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Multicopy Suppressors for Novel Antibacterial Compounds Reveal Targets and Drug Efflux Susceptibility

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Summary

Gene dosage has frequently been exploited to select for genetic interactions between a particular mutant and clones from a random genomic library at high copy. We report here the first use of multicopy suppression as a forward genetic method to determine cellular targets and potential resistance mechanisms for novel antibacterial compounds identified through high-throughput screening. A screen of 8640 small molecules for growth inhibition of a hyperpermeable strain of *Escherichia coli* led to the identification of 49 leads for suppressor selection from clones harboring an *E. coli* genomic library. The majority of suppressors were found to encode the multidrug efflux pump AcrB, indicating that those compounds were substrates for efflux. Two leads, which produced clones containing the gene *folA*, encoding dihydrofolate reductase (DHFR), proved to target DHFR in vivo and were competitive inhibitors in vitro.

Introduction

Phenotype-based screens for small molecules with biological activity have accounted for an overwhelming majority of pharmaceutical drugs in use today and are finding increasing use in a chemical biology research paradigm emerging in academic circles. The latter includes a number of recent examples of success in the discovery and optimization of novel molecules found in synthetic and natural product libraries that function as powerful probes of biological systems (reviewed in [1–4]). Such efforts have been punctuated by the ambitious goal of one selective, cell-permeable small molecule ligand for every protein in a cell [5].

One of the most significant hurdles to the use of phenotype-based small molecule screens is the identification of the macromolecular target in the cell. In contrast, the discovery and optimization of inhibitors of protein function using target-based biochemical screens is plagued with dilemma of ultimately creating molecules that penetrate and persist in cells in order that they have biological activity. Nowhere is the issue of cell permeability more acute than in antimicrobial research where the principles governing access of a given compound to the intracellular space of a bacterial or fungal cell are poorly understood. Nevertheless, there is growing appreciation that small molecule permeability in bacteria is governed to an extent by ubiquitous multidrug efflux pumps [6], whose substrate specificities are very broad and hard to define [7]. Hence phenotype-based screens have a particular advantage in that bioactive molecules identified already have physical-chemical properties that are compatible with microbial cell permeation. What are needed, however, are robust forward genetic approaches for the identification of protein targets of small molecules identified in phenotype-based small molecule screens of microbes.

Since the availability of the first plasmid-encoded genomic libraries [8], geneticists have exploited the effects of increasing gene dosage to identify genetic interactions. The technique is referred to as high-copy or multicopy suppression and involves the creation of a plasmid-encoded random genomic library followed by a selection or screen for clones that have a suppressor phenotype. More recently, multicopy suppression has found utility in the identification of genes capable of suppressing the activity of antibiotics with antibacterial [9, 10], antifungal [11–14], and antiparasitic [15, 16] activities. High-copy suppressors have likewise been identified for anticancer drugs [17]. From these studies it is understood that overexpression of a protein will often lead to resistance to the chemotherapeutic agent owing to two general mechanisms. In one mechanism, perhaps the most common, overexpression of a protein involved in the modification or efflux of the chemotherapeutic agent leads to suppression of the lethal phenotype. Alternatively, overexpression of the protein target itself can lead to resistance. Multicopy suppression has thus found some utility in identifying suppressors of the action of pharmaceutical agents of unknown mechanisms. We report here on the first use of multicopy suppression as a forward chemical genetic method to determine cellular targets and potential resistance mechanisms, particularly efflux, for novel antibacterial leads identified with high-throughput screening.

Results

Screening for Growth Inhibitory Small Molecules

Our work began with a high-throughput screen to identify compounds in a small molecule screening library (8640 molecules) that had growth inhibitory activity against *E. coli* strain MC1061, a hyperpermeable rough lipopolysaccharide mutant [18], at a concentration of 50 μM in rich liquid media. The compound library was from Maybridge plc (Cornwall, England), had an average molecular mass of 325 g/mol, and was chosen for its high quality, diversity, drug likedness, and resupply rate [19]. Figure 1 shows a replicate plot of the screening data where the quality of the screen is evident in the high correspondence of duplicate determinations. We calculated Z' statistical values of 0.78 and 0.73 for repli-

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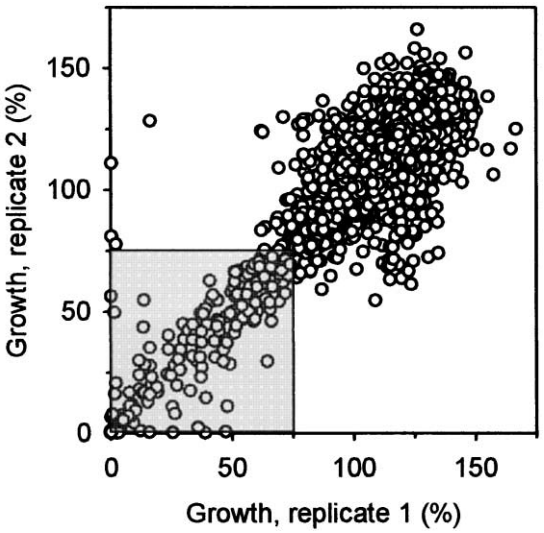


