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Chemical Probes of *Escherichia coli* Uncovered through Chemical-Chemical Interaction Profiling with Compounds of Known Biological Activity

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SUMMARY

While cell-based screens have considerable power in identifying new chemical probes of biological systems and leads for new drugs, a major challenge to the utility of such compounds is in connecting phenotype with a cellular target. Here, we present a systematic study to elucidate the mechanism of action of uncharacterized inhibitors of the growth of *Escherichia coli* through careful analyses of interactions with compounds of known biological activity. We studied growth inhibition with a collection of 200 antibacterial compounds when systematically combined with a panel of 14 known antibiotics of diverse mechanism and chemical class. Our work revealed a high frequency of synergistic chemical-chemical interactions where the interaction profiles were unique to the various compound pairs. Thus, the work revealed that chemical-chemical interaction data provides a fingerprint of biological activity and testable hypotheses regarding the mechanism of action of the novel bioactive molecules. In the study reported here, we determined the mode of action of an inhibitor of folate biosynthesis and a DNA gyrase inhibitor. Moreover, we identified eight membrane-active compounds, found to be promiscuously synergistic with known bioactives.

INTRODUCTION

Phenotype-based small molecule screening has emerged as a dominant approach for the discovery of new probes of complex biology and of leads for new drugs. While cell-based screens have considerable power in the discovery of new chemical matter with biological activity, the major challenge to the utility of such molecules is an understanding of mechanism of action.

Nowhere is the discovery of new bioactive chemical matter more important than in antibacterial research. With existing antibiotics directed at a small number of targets, principally cell wall, DNA and protein biosynthesis, multidrug resistance among bacterial pathogens is thought to be due in large part to the limited repertoire of antibacterial chemical matter that eradicate bacteria using a narrow range of mechanisms. Indeed, multidrug resistance in bacteria continues to be a health-care burden in both hospital and community settings where strains of some pathogens, e.g., *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Mycobacterium tuberculosis,* resist the action of every antibiotic in use (Boucher et al., 2009).

In addition to the well-recognized value of small bioactive molecules as leads for new drugs, there is an emerging demand for new chemical perturbants of biological complexity (Peterson, 2008). While genetic perturbation, either by mutagenesis or targeted gene deletion, is the conventional route to probe cellular function it has drawbacks (Alaimo et al., 2001; Stockwell, 2000). Genetic inactivation is permanent, frequently ''all or none'' in scope and for genes that are essential is fraught with the difficulty of creating conditional alleles. Further, the introduction of multiple mutations in the same cell is tedious in even the most tractable systems.

A considerable obstacle to the use of small molecules as probes of biological systems is the limited availability of highly characterized probes. While cell-based screens have considerable power in identifying new chemical perturbants, a major challenge to the utility of such probes is in understanding mechanism of action (Burdine and Kodadek, 2004). There is simply a paucity of systematic methods to reveal the cellular target or mechanism of action of phenotype-altering small molecules. Classically, protein targets have been identified biochemically using labeled or immobilized molecules. Among the most exciting advances in systematic approaches has been the development of a competitive growth assay using a pool of barcoded genome-wide heterozygous yeast strains to identify mutants that fail to grow in the presence of growth inhibitory drugs (Baetz et al., 2004; Giaever et al., 1999; Lum et al., 2004b; Parsons et al., 2004). More recently, with the explosion of genomic sequence information and associated tools, efforts to identify cellular targets have turned to genome-scale clone sets for the systematic identification of protein targets of small molecules of interest (Hillenmeyer et al., 2008; Pathania et al., 2009). Such approaches have largely been limited to model microbes (Baetz et al., 2004; Giaever et al., 1999; Lum et al., 2004a; Parsons et al., 2004), and in a recent application, to the pathogen *Staphylococcus aureus* (Donald et al., 2009). While the aforementioned tools have proved their utility in characterizing both existing and novel bioactives, their biggest drawback lies in the requirement for

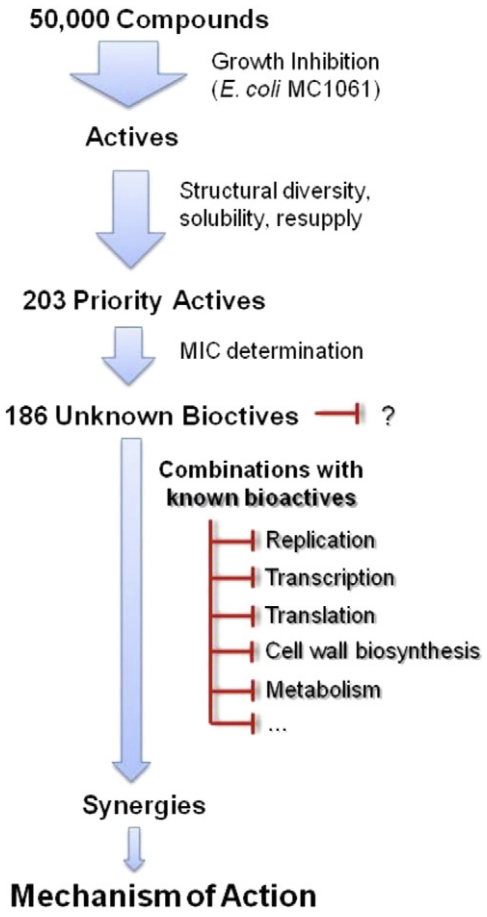


Figure 1. Chemical-Chemical Interaction Profiling to Characterize Novel Growth Inhibitory Compounds Derived from Small Molecule Screening

Summary of the approach to understand mechanism(s) of action of a novel active chemical matter. Synergies uncovered through combination studies, where priority actives are systematically combined with a panel of known bioactive compounds of diverse mechanism and chemical class, provide clues about the pathways and targets. See also Table S1.

genome-scale clone sets. These approaches are specific to the organisms under study, and are virtually impossible to transfer to even closely related species, highlighting the need for systematic methods that are universally applicable to understanding the mechanism of action of small molecules, independent of the biological system of interest.

Out of recognition of the complexity and redundancy of biological networks, chemical combinations are increasingly touted as having special utility as both therapies and probes of biological systems (Lehár et al., 2008a; Zimmermann et al., 2007). The biological impact of combinations of chemicals can be classified as synergistic, additive, or antagonistic, depending on whether the combined effect of the compounds is larger than, equal to or smaller than the effects that might be predicted from the individual drugs, respectively. The potential for efficacious drug synergy has long led to the routine testing and use of drug combinations, especially in antimicrobial therapies, but largely as an afterthought to the discovery of antibiotics (Moellering, 1983). A renaissance in interest in exploiting the power of chemical combinations in drug discovery has been accompanied by an emerging awareness of the value of simultaneous application of two molecular probes to gain biological insight (Lehár et al., 2008b; Yeh and Kishony, 2007; Yeh et al., 2006). It is nevertheless the early days in chemical combination research and there have been no systematic applications of chemical-chemical interaction profiling to understand the mechanism of action of novel bioactive molecules discovered in high-throughput screening.

