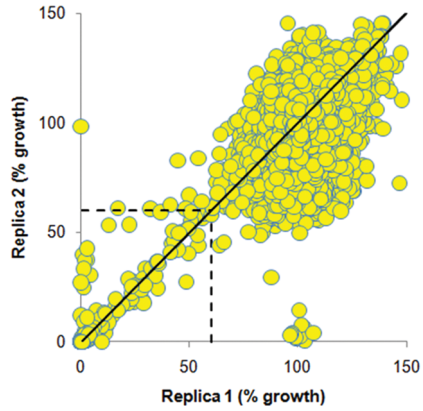

Figure 2. Replica plot of primary screening results. Plot of duplicate screen of of *E. coli* BW25113 cells grown in M9 minimal media for 22 h at 37 °C in the presence 256 μg/mL novobiocin against 29,520 compounds from the Canadian Compound Collection at 10 μM. Solid line: replication line. Dashed line: hit zone. Data points that do not align across the diagonal are the result of stochastic error; all were reassayed independently in duplicate to confirm actual values.

The 283 hits were next subjected to a secondary screen, where cell growth inhibitory activity was evaluated in both the presence and the absence of novobiocin. This secondary screen served two purposes: it confirmed the molecules as *bona fide* hits and eliminated compounds that had intrinsic antibacterial activity against *E. coli* regardless of the presence of novobiocin. As a result, 11 small molecules were identified as hits for further analysis (Figure 3).

The hits consisted of molecules with known bioactivity and those of unknown function. Compounds **1**, **2**, **3**, and **4** could not be linked to any known function from previous studies. Compound **5** is a known molecule (A22) that has been characterized as an inhibitor of MreB, a bacterial homologue of actin, required for maintenance of cell shape in rod-shaped bacteria including *E. coli*.[21,22] For consistency with the literature, compound **5** is referred to as A22 throughout the remainder of this text. Compound **1** and A22 are related molecules, varying only in the position of one chlorine on the dichlorobenzylthio ring and the presence of a triazine ring on **1** compared to the thiourea of A22.

The remaining six hits were known bioactive molecules: cisplatin, rosamicin, rifabutin, pivmecillinam, and niridazole. Cisplatin is an anticancer drug that cross-links DNA and is known to inhibit *E. coli* cell division.[23] Due to the non-specific and cross kingdom nature of cell killing in addition to nephrotoxic effects observed when administering cisplatin to patients, no further analysis was performed on this compound.[24] Rosamicin, a macrolide antibiotic with improved activity against

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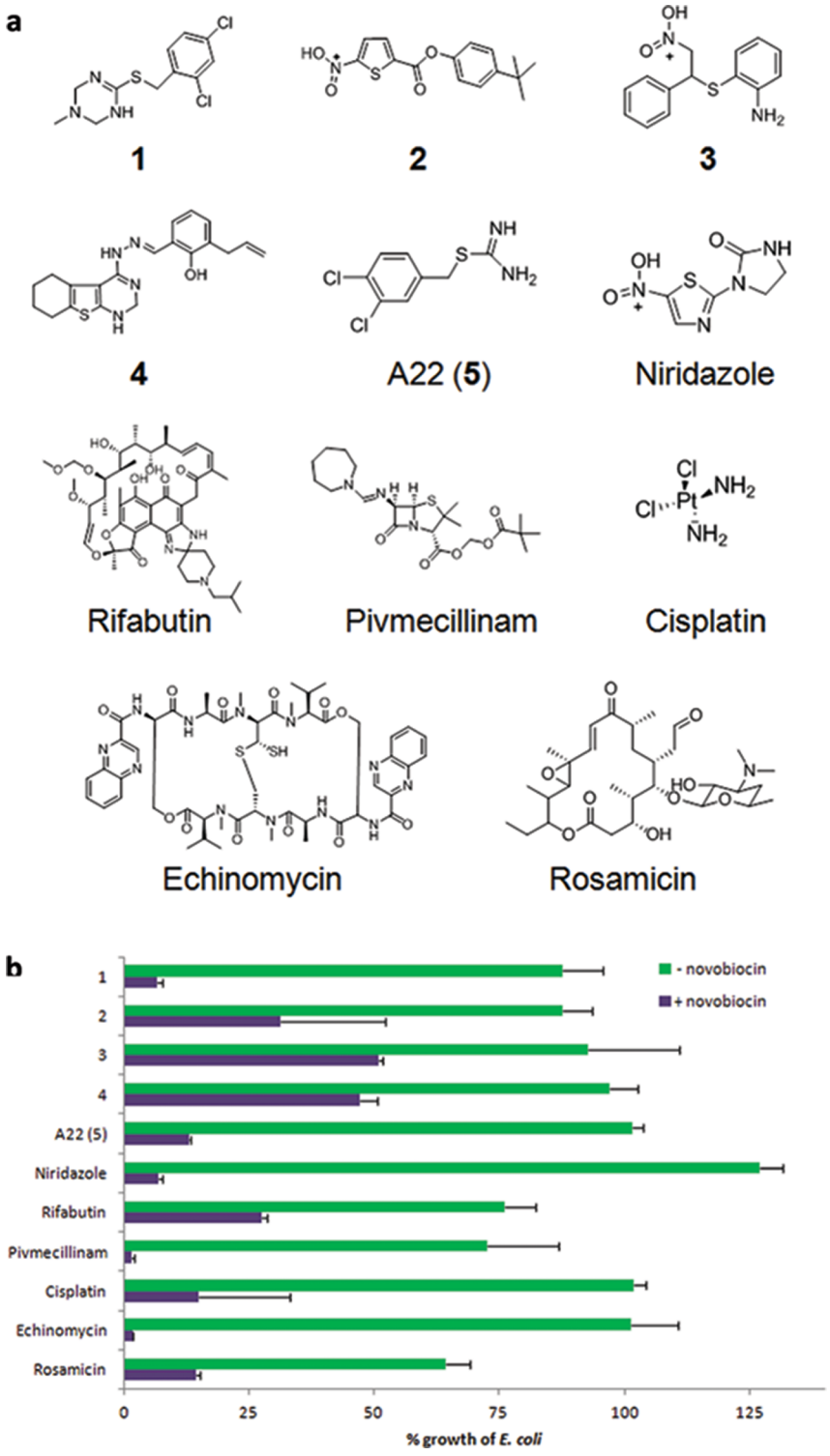