Figure 1. Primary Screen for Compounds with Growth Inhibitory Activity

Shown is a replicate plot for the test wells of a duplicate screen of 8640 compounds. Growth in the test wells is expressed as percentage of that in control wells. A total of 301 actives were identified in the hit zone (gray inset) that was established at a threshold of 75% growth relative to the control wells.

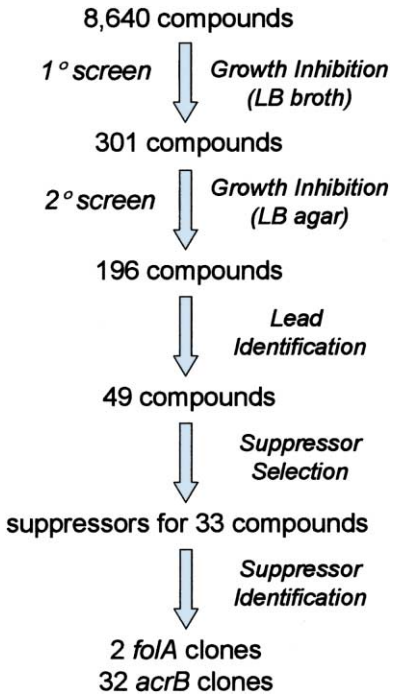


Figure 2. Outline of Screening Process

cates 1 and 2, respectively. Z′ is a measure of the quality of a small molecule screening campaign [20] and is defined as

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}, \quad (1)$$

where σ_{c+}, σ_{c-}, μ_{c+}, and μ_{c-} are the standard deviations (σ) and averages (μ) of the high ($_{c+}$) and low ($_{c-}$) controls. The Z′ value also suggests that this was a high-quality screen since a Z′ of 0.5 or greater is indicative of a data with an acceptable signal window and errors associated with the high and low controls. Molecules that demonstrated 25% growth inhibition were judged to be hits in the primary screen resulting in the selection of 301 compounds (3.5% hit rate) for further analysis.

Figure 2 summarizes the approach to arrive at a small number of growth inhibitory leads for multicopy suppression and subsequent follow up. Of the 301 compounds selected from primary screening, 196 showed complete growth inhibition on rich solid media at 500 μM. This secondary screen was necessarily on solid media and at a higher concentration to test for complete inhibition of growth because of the nature of downstream multicopy suppression experiments. Those experiments required the selection and isolation of clones resistant to otherwise lethal concentrations of the leads and where the minimum inhibitory concentrations (MIC) of the leads were frequently in the range of 50–500 μM. Classification of these 196 molecules according to structural features facilitated the selection of a representative subset of 49 leads for further analysis. Subsequent MIC analysis on these 49 leads revealed the minimum concentration necessary to inhibit bacterial growth for each active molecule. Having established growth-retarding concentrations of each novel molecule, we set about to select for suppressors using a pool of clones harboring a multicopy genomic library of *E. coli*.

Selection for Multicopy Suppressors

As proof of principle we first selected for suppressors of the activity of three well-known antibiotics. Figure 3 shows a typical experiment where we systematically exposed a control strain and a pool of clones harboring the genomic library to increasing concentrations of the antibiotic trimethoprim. While growth inhibition was seen in the control at 0.078 μg/ml of trimethoprim, suppressor clones were evident at almost ten times that concentration (0.64 μg/ml). Similarly, suppressors were isolated for growth inhibition by cycloserine and fosfomycin (Table 1). Sequencing of the plasmid DNA contained in these clones revealed inserts in the cloning site of pGEM7 corresponding to genomic fragments encoding the targets of these three antibiotics. Trimethoprim produced clones with the fragment *yabF-kefC-folA* where *folA* encoding dihydrofolate reductase is the target of trimethoprim [21]. Fosfomycin yielded the fragment *ispB-sfsB-murA-yrbA* where *murA* encodes its target UDP-*N*-acetylglucosamine enolpyruvyltransferase [22]. Cycloserine yielded two different clones containing the fragments *yaiW-yaiY-YaiZ-ddlA-yaiB-phoA-psiF* and *yaiY-YaiZ-ddlA-yaiB* where *ddlA* encodes the target D-ala-D-ala ligase [23].