In the work described herein, we have taken a systematic approach to elucidating the mechanism of action of uncharacterized inhibitors of the growth of *Escherichia coli* through meticulous analyses of interactions with compounds of known biological activity. We have examined growth inhibition of *E. coli* using a collection of 200 novel antibacterial compounds of unknown mechanism when combined with a panel of 14 known bioactive antibiotics of diverse mechanism and chemical class. Our work revealed a surprising frequency of synergistic chemical-chemical interactions where the interaction profiles were unique to the various compound pairs. Thus, these studies revealed that chemical-chemical interaction data can provide a fingerprint of biological activity and testable hypotheses regarding the mechanism of action of the novel bioactive molecules. We determined the mode of action of two novel antibacterial compounds. One molecule was found to be an inhibitor of folate biosynthesis and the other a DNA gyrase inhibitor. Further, the method allowed for the identification of membrane-active compounds. These compounds showed promiscuous synergistic behavior in combination with various known bioactives. Of interest, we identified eight compounds that were capable of depolarizing the membrane of *E. coli*.

RESULTS

A Screen for Growth Inhibitory Small Molecules

Our work began with a high-throughput screen to identify bioactive molecules from a library of approximately 50,000 small molecules that were growth inhibitory against *E. coli* strain MC1061 (Li et al., 2004). *E. coli* MC1061 is an outer membrane hyperpermeable mutant, making it hypersensitive to known antibiotics (Casabadan and Cohen, 1980). A subset of actives, namely, 203 compounds (see Table S1 available online), was selected based on structural diversity, solubility, and resupply (Figure 1). Further prioritization based on minimum inhibitory concentration (MIC) determination against *E. coli* MC1061 excluded 17 compounds with high minimum inhibitory concentrations ($\geq$102.4 μg/ml) (Figure 1). These remaining 186 molecules were further subjected to combination profiling with known bioactives, 14 antibiotics of diverse mechanism and chemical class, to elucidate mechanism of action(s).

Combination Profiling Screen

This comparatively small number of priority actives (Figure 1) generates a large number of possible experiments when combined with 14 known bioctives, namely, 2604 pairwise combinations. Indeed, chemical-chemical interaction studies to detect synergy typically employ standard checkerboard methodology using a 64-point dose matrix (Krogstad and Moellering, 1986).

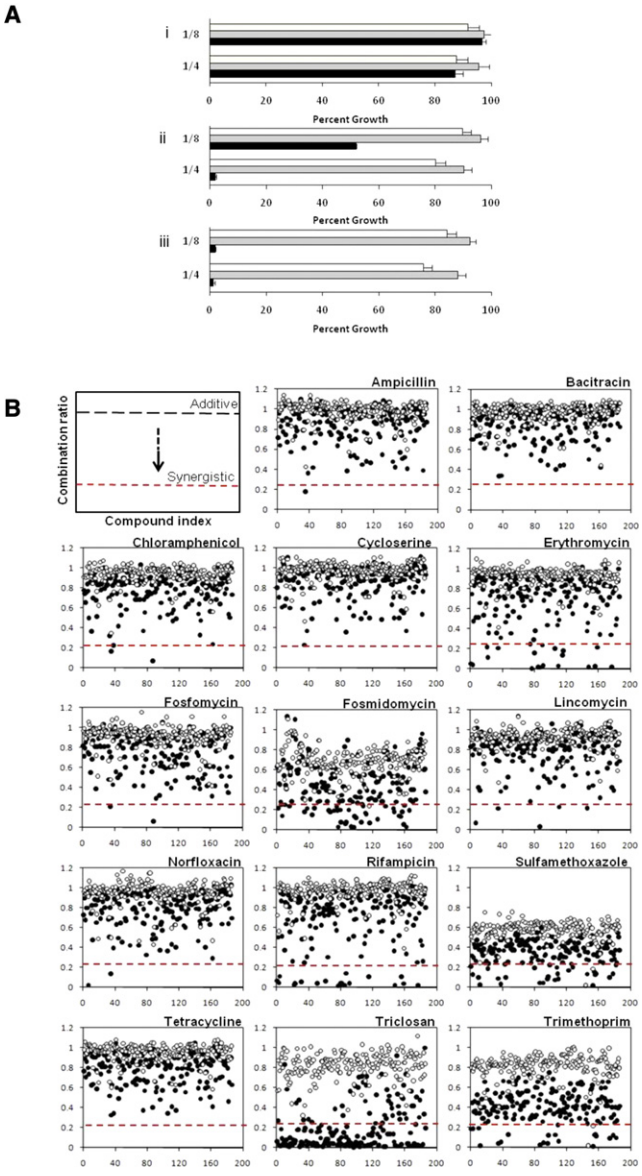


Figure 2. Chemical-Chemical Interaction Profiling Screen with Known Bioactives

(A) Sample two-point dose matrix data showing three possible outcomes of chemical-chemical interaction screens. The panels depict the effect of combining a known bioactive compound with a priority active. Growth of the combinations is represented by a black horizontal bar where the compound concentrations are at one-quarter (1/4) and one-eighth (1/8) of their MICs as indicated. Growth of the known bioactive compounds alone is described by a white horizontal bar and that of the novel priority active alone is a gray bar. All growth is compared with uninhibited controls and expressed as percentage growth. (i) Example of an indifferent interaction, where the growth of the combination of ampicillin and MAC-0002408 was not altered significantly relative to that obtained with the compounds individually. (ii) A highly synergistic interaction is observed when norfloxacin and MAC-0003199 are combined at one-quarter of their MICs. (iii) MAC-00038968 and sulfamethoxazole show profound synergy at both at 1/4 and 1/8 of their MICs.

(B) Result of chemical-chemical interaction profiling of 2604 possible pairwise combinations of 186 priority actives with 14 known antibiotics tested at one-quarter (●) and one-eighth (○) MIC values to identify synergistic interactions. Graphed are the average combination ratios (triplicate data) where percentage growth recorded for the compound combinations is normalized to the percentage growth found for the known bioactives as single agents for all 186 test compounds. A statistical threshold of inhibition of 75% (normalized ratio of 0.25) was established some three standard deviations away from the mean of the high controls (DMSO). Compounds found below this line were judged as hits.

See also Figure S1.