Figure 3. Summary of hits. (a) Chemical structures of the 11 compounds that inhibit the growth of *E. coli* BW25113 only in combination with 256 μg/mL novobiocin. (b) Each compound was evaluated for its ability to impair growth of *E. coli* BW25113 in M9 minimal media in the absence (0 μg/mL, green) and presence (256 μg/mL, purple) of novobiocin.

Gram-negative bacteria,[25] was also eliminated from further study due to lack of availability. For comparison, the related erythromycin, although present in the library, was not a hit in this screen, devaluing rosamicin as a priority hit. Rifabutin is a member of the RNA polymerase-inhibiting rifamycin family of antibiotics and is used in the treatment of tuberculosis.[26] Echinomycin has known antibiotic and antitumor activity based on its ability to intercalate double-stranded DNA.[27] Though this molecule has demonstrated toxicity, recent efforts look to develop less toxic analogs.[28] Pivmecillinam is a pro-drug of mecillinam, a β-lactam antibiotic that specifically binds penicillin binding protein 2 (PBP2) in *E. coli*.[29] Niridazole has broad spectrum antimicrobial activity and can also be used as an antiparasitic to treat schistosomiasis, a helminthic disease caused by flatworms.[30,31]

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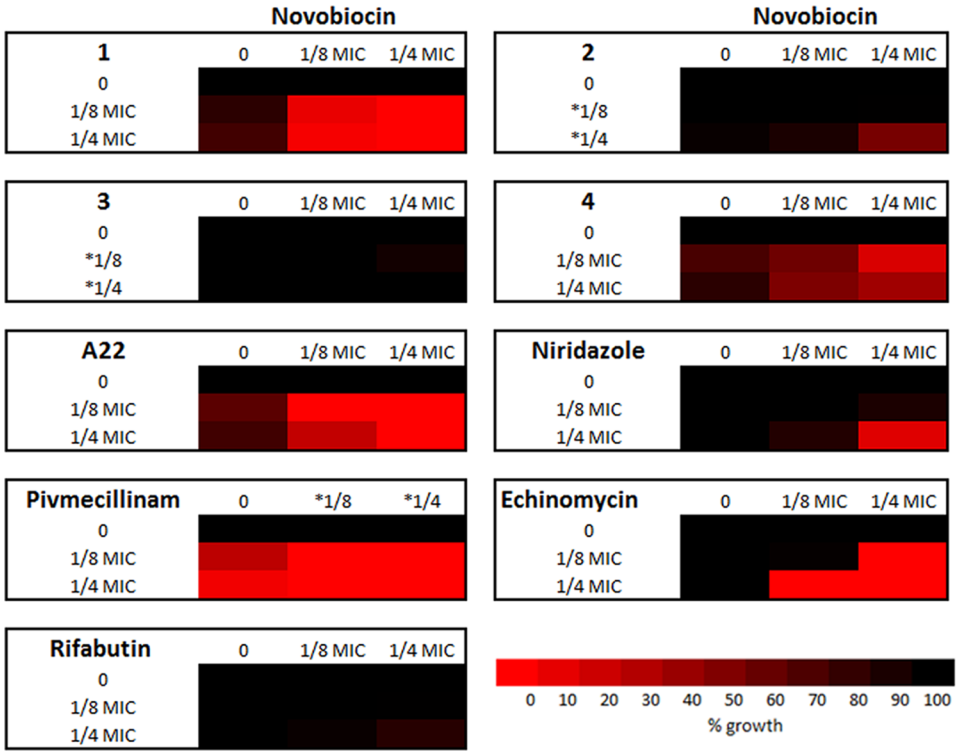