Of the 49 leads selected from our screen for novel growth inhibitory molecules, suppressor clones could be isolated for 33 of the compounds, while the remainder of the compounds simply did not produce resistant clones. Clones suppressing the activity of two of the compounds, **1a** and **2a**, were found to contain *folA* en-

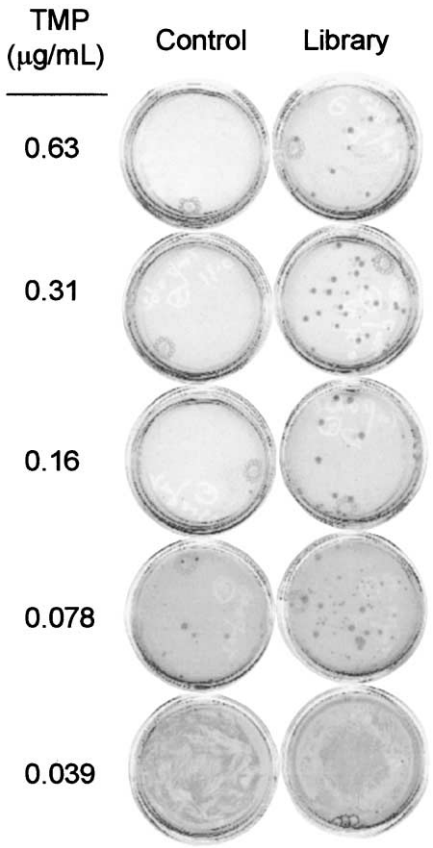


Figure 3. Identification of Suppressor Clones for the Growth Inhibitory Activity of Trimethoprim

Shown are petri plates from a proof of principle suppression experiment where 10^5 cells of *E. coli* MC1061 were exposed to increasing concentrations of trimethoprim in LB-Str-Amp agar. The cells were from a selection pool, containing a multicopy genomic library cloned into pGEM7, and a control pool, containing empty vector pGEM7. Suppressors were those clones from the library showing resistance not seen in the control pool. These colonies were found to contain identical clones containing DNA coding for the neighboring genes *yabF*, *kefC*, and *folA* at the cloning site of pGEM7.

coding DHFR (Table 1). While all of the suppressors isolated for the activity of **1a** contained the gene *folA*, two different suppressors isolated for **2a** and at a similar frequency. Those suppressors contained either *folA* or *acrB*, encoding the membrane component of the acridine efflux pump [24]. Indeed, all of the suppressors that were isolated for the remaining 31 molecules contained a clone with a single open reading frame for *acrB* in the cloning site of pGEM7. We reasoned that the antibacterial action of these compounds was abrogated by overexpression of this cellular pump from the high-copy plasmid pGEM7 resulting in resistance to otherwise toxic molecules.

Growth Inhibition Is Dependent on the Expression of Dihydrofolate Reductase

To further confirm that the antibacterial activities of compounds **1a** and **2a** were related to inhibition of DHFR, we subcloned *folA* into an arabinose inducible expression system, vector pBAD18 [25], and placed this plasmid in an *E. coli* host, strain CW2553 containing pAK01, that allowed for incremental control of protein expression [26]. With this system, increasing inducer concentrations led to a steady increase in MIC for the control molecule trimethoprim without any impact on the MIC for tetracycline (Figure 4A). Similarly, the MIC values for **1a** and **2a** demonstrated an inducer dependence (Figure 4B) that was consistent with the conclusion that growth inhibition by these molecules was due to inhibition of DHFR.

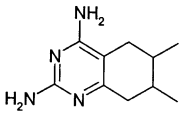
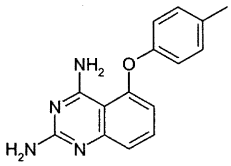
2,4-Diaminopyrimidine- and 2,4-Diaminoquinazoline-Containing Analogs

Compounds **1a** and **2a** can be broadly classified as 2,4-diaminopyrimidine- and 2,4-diaminoquinazoline-containing molecules, respectively, where trimethoprim falls into the former structural class. Interestingly, we previously reported on the discovery of such molecules in a biochemical screen of 50,000 small molecules against recombinant *E. coli* DHFR [27], where the 8,640 compound library used in this work was a subset of those screened for inhibition of the enzyme. In the work reported here, we have sampled analogs of **1a** and **2a** from our compound library that were previously identified as inhibitors of DHFR activity and characterized these for their MIC and for their kinetic inhibition constants (Table 2). The latter involved a systematic analysis of the steady-state kinetic behavior of DHFR at a range of dihydrofolate and inhibitor concentrations to reveal competitive inhibition mechanisms for all of the diaminopyrimidines (including trimethoprim) and diaminoquinazolines (data not shown). The diaminopyrimidines **1a** and **1b** differ by only a methyl group, have comparable K_i values (1.0 and 1.1 μM, respectively), and showed a 4-fold difference in MIC (16 and 64 μg/ml). Likewise, the MIC values of the diaminoquinazolines **2a**, **2b**, **2c,** and **2d** did not track with K_i. For example, **2a** and **2d** differ in the bridging position Y (O or S, respectively) and in the phenyl substitution Z (CH_3 and Cl, respectively) and demonstrated a greater than 6-fold difference in affinity (230 and 38 nM, respectively) but had identical MIC values of 4 μg/ml. The lack of correspondence of MIC and K_i values seen here, despite minor variations in structure, is illustrative of a common difficulty in antimicrobial research of translating gains in biochemical inhibition into increased cellular potency.