Thus, a single replicate of the checkerboard methodology would require more than 166,000 wells, excluding controls, to examine our 186 priority actives in combination with 14 known bioactives of diverse chemical class and antibacterial mechanism. Instead, we developed a high-throughput method for the efficient identification of synergistic interactions, whereby two small molecules at sublethal concentrations become growth inhibitory when combined. We opted to combine compounds only at a quarter and eighth of their MIC. This stems from the widely recognized definition of synergy, as requiring a minimum of fourfold reduction in the MIC of both compounds in combination, compared with each used alone (Krogstad and Moellering, 1986). Combining bioactives at an eighth of their MICs allows for the ready identification of highly synergistic interactions. Additionally, since there is an inherent one-dilution variability when determining the MICs of the compounds alone (Rand et al., 1993), combinations at an eighth of the MIC allow for a more conservative approach. This systematic two-point dose matrix allowed us to test for synergistic interactions for all of the priority actives when combined with the known bioactives in just 5200 wells. In addition, all combinations were tested in triplicate, allowing the assignment of a standard error to all percentage growth values, and the inclusion of controls accounting for 20% of all test wells ensured that all test samples could be normalized on a plate by plate basis to cells to high controls. To check that this high-throughput approach was as sensitive as the checkerboard method in detecting synergy, combination studies using both methods were conducted on a random subset of 240 pairs of small molecules and a 96% rate of agreement was calculated, revealing the reliability of the two-point dose matrix in detecting synergy (Figure S1).

Figure 2 highlights our two-point dose matrix approach (Figure 2A) and shows average data from the combination profiling screen of combinations at both 1/4 and 1/8 MIC (Figure 2B). For this work, we defined the "combination ratio" as the ratio of the average percentage growth (from three replicates) of cells exposed to the various combinations divided by the average percentage growth in the presence of only the known bioactives. Although the data were normalized to the percentage growth in the presence of the known bioactive as a single agent, the activity of each of the 186 priority actives alone was also controlled for in the assay. In all cases, growth in the presence of the priority actives as single agents resulted in over 85% growth relative to the high control. A pair of compounds with a combination ratio of 0.25 or lower was considered synergistic. This represents a growth inhibition of at least 75%, corresponding to the statistical threshold based on the high controls in the screen (Zlitni et al., 2009). Figure 2A shows detailed sample data from the two-point dose matrix approach, where three possible chemical-chemical interaction scenarios are depicted. Figure 2Ai shows an instance where the

combination of the priority active and known bioactive have no interactions. In Figure 2Aii, synergy is manifested at one-quarter of the MIC for the two compounds but not at one-eighth of the MIC, and in Figure 2Aiii, synergy is evident at both one-quarter and one-eighth of the MIC. This analysis allowed a straightforward assessment of the various chemical-chemical interactions. Synergistic pairings were evident when the effect on percentage growth was significantly reduced when in combination, as compared with their effects individually. These compounds were considered hits and further evaluated in a full fingerprint of biological activity with the other known bioactives.

Figure 2B shows average combination ratios for each of the 186 priority actives with each of the 14 known bioactives. For the most part, combinations led to only occasional synergy, evident as a combination ratio of less than 0.25. Interestingly, triclosan was found to be synergistic with a large number of molecules, particularly at one-quarter MIC. This promiscuous behavior is presumably due to the mechanism of triclosan, well known as disruptor of bacterial membranes (Schweizer, 2001). The next greatest preponderance of synergistic interactions of the priority actives was with fosmidomycin, sulfamethoxazole, and trimethoprim, where combination ratios were comparatively low overall relative to the other known bioactives. We attribute this trend to the shape of the dose-response curves for these known bioactives which revealed a gradual inhibitory effect compared with steeper dose-response curves for the other known bioactive compounds (Figure S2A). The shallower dose-response curve makes these compounds more prone to synergistic interactions. At a concentration of one-quarter MIC, the activity of the known bioactives would fall within the slope of the dose-response curve such that it would be more inclined to a drastic change in inhibitory activity upon combination with a second agent. And while the combination ratios of fosmidomycin, sulfamethoxazole, and trimethoprim are relatively low, the effect of the combination is not strong enough to fall into our statistically defined zone of synergy (<0.25). Indeed, we confirmed that this dose-response behavior leads to more frequent synergy when using the standard 64-point dose matrix checkerboard analyses (Figure S2B).

Evaluation of Uncovered Synergistic Interactions

The combination profiling screen revealed that 45 of the 186 priority compounds had synergistic interactions with the 14 known bioactive compounds. At one-quarter MIC, a total of 112 compound combinations (excluding triclosan) were shown to be capable of reducing the growth of *E. coli* MC1061 by at least 75%. Triclosan showed an additional 143 synergistic combinations. These results are presented in the form of a heat map, where interactions of the priority actives with the panel of known bioactives are colored based on the extent of synergy measured (Figure 3). The heat map reveals a rich interaction matrix of the 45 synergistic priority actives with known bioactives consistent with the thesis that chemical-chemical interactions can uncover valuable functional connections for uncharted small molecule inhibitors of bacterial growth.

When the known bioactives were combined with themselves, very few combinations were synergistic. This was not unexpected, however, as these compounds were chosen to probe diverse aspects of bacterial physiology. However, a small number of known bioactives, fosmidomycin, sulfamethoxazole, trimethoprim, and triclosan, were found to yield synergies. Among these was trimethoprim and sulfamethoxazole, a signature interaction that has been long exploited in antibacterial therapy (Rubin and Swartz, 1980). Additionally, fosmidomycin was found to be synergistic with both trimethoprim and sulfamethoxale (Neu and Kamimura, 1982).

Having selected for priority actives that were synergistic with known bioactives, the heat map details a high density of interactions for these 45 molecules. Many of these are unique interactions that reveal a fingerprint of selective biological activity. On the other hand, many of the compounds demonstrated promiscuous synergistic interactions with several known antibiotics. For example, MAC-0010522 was strongly synergistic with all chosen known antibiotics. Such behavior suggested that this compound might act on multiple pathways or affect cellular permeability, perhaps enhancing the uptake of the known bioactives. Thus, our screening approach had an ability to identify nonselective molecules that would otherwise prove quite challenging in follow-up experiments to identify cellular target(s). The promiscuous nature of these molecules can limit their utility as chemical probes or as leads for drugs. Such molecules were further assessed for their ability to permeabilize bacterial membranes as described below.