Figure 4. Synergy between hits and novobiocin in *E. coli*. Three-point matrix heat plots showing synergistic growth inhibition of *E. coli* BW25113 by novobiocin in combination with hits derived from the primary screen. Compounds **1**, A22, pivmecillinam, and echinomycin each show synergy with novobiocin according to this test, where black represents 100% growth and red represents 0% growth, normalized to growth controls.

Evaluation of Compound Synergies. The nine hits of interest were evaluated for the ability to synergize with novobiocin using 3-point dose matrices.[32] Using the fractional inhibitory concentration index (FIC), synergy occurs when a combination of molecules has an FIC ≤ 0.5. By definition, this is achieved when enhanced activity is observed and both compounds are present at a concentration of 1/4 MIC or less. By evaluating each combination at 0 μg/mL and 1/4 and 1/8 MIC, a synergistic effect can accurately be determined using minimal reagents, as compound pairs inhibiting growth at 1/4 MIC can be deemed synergistic, and at 1/8 MIC highly synergistic. Figure 4 shows that four of the hits, **1**, A22, pivmecillinam, and echinomycin, are synergistic with novobiocin, while the remainder demonstrated additivity in these conditions. We note that while pivmecillinam met the definition of synergy with novobiocin, this pro-drug did reduce the level of cell growth even at low concentrations (Figure 4), consistent with an intrinsic basal antibiotic activity even in the absence of novobiocin. This observation was consistent over numerous trials.

Assessing the Antibiotic Spectra of Novobiocin Adjuvants. In order to establish whether synergy was specific to the aminocoumarin novobiocin or was generally applicable to other "Gram-positive" antibiotics, we combined the four priority hits, **1**, A22, pivmecillinam, and echinomycin, (as well as compound **2** and niridazole, which both showed a strong additive action, as indicated by partial growth inhibition (Figure 4; Supplementary Table S1)) with a panel of antibiotics with limited activity against Gram-negative bacteria (Figure 5). None of the compounds demonstrated synergy with the macrolide erythromycin against *E. coli* BW25113. Compound **2** and lincomycin were selectively synergistic with each other. Rifampin showed a synergy profile similar to that of novobiocin, with the exception of echinomycin, where additivity was found.

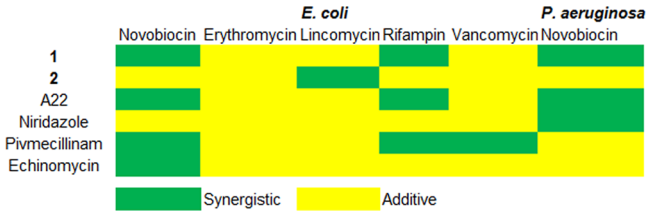

Figure 5. Interaction profile of various antibiotics against hits that demonstrated potential to act synergistically against *E. coli* BW25113 or *P. aeruginosa* PA01. Combinations were shown to act synergistically (green), or additively (yellow).

The glycopeptide vancomycin was synergistic only with pivmecillinam. This demonstrated that synergy is likely the result of specific chemical genetic interactions and not the result of, for example, a nonspecific increase in membrane permeability as seen with other compounds such as polymyxin and other cationic peptides.

The compounds were also tested for synergy with novobiocin in the opportunistic Gram-negative pathogen *P. aeruginosa* PA01, to determine if the results were specific to *E. coli* (Figure 5). Both **1** and A22 were synergistic with novobiocin against *P. aeruginosa*; however, pivmecillinam and echinomycin were not. On the other hand, niridazole was synergistic with novobiocin in this organism.

A22 + Novobiocin Synergy Is Dependent on MreB. A22 has been previously shown to be an inhibitor of the actin homologue MreB.[21] Using a strain of *E. coli* that has a single point mutation in the *mreB* gene that renders it resistant to A22,[33] we tested whether synergy between A22 and novobiocin was maintained in this background (Figure 6). While A22 and novobiocin were synergistic in the A22-sensitive strain of *E. coli*,

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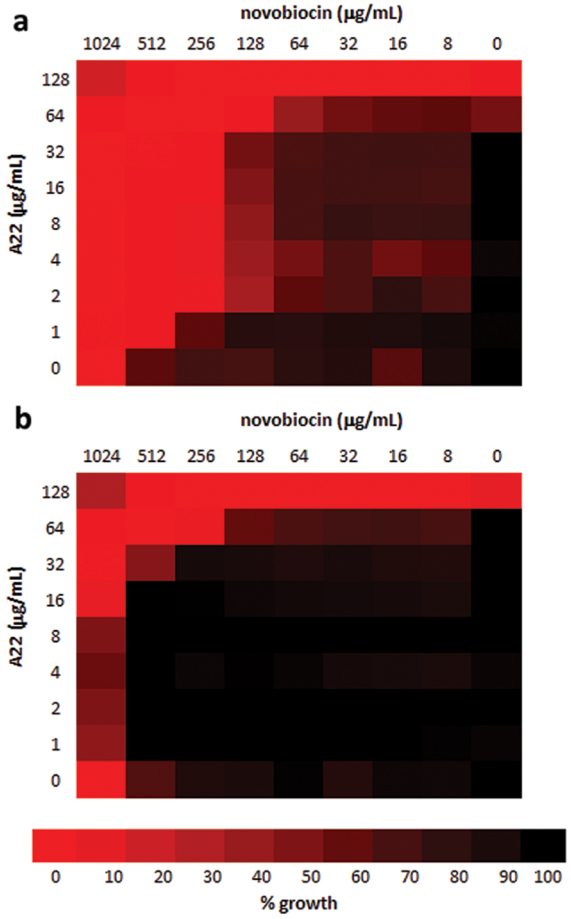

Figure 6. Comparing A22/novobiocin synergy in A22 sensitive and resistant *E. coli*. Heat plots showing growth inhibition of *E. coli* expressing *mreB* (a) sensitive and (b) resistant to A22, respectively, in the presence of varying concentrations of novobiocin and A22. Synergy was apparent in the A22 sensitive strain with an FIC of 0.27, compared to an FIC of 0.75 in the resistant strain.

with an FIC of 0.27, synergy was absent in the A22-resistant strain (FIC 1.0). Similar results were seen for the compound **1** + novobiocin combination, with FIC values of 0.51 and 1.0 against the sensitive and resistant strains, respectively.

Novobiocin Synergizers Alter *E. coli* Cell Shape. The role of MreB in A22 + novobiocin synergy led us to explore impacts on cell shape by these compounds. A22 induces a spherical cell shape in the A22-sensitive strain, regardless of the presence of novobiocin, while the A22-resistant strain maintained a rod shape consistent with the control cells. The addition of novobiocin had no discernible effect on cell shape at any concentration (Figure 7a). Similarly, pivmecillinam also induced a rod to sphere shape change (Figure 7B). This result encouraged us to explore novobiocin synergy with other β-lactam antibiotics such as the penicillins carbenicillin and metampicillin, which do not alter cell shape (Figure 7b), and the carbapenem Meropenem, which does induce a spherical cell shape change. With these compounds, synergy consistently tracked with rod to sphere shape change. The novobiocin synergizer echinomycin also induced a change in cell shape, but in the form of long filaments rather than rounding. A control compound that is not synergistic with novobiocin, the GyrA inhibitor ciprofloxacin, induces a change to filamentous cell shape but does not synergize with novobiocin. Therefore this shape transition is not indicative