Clustering of Chemical-Chemical Interaction Profiles

To assess whether there was a correlation of chemical-chemical interaction profiles and chemical structures, we performed hierarchical clustering of the priority synergistic actives based on their profiles (Figure 3) and on chemical similarity (Figure S3). More than half the time, clustering by structural similarity tracked with clustering by chemical-chemical interaction profile (Figure S3). Examples of related compounds are shown in Table 1. MAC-0007715 and MAC-0007720 share chemical functionality, only differing in one of the thiourea side chains possessing either a morpholine or 2-methylpiperdine ring, and show a unique interaction fingerprint that includes erythromycin, rifampicin, sulfamethoxazole, and triclosan. MAC-0019671 and MAC-0020001 show a similar profile but with an additional signature interaction with fosmidomycin, and only differ in their substituents in the *para* position of the *N*-phenylurea functionality. MAC-002303 and MAC-0024645 share a 1-(4-chlorophenyl)urea moiety and interacted uniquely with fosmidomycin, sulfamethoxazole, and triclosan. These observations reinforce a well-established concept that chemical structure dictates biological activity and reveal the predictive power of our approach in assigning the potential chemical-chemical interaction profile of a novel molecule. There are, however, examples of molecules showing similar chemical-chemical interactions when in combination, without being structurally similar.

In this work, we were especially interested in following up on compound combinations that showed unique synergies. Two high interest compounds, MAC-0038968 and MAC-0003199, were selected for follow-up experiments to characterize cellular targets and mechanisms of action. We also investigated the activity of a subset of priority actives that were found to be promiscuously synergistic.

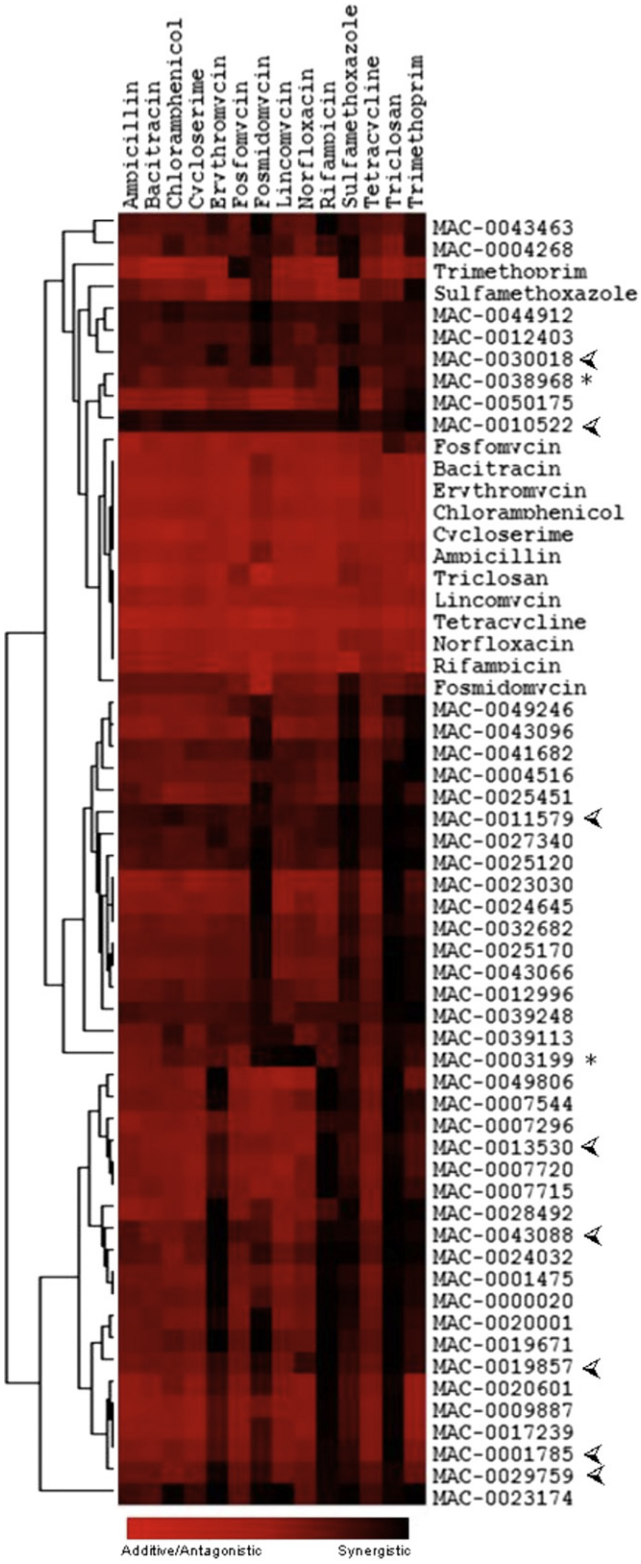


Figure 3. Hierarchical Clustering of Chemical-Chemical Interaction Profiles

Priority actives found to be synergistic with at least one known bioactive are clustered according to their response when combined with the panel of known antibiotics. Hierarchical cluster analysis was performed based on relative percentage residual growth using Cluster software and displayed using Treeview software (Eisen et al., 1998). Highly synergistic interactions are represented in black. The asterisks (*) denote the two molecules of high interest in this work: MAC-0038968, a dihydrofolate reductase inhibitor, and MAC-0003199, a DNA gyrase inhibitor. The arrows to the right of the fingerprints represent the molecules found to be membrane active as judged by the $DiSC_3$ fluorescence assay described herein.
See also Figures S2–S4.

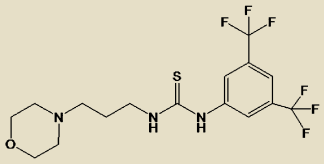
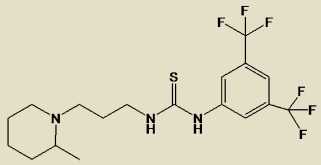
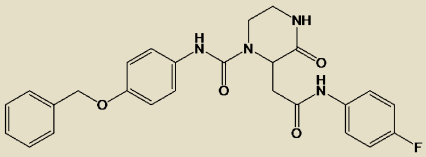
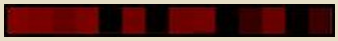
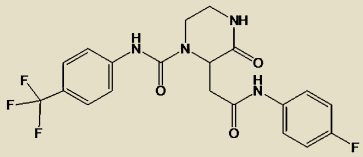
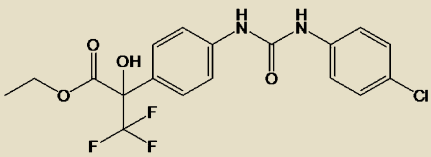
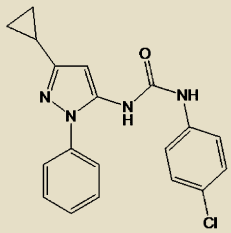
MAC-0038968 Is Synergistic with Sulfamethoxazole and Active against Dihydrofolate Reductase