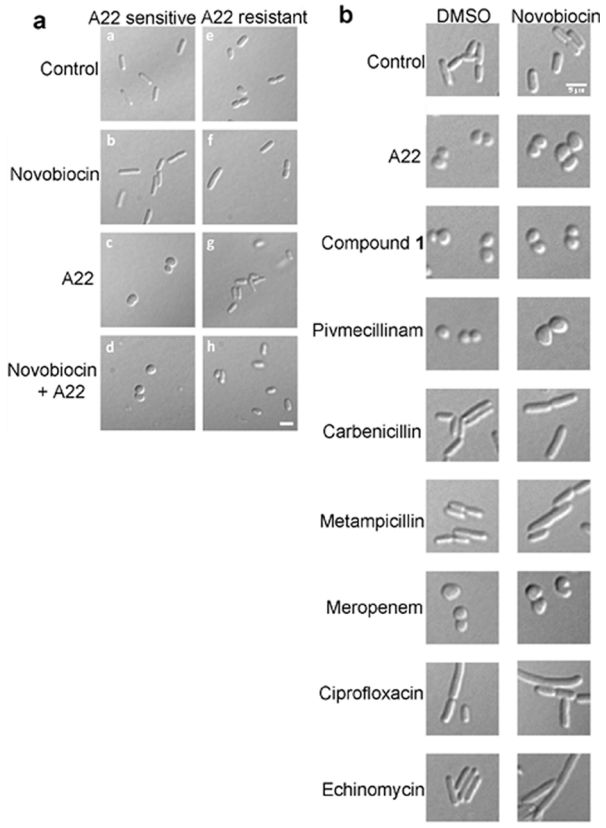

Figure 7. Effect of novobiocin synergizers on *E. coli* cell shape. (a) A22 sensitive (a–d) and A22 resistant (e–h) strains of *E. coli* were grown in the presence of 0 μg/mL A22 and novobiocin (a, e), 0 μg/mL A22 + 512 μg/mL novobiocin (b, f), 16 μg/mL A22 + 0 μg/mL novobiocin (c, g), and 16 μg/mL A22 + 128 μg/mL novobiocin (d, h). Size bar indicate 5 μm. (b) *E. coli* BW25113 cells were grown in the presence and absence of 256 μg/mL novobiocin, 32 μg/mL A22 ± 128 μg/mL novobiocin, 16 μg/mL ± 128 μg/mL novobiocin, 8 μg/mL pivmecillinam ± 256 μg/mL novobiocin, 8 μg/mL carbenicillin ± 256 μg/mL novobiocin, 1 μg/mL metampicillin ± 256 μg/mL novobiocin, 0.008 μg/mL Meropenem ± 128 μg/mL novobiocin, 0.004 μg/mL ciprofloxacin ± 256 μg/mL novobiocin, and 4 μg/mL echinomycin ±128 μg/mL novobiocin. Size bar indicates 5 μm.

of synergy. These results were comparable in two *E. coli* strains, BW25113 and WA220.

Novobiocin Synergizers Induce Changes in Small Molecule Transport. The impact on cell shape and synergy with novobiocin led us to explore the impact of the synergizers on small molecule transport in the cell. Here we used a standard ethidium bromide accumulation assay as a measure of compound permeability. Novobiocin, like the control ciprofloxacin, had no effect on ethidium bromide entry into the cell. On the other hand, compounds that synergized with novobiocin and induce changes in cell shape enhanced the accumulation of the dye in *E. coli* cells (Figure 8).

DISCUSSION

The crisis of multi-drug resistance in Gram-negative pathogens and the lack of suitable new antibiotics require the exploration of different approaches to address this pressing clinical need. Here we investigated the systematic combination of a 30,000 compound library with novobiocin, a hydrophobic aminocoumarin antibiotic that blocks the ATP binding site of bacterial DNA gyrase subunit B, blocking DNA supercoiling.[34] Novobiocin, and