One compound of interest uncovered in our screen was MAC-0038968 (Figure 4A). This small molecule was found to be uniquely synergistic with sulfamethoxazole, an antibiotic that inhibits tetrahydrofolate biosynthesis in bacteria by acting as an analog to one of the pathway intermediates (Walsh, 2003). A detailed checkerboard analysis of the compound combination confirmed strong synergy between MAC-0038968 with sulfamethoxazole with a fractional inhibitory index (ΣFIC) of 0.187 (Figure 4B). Interestingly, we previously reported on this compound in a biochemical screen to identify inhibitors of dihydrofolate reductase (DHFR), the enzyme responsible for the reduction of dihydrofolate to tetrahydrofolate but had not investigated its cellular activity (Zolli-Juran et al., 2003). The observed synergy served to validate the proposed method, as MAC-0038968, which was found to be highly synergistic with sulfamethoxazole, inhibits a later step in a common cellular pathway. To investigate the cellular target of MAC-0038968, we sought to suppress the action of this compound by expression of the putative target DHFR at high copy. Indeed, we have previously found high copy suppression to be a valuable chemical-genetic tool to probe mechanism of novel antibacterial compounds (Li et al., 2004; Pathania et al., 2009). Figure 4C reveals that increased expression of DHFR led to a steady increase in the MIC for MAC-0038968 and trimethoprim without any impact on the MIC of the control compound tetracycline (Figure 4C). These results are consistent with the conclusion that the growth inhibition by MAC-0038968 was due to inhibition of DHFR. Trimethoprim, one of the known bioactives in this study is of course a celebrated DHFR inhibitor (Baccanari and Kuyper, 1993). The lack of synergy with trimethoprim (Figure 3A) suggests that MAC-0038968 and trimethoprim bind to similar sites on DHFR; otherwise a synergistic interaction would be expected if the two bound to separate sites on the target (Krogstad and Moellering, 1986). In fact, we have previously shown that this DHFR inhibitor, like trimethoprim, is competitive with the substrate dihyrofolate and inhibits the enzyme with a reasonable potency (K_i = 65 nM) (Zolli-Juran et al., 2003). Importantly MAC-0038968 represents a new chemical class of DHFR inhibitors with cellular activity.

MAC-0003199 Is a DNA Gyrase Inhibitor, Uncovered through Synergy with Norfloxacin

From the combinatorial screen the quinoline carboxylic acid, MAC-0003199, was found to have a unique interaction fingerprint that included lincomycin, triclosan, and norfloxacin. The latter interaction was particularly noteworthy as this compound had a selective and profound interaction with this DNA gyrase inhibitor. MAC-0003199 represents a novel structure but is reminiscent of the quinolone family of synthetic antibiotics that

Table 1. Examples of Molecules with Similar Chemical Structures and Biological Fingerprints

Compound	Structure	Biological Fingerprints
MAC-0007715		
MAC-0007720		
MAC-0019671		
MAC-0020001		
MAC-0023030		
MAC-0024645		

Biological fingerprints derived from the combination profiling screen with a panel of known antibiotics for the various molecules are shown, where highly synergistic combinations are represented in black. The biological fingerprints were taken out of the heat map in Figure 3 to illustrate the correlation between activity and chemical structures.

are well known for their inhibitory action on bacterial DNA gyrase and topoisomerase IV and associated lethal impact on DNA replication and transcription.

A detailed checkerboard analysis of the interaction between norfloxacin and MAC-0003199 confirmed the synergistic interaction with a ΣFIC of 0.312 (Figure 5B). In an effort to assess the capacity of MAC-0003199 to induce DNA damage, we characterized the in vivo phenotypic response of *E. coli* in response to the compound, employing a DNA damage-inducible reporter construct that is based on LexA repression for regulation of

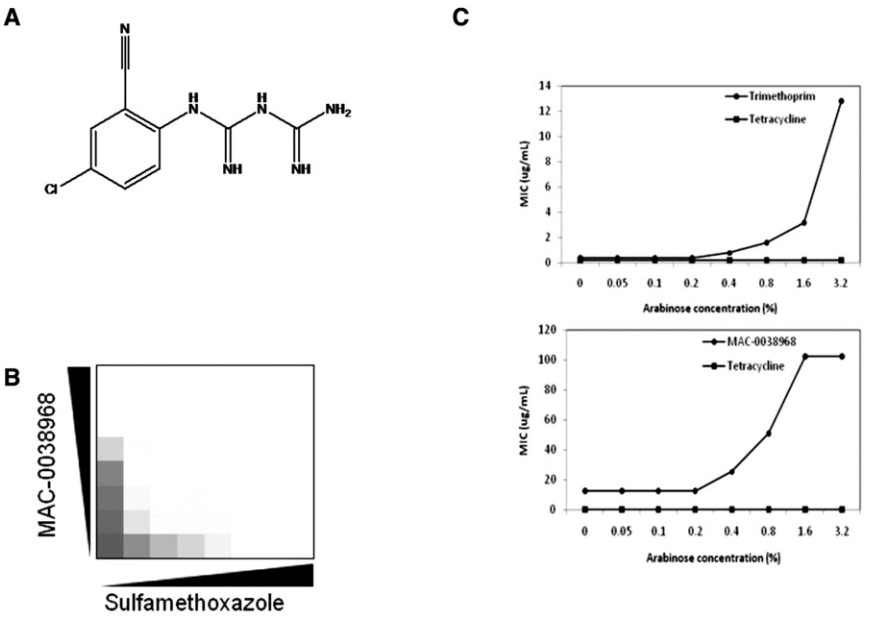


Figure 4. MAC-0038968 Is Uniquely Synergistic with Sulfamethoxazole and Active against Dihydrofolate Reductase (DHFR)

(A) Chemical structure of MAC-0038968.

(B) Checkerboard analysis of MAC-0038968 in combination with sulfamethoxazole, showing a strong synergistic interaction with a ΣFIC = 0.187.

(C) Suppression of growth inhibition by MAC-0038968 on increasing expression of DHFR encoded in the *folA* gene. Here, expression is manipulated by the arabinose-inducible promoter of copy of pBAD18-*folA* in *E. coli* strain MC1061. Arabinose dependence of the MIC is shown for the positive control trimethoprim (circles, top panel) and the test compound MAC-0038968 (circles, bottom panel). Also shown is the negative control tetracycline (squares).

gfp transcription (Dwyer et al., 2007). Thus, an output in fluorescence is indicative of RecA-stimulated autocleavage of LexA following recognition of DNA damage (Dwyer et al., 2007). The control compound norfloxacin led to the induction of high levels of fluorescence (Figure 5C). Norfloxacin is known to stabilize a tripartite interaction with DNA gyrase and cleaved DNA thus stimulating the formation of DNA breaks (Walsh, 2003). We also observed a large shift in fluorescence upon treatment of the cells with MAC-0003199 (Figure 5C), as compared with the negative control, tetracycline (Figure 5C). We next sought to determine whether MAC-0003199's mode of action in the cell was similar to that of norfloxacin, acting by inhibiting DNA gyrase's ability to supercoil DNA (Drlica and Zhao, 1997). MAC-0003199 inhibited DNA gyrase at a relatively high concentration, compared with the potent inhibitor norfloxacin (Figure 5D) but nevertheless was consistent with the relative cellular potencies of these compounds (6.4 μg/ml for MAC-0003199 compared with 0.025 μg/ml for norfloxacin).

Interestingly, the synergistic nature of the interaction between MAC-0003199 and norfloxacin suggests that the two molecules do not work with exactly the same mechanism. For example, it seems unlikely that the two compounds bind to exactly the same site on DNA gyrase as this would lead to an antagonistic interaction (Krogstad and Moellering, 1986). Further, MAC-0003199 was found to be synergistic with triclosan and lincomycin, while norfloxacin showed no such interaction. Triclosan is known to disrupt the inner membrane of bacteria (Schweizer, 2001), and this interaction may relate to inherent permeability problems for MAC-0003199. Lincomycin is an inhibitor of protein synthesis that binds the 50S ribosome and inhibits peptidyl transferase activity, thus preventing translation termination (Lin et al., 1997; Walsh, 2003). The mechanistic connection between lincomycin and MAC-0003199 remains unclear but suggests nevertheless that MAC-0003199 may not function simply as an inhibitor of DNA gyrase. Its striking similarity in structure to the quinolone class of molecules, however, strongly suggests that MAC-0003199 is likely an inhibitor of DNA gyrase, such that its interaction with norfloxacin would expectedly be antagonistic in nature. However, quinolones have a unique mechanism of action in that they inhibit both bacterial DNA gyrase and topoisomerase IV, with varying target preference, depending on the organism in question. In *E. coli*, the inhibition of topoisomerase IV becomes more apparent when DNA gyrase is mutated to be resistant to the quinolone (Khodursky et al., 1995). Moreover, quinolones can in fact prefer topoisomerase IV over DNA gyrase, and vice versa, or target both enzymes at nearly the same level (Takei et al., 2001). This dual-targeting property of quinolones may help explain the synergy between MAC-0003199 and norfloxacin, in that the observed antibacterial activities are involved not only in DNA gyrase inhibition but also in topoisomerase IV inhibition.

Promiscuously Synergistic Molecules

As discussed above, a particular advantage of systematic screening for chemical-chemical interactions was the identification of promiscuously synergistic molecules. In an effort to understand the mode of action of such molecules, we investigated the possibility that these were membrane active and resulted in a breach in bacterial membrane potential. This was assessed with a membrane potential-sensitive fluorescent probe, 3,3′-dipropylthiacarbocyanine ($DiSC_3$) (Sims et al., 1974). Once inside the cell, this dye becomes concentrated and self-quenches its own fluorescence. Membrane-active compounds that depolarize the $\Delta\psi$ component of the proton motive force will lead to the release of the dye and a consequent increase in fluorescence. Normally, this dye is incapable of penetrating Gram-negative bacteria; however, we were able to establish this assay using our screening strain (*E. coli* strain MC1061) because of its documented hyperpermeability. In fact, we tested all compounds found to be synergistic in our screen in our combination profiling screen for their capacity to alter membrane potential at MIC concentrations. All 14 of the known bioactives tested negatively in this assay, as expected, whereas polymyxin B, a lipopeptide known to be membrane

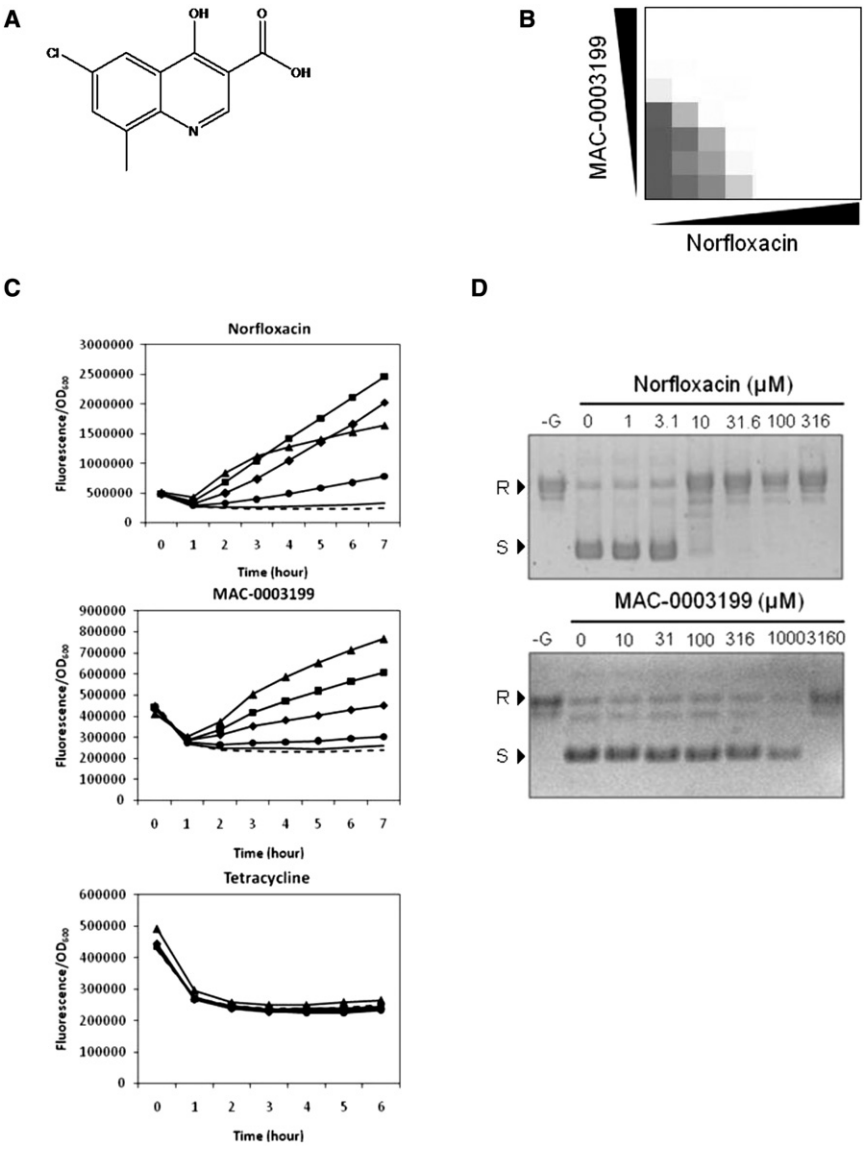


Figure 5. MAC-0003199, Synergistic with Norfloxacin, Inhibits DNA Gyrase

(A) Chemical structure of MAC-0003199.