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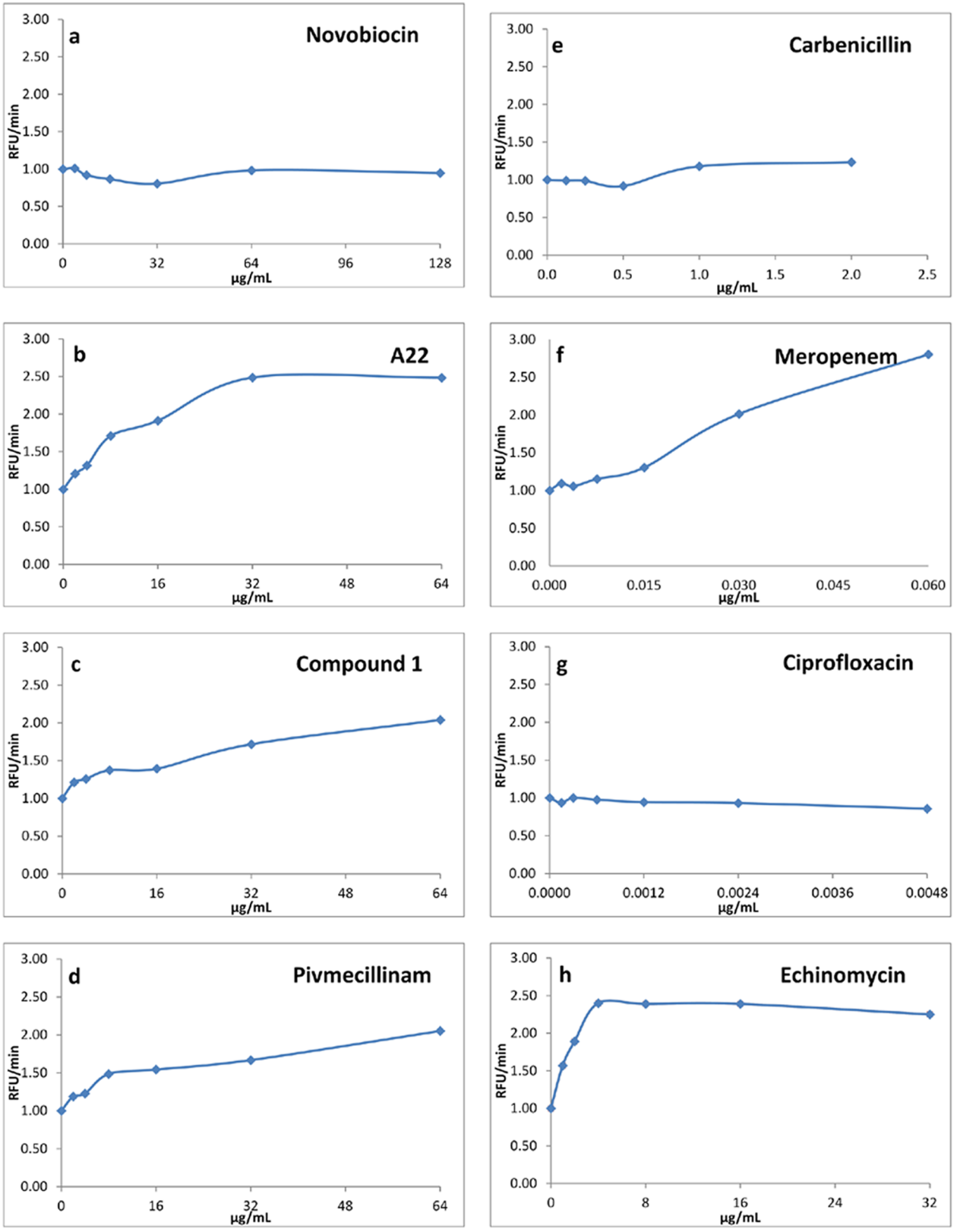

Figure 8. Effect of novobiocin synergizers on small molecule uptake in *E. coli.* Graphs showing the ability of compound treated *E. coli* to uptake EtBr. Cells were grown in the presence of increasing concentrations of novobiocin (a), A22 (b), compound 1 (c), pivmecillinam (d), carbenicillin (e), Meropenem (f), ciprofloxacin (g), and echinomycin (h). Compounds that are synergistic with novobiocin show an increased uptake of EtBr.

the aminocoumarins in general, show poor antibiotic activity against Gram-negative bacteria even though their gyrase B is sensitive to the antibiotic. Recent efforts to uncover the "intrinsic resistome"[35] of *E. coli* in a series of synthetic lethal screens,[17] suggested to us that there are multiple targets that can be inhibited by adjuvant molecules that would enhance the activity

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of antibiotics, even those such as novobiocin that have little activity against Gram-negative bacteria.

We identified four compounds that synergized with novobiocin in this screen of 30,000 small molecules. Echinomycin is a DNA intercalator that blocks hypoxia-inducible factor-1, which is a transcription factor important in tumor growth.[36] This molecule has no known protein target but rather blocks DNA metabolism through indirect means.

On the other hand A22 (and by analogy compound **1**) block the activity of MreB.[21] This actin-like protein is of great interest as a component of the bacterial cytoskeleton that scaffolds key cell wall assembly proteins and is therefore vital to cell division and growth (reviewed in refs 37−39). Inhibition of MreB resulted in alteration of cell shape toward a round cell phenotype, increased accumulation of ethidium bromide, and synergy with novobiocin and rifampin in both *E. coli* and *P. aeruginosa*.

Similarly, pivmecillinam and Meropenem also promote the rounded cell phenotype, increased accumulation of ethidium bromide, and showed synergy with novobiocin and rifampin. Pivmecillinam, a prodrug of mecillinam, inhibits PBP2, a transpeptidase involved in the insertion of new polymer strands into the peptidoglycan layer that is also required for maintaining cell shape.[40,41] This cell wall synthesis enzyme is known to be a component of the MreB-associated cell wall assembly platform.[42] On the other hand, Meropenem is an inhibitor of PBP7 and its proteolytic cleavage product PBP8,[43] which has been associated with changes in cell shape[44] but has no known interaction with MreB.

Bacterial cell shape has been shown to be important in a myriad of cell functions, including the regulation of cell differentiation, attachment, motility, and polar differentiation, among others.[45] Cell shape is determined by the rigid structure of peptidoglycan, a matrix of peptide linked glycan polymers that varies in thickness between species, and disruptions in peptidoglycan synthesis can result in cell shape abnormalities, such as conversion from rod to spherical shape in *E. coli*, where roughly 80% of the peptidoglycan in the cell wall is contained in a single layer.[46,47] Similarly, disruption of the cytoskeleton components such as MreB also contributes to alterations to cell shape. Our results add altered transport of small molecules upon disruption of cell shape and sensitivity to "Gram-positive" antibiotics. We hypothesize that direct or indirect disruption of cell shape *via* cytoskeleton proteins and/or peptidoglycan biosynthesis alters various components of the influx/efflux machinery of Gram-negative bacteria, thereby enabling the accumulation of otherwise excluded toxic antibiotic molecules. This provides an attractive orthogonal strategy for the development of new anti-Gram-negative therapies that leverage existing antibiotics with a growing understanding of microbial physiology.

METHODS

Bacterial Strains, Reagents, and General Methods. *E. coli* BW25113 (Δ(*araD-araB*)*567*, Δ*lacZ4787*(::rrnB-3), lambda$^-$, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*) was used for all experiments, unless otherwise noted. Liquid M9 minimal media (M9 salts [4.78 mM Na_2PO_4, 2.2 mM KH_2PO_4, 0.86 mM NaCl, 1.87 mM NH_4Cl], 0.4% glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$) was the growth media for all experiments, unless otherwise noted. The Canadian Compounds Collection (CCC) was used in the screen. This collection of 29,520 small molecules is unique to the McMaster University High-throughput Screening Facility and is compiled from a variety of vendors, including Maybridge (Cornwall, England), ChemBridge (San Diego, CA), MicroSource Discovery Systems, Inc. (Gaylordsville, CT), Biomol−Enzo Life Sciences (Farmingdale, NY), and Prestwick Chemical (Illkirch, France). Compounds were dissolved at an average concentration of 1 mM in DMSO and screened at a final concentration of 10 μM.

Minimum Inhibitory Concentration Determination. The minimum inhibitory concentration (MIC) of all antibiotics and compounds of interest were determined to evaluate their inhibitory activity. *E. coli* BW25113 was grown overnight in 5 mL of M9 minimal media at 37 °C with shaking at 250 rpm. The cell culture was then diluted in fresh media to an OD_{600} of 0.1 and further diluted 1:200. In 96-well round-bottom plates, 99 μL of diluted cells was mixed with 1 μL of 2-fold serial dilutions of the compounds/antibiotics, in DMSO or water as necessary, with various final concentrations ranging from 0 to 1024 μg/mL, depending on the compound. Growth controls contained 1 μL of DMSO or water in place of compound, and sterility controls contained 99 μL of non-inoculated media. Plates were incubated at 37 °C for 22 h, followed by determination of the OD_{600}. The MIC was determined to be the concentration at which growth, as determined by OD_{600}, was 10% or less than that of the growth controls, calculated using eq 1, where $\mu OD_{600\,high}$ represents the mean of the growth controls, and $\mu OD_{600\,low}$ represents the mean of the sterility controls.

$$\%\ \text{growth} = [(OD_{600} - \mu OD_{600\,low})/(\mu OD_{600\,high} - \mu OD_{600\,low})] \times 100 \quad (1)$$

Novobiocin Combination Screen. *E. coli* BW25113 was grown and diluted in M9 minimal media as described above. Novobiocin was added at a concentration of 256 μg/mL (1/4 MIC). Using a Beckman Biomek FX liquid handler (Beckman Coulter Inc., Fulleron, CA) 2 μL from a 1 mM stock of each compound, dissolved in 100% DMSO, was dispensed into unique wells of a 96-well flat-bottom plate, to achieve a final concentration of 10 μM. This was followed by 198 μL of diluted cell culture containing novobiocin. Alternating controls were set in rows 1 and 12 of each plate containing a final concentration of 1.0 μg/mL colistin (positive control) or DMSO (negative control) in place of CCC compounds. All compounds were screened in duplicate. The $OD_{600\,nm}$ (initial read) of each plate was read prior to incubation at 37 °C, and the $OD_{600\,nm}$ (final read) was measured again after 22 h incubation. The initial read served as a background absorbance measurement and was subtracted from the final read to generate net $OD_{600\,nm}$ values. Growth controls were used to generate percent growth, and replicates were plotted against each other to determine hits from the screen. The robustness of the screen was measured by calculating the Z′-score.[48] A Z′-score of 0.72 was obtained in this experiment in the presence of 1/4 MIC of novobiocin, using eq 2:

$$Z' = 1 - [3(\sigma_p + \sigma_{n)})/|\mu_p + \mu_n|] \quad (2)$$

where σ_p and σ_n are the standard deviations of the positive and negative controls, respectively, and μ_p and μ_n are the means of the positive and negative controls, respectively.