(B) Checkerboard analysis of MAC-0003199 in combination with norfloxacin, showing synergy with a ΣFIC = 0.312.

(C) *E. coli* MC1061 harboring pL(lexO)-GFP, a promoter-GFP reporter gene construct used to report on DNA lesion formation, was grown to an OD_{600} of 0.3–0.4 and treated with various concentration of norfloxacin (positive control), MAC-0003199 and tetracycline (negative control) (dashed line: no drug; solid line: 1/4 MIC; circles: 1/2 MIC; diamonds: MIC; squares: 2× MIC; triangles: 4× MIC). Optical density at 600 nm and fluorescence at 535 nm were monitored for 7 hr. Induction of DNA damage was evident for norfloxacin and MAC-0003199.

(D) Inhibition of supercoiling of DNA by MAC-0003199 and norfloxacin. *E. coli* gyrase was incubated with relaxed pBR322 in the presence of various concentrations of norfloxaxin (top panel) and MAC-0003199 (bottom panel).The lane labeled –G is relaxed pBR322 in the absence of *E. coli* gyrase . R and S represent relaxed and supercoiled pBR322, respectively.

active (Daugelavicius et al., 2000), led to a significant increase in $DiSC_3$ fluorescence in our assay (Figure S4A). Of the 45 synergistic priority actives, 8 molecules caused a significant increase in $DiSC_3$ fluorescence, suggesting that these molecules have the capacity to depolarize the cytoplasmic membrane. These are highlighted with an arrow in Figure 3 to the right of their respective chemical-chemical interaction profiles and the data are presented in Figure S4B.

It is tempting to conclude that these eight molecules represent nonselective compounds that are unsuitable probes of biology or as leads for new drugs. Membrane-active molecules, such as the peptide-drugs, daptomycin, and polymyxin B, have proved invaluable in treating drug resistant infections (Evans et al., 1999; Fuchs et al., 2002; Giamarellou and Poulakou, 2009; King and Phillips, 2001). Regardless of the disposition toward membrane-active and promiscuous chemical matter, the combination profiling approach used here was quite successful in identifying strong candidates for membrane depolarizing activity among priority actives.

DISCUSSION

The systematic determination of the mechanism of actives derived from phenotype-based small molecule screening remains a very significant challenge to the discovery of useful probes of biology or leads for new drugs. Chemical-genetic approaches have dominated for this purpose in recent years and, while the success of these has been tangible, there remain some significant drawbacks. Forward chemical-genetic methods rely on enhancement or suppression of growth phenotypes associated with novel actives by genetic perturbation. The latter has the potential to inform on mechanism and provide testable hypotheses particularly when there is depth of knowledge on the interacting genes. Classical chemical-genetic approaches are powerful but require the tedious isolation and characterization of suppressing or enhancing mutants (Eggert et al., 2001; Gitai et al., 2005; Heitman et al., 1991). Modern chemical-genomic approaches have typically relied on genome-scale deletion or overexpression clone-sets in model microbes such as yeast or *E. coli* to screen for suppressing or enhancing genotypes (Baetz et al., 2004; Giaever et al., 1999; Li et al., 2004; Lum et al., 2004b; Pathania et al., 2009). Such clone sets are currently unavailable in all but the most tractable model microbes.

Herein, we describe an alternative and complementary approach where a small library of known bioactives is systematically combined with actives to detect chemical-chemical

interactions. Almost 200 growth inhibitory and novel active compounds were systematically combined with a library of some 14 well-known antibiotics of diverse chemical class and mechanism. Combination profiles so discovered were examined in the context of a deep knowledge of the mechanism of interacting chemicals. These profiles generated hypotheses that were testable with biochemical and physiological experiments and revealed new probes of folate metabolism, DNA gyrase, and the cell membrane. As such, chemical-chemical interaction profiles can be generated for any phenotype-inducing compound in any biological system without a need for genetic manipulations provided there are existing well-characterized chemical perturbants available.

The work reported here suggests that phenotypic responses to combinations of known and novel bioactives can reveal information about the pathways and targets affected by the latter. The approach was data rich but nevertheless allowed for efficient testing of combinations of chemicals, making it readily adaptable as a secondary screening approach in high-throughput screening efforts. In a validating discovery, the bi-guanidine-containing compound MAC-0038968 was found to be synergistic with sulfamethoxazole. Sulfamethoxazole limits the supply of PABA precursor available to subsequent steps in the tetrahydrofolate biosynthesis pathway and is well known for its synergy with trimethoprim, an inhibitor of DHFR (Rubin and Swartz, 1980). Further, we demonstrated that MAC-0038968, previously shown to be a potent inhibitor of DHFR in vitro (Zolli-Juran et al., 2003), could be suppressed by high copy DHFR, demonstrating that his compound had cellular activity and was on target. Interestingly, MAC-0038968 showed no synergy with trimethoprim, presumably because these compounds bind to the same site on DHFR. MAC-0003199, a quinoline carboxylic acid, was shown to be a DNA-damaging agent that targets DNA gyrase. This molecule, similar in structure to quinolones, was suitably found to be highly synergistic with norfloxacin, a potent inhibitor of DNA gyrase. The two novel inhibitors of bacterial physiology uncovered through the use of chemical-chemical interactions highlight the utility of the proposed approach in facilitating the search for cellular targets of novel biological probes.

In cases where a novel compound shows promiscuous interactions with multiple known bioactives, this nonselective behavior can be used as a filter to eliminate nuisance compounds. Other methods to identify such molecules include computational means based on chemical functionality (Roche et al., 2002; Walters et al., 1999). Another is a biochemical approach that uses a detergent-based assay to counterscreen for nonselective aggregating compounds (Feng et al., 2005; McGovern et al., 2002; Seidler et al., 2003). There are, however, few systematic counter screens for nonselective compounds that are cell based. Interestingly, further study of such compounds in the work reported here led to the discovery of a subset of compounds capable of dissipating the transmembrane potential of *E. coli*. Given recent clinical success of the antibiotic daptomycin, also know to depolarize membranes (Silverman et al., 2003), promiscuous behavior might well be viewed as both a strength and a weakness. Nevertheless, combination profiling proved to be a powerful tool in identifying these molecules.

Interactions between bioactive chemicals can be synergistic or antagonistic (Krogstad and Moellering, 1986). In this study, we limited our focus to synergistic interactions. In the same manner that synergies were specifically used to elucidate mechanisms of action, a screen looking for antagonistic interactions with known bioactives could also yield rich information about associated cellular pathways. In brief, antagonistic interactions are most often observed when two compounds compete between binding sites or when one alters the binding site of the other (Krogstad and Moellering, 1986). Antagonism might also be the result of complex genetic interactions propagated through the cellular genetic network (D'Elia et al., 2009; Motter et al., 2008). Thus, looking for antagonistic interactions among novel and know bioactives, though rare, could surely also provide useful mechanistic insights and testable hypotheses regarding the mechanism of action of novel actives.