The hits were defined statistically using 3σ from the mean of the data set (60% growth). Thus, any compounds showing less than 60% growth, calculated using eq 1, in the presence of 1/4 MIC of novobiocin, in duplicate, were determined to be hits. For confirmation and validation, hits were rescreened as described above, in duplicate, in the presence of 0 and 256 μg/mL novobiocin.

Synergy Analysis Using Dose Dependence Checkerboards. Once hits were validated, they were analyzed for synergistic activity with novobiocin. Cell cultures were grown and diluted in minimal media, as described above, and added to a 3 point dose matrix, as described previously.[32] This method allowed for identification of synergy in duplicate using minimal reagents. Select combinations were verified using full checkerboards. To evaluate the impact of the compound combinations, fractional inhibitory concentration (FIC) indices were calculated using eq 3,

$$\text{FIC} = [(\text{FIC}_A/\text{MIC}_A) + (\text{FIC}_B/\text{MIC}_B)] \quad (3)$$

where FIC_A and MIC_A are the fractional inhibitory concentration and minimal inhibitory concentration of compound A, and FIC_B and MIC_B are the fractional inhibitory concentration and minimal inhibitory

concentration of compound B. Combinations with FIC values >4 are considered antagonistic, >0.5−4 are considered additive, and ≤0.5 are considered synergistic. To test MreB as a target of synergy between A22 and novobiocin, synergy analysis was conducted as described above using A22-resistant (WA221) and -sensitive (WA220) strains of *E.* coli.[33]

Microscopic Analysis. *E. coli* WA220 and WA221 were grown in the presence of various concentrations of novobiocin and/or A22 for 15 h, as described above. Similarly *E. coli* BW25113 was grown in the presence of novobiocin in combination with compounds using 3 point matrices described above. In all cases, cells for microscopic analysis were taken from the well directly adjacent to the synergistic concentrations. At this time, 10 μL of cells was transferred to a slide, 10 μL of melted, cooled 1% low melt agarose was transferred to the drop of cells and immediately covered with a coverslip. Slides were compressed for 10 min to ensure a single layer of cells. Nail polish was used to seal the coverslip to prevent the sample from drying out. All slides were imaged using a Leica DMI 6000 B microscope at 100X magnification under oil immersion. Images were captured using a Hamamatsu Orca 1394-ERA camera and Volocity 5.2.0 software.

Ethidium Bromide Accumulation Assay. A single colony of *E. coli* BW25113 was inoculated in 3 mL of M9 minimal media and grown overnight at 37 °C with shaking at 250 rpm. The cell culture was then diluted 30 times in 5 mL of M9 minimal media in the presence and absence of serial dilutions of test compounds. The bacterial suspensions were grown until they reached an $OD_{600\ nm}$ of 0.6. Cells were then centrifuged for 2 min at 15,000 rpm and then pelleted and washed twice with 1× PBS. The OD_{600} of the cellular suspension was adjusted to 0.6 in 1× PBS. Then 50 μL of each cell suspension, 25 μL of 20 μg/mL EtBr, and 25 μL of 100 μg/mL chlorpromazine (efflux inhibitor) were transferred to a 96-well flat bottom black plate (Corning Life Sciences, USA). The EtBr fluorescence was measured continuously for 180 min using a Synergy 4 plate reader (BioTek, Winooski, VT, USA) with wavelength excitation at 518 nm and emission of 605 nm. The EtBr accumulation rates were calculated by measuring the change of relative fluorescence units (RFU) over time during the linear range of the time course. Each accumulation rate was then normalized with the no compound control by dividing the sample RFU/min by the control rate. The compound concentrations tested (increasing by 2-fold) were as follows: Compounds 1, between 2 and 64 μg/mL; A22, between 2 and 64 μg/mL; pivmecillinam between 2 and 64 μg/mL; echinomycin between 1 and 32 μg/mL; novobiocin between 4 and 128 μg/mL; ciprofloxacin, between 0.0001 and 0.0048 μg/mL; Meropenem, between 0.002 and 0.06 μg/mL; and carbenicillin, between 0.12 and 2 μg/mL.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

MICs and the FIC indices against novobiocin (in *E. coli* BW25113) for compounds **1**, **2**, **3**, **4**, A22, pivmecillinam, echinomycin, niridazole, and rifabutin. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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