Herein we have demonstrated real promise for chemical-chemical interactions in understanding mechanism of the interacting components. So characterized, these interactions also have the potential to perturb the complex and redundant nature of biological pathways. It is increasingly becoming evident that biological systems are composed of dense networks of interacting components that are characterized by redundancy (Ho et al., 2002; Tong et al., 2004). Thus, the simultaneous use of multiple perturbants will ultimately be required to adequately describe this complex cellular matrix. As the compendium of interacting combinations grows, these will become an increasingly powerful tool for assessing mechanism of action.

SIGNIFICANCE

Phenotype-based screening provides a powerful tool for the discovery and characterization of new probes of biology and leads for new drugs; however, significant challenges remain for connecting compounds to their targets. Here, we report the application of systematic chemical-chemical combination profiling to understand the mechanism of action of antibacterial compounds. The interaction of newly discovered compounds with a panel of known bioactives of diverse mechanism and chemical class was used to gain insight into mode of action. Combination profiling for synergistic chemical-chemical interactions resulted in a data-rich map of interactions and led to testable hypotheses regarding the mechanism of action of the bioactive molecules. One such molecule was found to be an inhibitor of folate biosynthesis, (MAC-0038968) and another was shown to be a DNA gyrase inhibitor (MAC-0003199). The approach also proved to be useful in identifying nonselective molecules that showed promiscuous interaction behavior where a subset of these compounds was shown to depolarize the bacterial membrane.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Reagents, and General Methods

E. coli MC1061 was used for all experiments and follow-up work. Growth media was liquid or agar Luria-Bertani Broth (LB). The small molecule library was purchased from Maybridge (Cornwall, England) and was dissolved in DMSO at a concentration of 6.4 mg/ml. All chemicals were purchased from Sigma Aldrich (Oakville, ON).

Determination of Minimum Inhibitory Concentrations of Chemical Compounds using *E. coli* Strain MC1061

The minimum inhibitory concentrations of priority actives and known antibiotics were determined to characterize their growth inhibition. Typically, *E. coli* MC1061 was grown overnight in 5 ml of LB media. The cells were then diluted 1:100 in fresh media and allowed to grow until the OD_{600} reached 0.4. The cells were then diluted 1:100,000 and exposed to 2-fold serial dilutions of the compounds at final concentrations ranging from 0 to 204.8 μg/ml from stock solutions of 6.4 mg/ml. These were tested in 96-well microwell plates with a total volume of 200 μl and incubated at 37°C with 80% humidity for 18 hr before determining optical density at 600 nm. The concentration where the optical density was less than 0.05 was deemed the MIC of the test compound.

Combination Profiling Screen

To screen the various combinations, 80-fold stock solutions of chemicals were placed into polypropylene 96-well master plates in two consecutive columns. Aliquots of 2.5 μl from both master plates were dispensed into the assay plate using a Biomek FX liquid handler (Beckman Coulter Inc., Fullerton, CA) in the McMaster High Throughput Screening Laboratory. Subsequently, 195 μl of a mid-log subculture of *E. coli* MC1061 cells diluted 1:100,000 were added. These plates were incubated for 18 hr at 37°C before being read for optical density at 600 nm. All combinations were done in triplicate, allowing the assignment of a standard error to all percentage growth values.

Checkerboard Analysis of Synergy

A mid-log subculture of *E. coli* MC1061 cells was diluted 1:100,000 before adding to an 8 × 8 matrix of priority actives and known antibiotics. The plates were incubated for 18 hr at 37°C before reading for optical density at 600 nm. All combinations were done in duplicate. Heat maps of the averages were created in Excel and used to illustrate growth compared with the high controls (DMSO only). To evaluate the effect of the combinations, the fractional inhibitory concentrations (ΣFIC) indices were calculated. This metric is defined as the sum of the MIC of each drug when used in combination divided by the MIC when used alone. Chemical-chemical interactions with ΣFIC of less than 0.5 were deemed synergistic.

High Copy Suppression of Growth Inhibition

E. coli MC1061 harboring pBAD18-*folA* was grown overnight in LB supplemented with 100 μg/ml ampicillin, subcultured the following day (1:100 dilution in the same media) and grown to mid log (OD_{600} = 0.4) with aeration at 250 rpm at 37°C. The cells were then diluted 1:100,000 and exposed to 2-fold serial dilutions of the compounds ranging from 0 to 102.4 μg/ml in the presence of various concentration of arabinose (0%–3.2%). These were tested in 96-well microwell plates (total volume of 200 μl) and incubated at 37°C with 80% humidity for 18 hr before determining optical density at 600 nm. The concentration where the optical density was less than 0.05 was deemed the MIC.

Promoter-Reporter Construct Experiments

The DNA-damage reporter construct was a kind gift from Dr. James Collins (Boston University). In all experiments, *E. coli* MC1061 harboring pL(lexO)-GFP were grown overnight and then diluted 1:100 in 50 ml LB supplemented with 100 mg/ml ampicillin. Cells were grown at 37°C, 250 rpm, until an OD_{600} of 0.3–0.4. Cells were aliquoted into black clear bottom 96-well plates (Corning Life Sciences, Corning, NY) and drug, previously serially diluted in stock plates, was added at various concentrations. Optical density at 600 nm and fluorescence at 535 nm were monitored for 7 hr using the EnVision from Perkin Elmer (Waltham, MA).

DNA Supercoiling Assay

E. coli gyrase was incubated with 0.5 μg of relaxed pBR322 DNA in a 30 μl reaction mixture at 37°C for 1 hr under the following conditions: 35 mM Tris-HCL (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.75 mM ATP, 5 mM spermidine, 0.1 mg/ml BSA, and 6.5% glycerol. Reactions were analyzed by electrophoresis through a 0.8% agarose gel (run at 130V for 1.5 hr).

Dissipation of Transmembrane Potential

The transmembrane potential was determined with the fluorescent probe, 3′-dipropylthiacarbocyanine ($DiSC_3(5)$). *E. coli* MC1061 cells were washed twice with Tris buffer (10 mM [pH 7.5]), and then resuspended to an optical density at 600 nm of 0.35. $DiSC_3(5)$ was added at a final concentration of 0.4 μM, and the cells were incubated, with constant stirring to let the dye stabilize. Compounds were then injected. Fluorescent traces were measured in a fluorimeter (Photon Technology International) at the excitation and emission wavelengths of 622 and 660 nm, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at doi:10.1016/j.chembiol.2010.06.008.

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