Quantitative Analysis of Suppression by *folA* and *acrB* Containing Clones

Struck by the frequency with which we isolated suppressors containing the multidrug efflux pump, *acrB*, we endeavored to make a comparative analysis of the suppression of the activity of compounds **1a**, **2a**, and analogs by clones containing *acrB* and *folA*. Figure 5 shows the fold suppression of the activity of these molecules by clones containing pGEM7-*acrB* and pGEM7-*yabF-kefC-folA* relative to a clone containing empty vector pGEM7. The controls trimethoprim and ciprofloxacin showed selective suppression as expected by clones encoding *folA* and *acrB*, respectively. Suppression of the activity of ciprofloxacin, for example, was 4-fold by the pGEM7-*acrB*-containing clone, consistent with

Table 1. Outcome of Selections for Multicopy Suppressors for Controls and Two Test Compounds

Compound	MIC[a] (μg/ml)	Suppressor Clones[b]
Fosfomycin	80	*ispB-sfsB-murA-yrbA*
Trimethoprim	0.078	*yabF-kefC-folA*
Cycloserine	80	(i) *yaiW-yaiY-YaiZ-ddlA-yaiB-phoA-psiF* (ii) *yaiY-YaiZ-ddlA-yaiB*
 **1a**	16	*yabF-kefC-folA*
 **2a**	4.0	(i) *yabF-kefC-folA* (ii) *acrB*

[a] Minimum growth inhibitory concentrations (*E. coli* MC1061/pGEM7) for the compounds indicated. Cells were grown on LB agar containing ampicillin (50 μg/ml) and streptomycin (50 μg/ml).
[b] Indicated are the identities of compete open reading frames present in the cloning site of pGEM7 found by sequencing suppressor clones isolated from the random genomic library. In the case where two types of suppressor clones were identified they are listed (i) and (ii).

a well-documented role for *acrB* in fluoroquinolone resistance [6]. Given that compound **1a** failed to select for clones containing *acrB* in our high-copy suppression screen, it is perhaps not surprising that we saw no suppression by pGEM7-*acrB* and 8-fold suppression with the *folA*-containing clone. Compound **1b**, on the other hand, differs only by a methyl group and showed a 4-fold suppression by *acrB*. The diaminoquinazoline **2a**, showed an equal propensity to produce clones encoding *folA* and *acrB* in our multicopy suppression selection. Compound **2a** likewise demonstrated a 4-fold suppression with clones containing either pGEM7-*yabF-kefC-folA* or pGEM7-*acrB*, and similar behavior was evident with the diaminoquinazoline analogs **2c** and **2d**. In contrast, the *acrB*-containing clone showed no capacity for suppressing the activity of analog **2b** while demonstrating 64-fold suppression by *folA* at high copy.

While there have been no systematic investigations of the substrate specificity of *acrB*, the anecdotal evidence [6] and that of recent costructures of four such ligands with AcrB [7] have pointed to compound hydrophobicity as an important determinant. In data not shown, we examined the fold suppression of growth inhibition by *acrB* at high copy (pGEM7-*acrB*) for all 49 growth inhibitory compounds short listed in this work and noted a positive correlation between fold suppression and calculated LogP, a commonly used molecular descriptor for general hydrophobicity [28].

Discussion

Classically, the identification of protein targets for phenotype-perturbing small molecules has been accomplished biochemically using labeled or immobilized molecules. Recent advances in forward chemical genetic approaches have included DNA [29] and protein [30] microarrays, three-hybrid systems [31], and compound library annotation by biological mechanism profiling [32]. A competitive growth assay using a pool of barcoded genome-wide heterozygous yeast strains was recently used to identify mutants that fail to grow in the presence of growth inhibitory drugs [33, 34]. The procedure, termed drug-induced haploinsufficiency, has been shown to be effective in identifying the targets of well-characterized clinical and agricultural agents. Most recently, Parsons et al. [35], using the complete set of yeast haploid deletion mutants, generated drug-hypersensitivity profiles for 12 compounds to identify target proteins, pathways, and multidrug resistance mechanisms.

Here we report on the first use of multicopy suppression to identify the targets of novel antibacterial compounds discovered with high-throughput screening. Unlike genomic methods that require extensive screening of transcripts, such as transcriptional profiling, multicopy suppression is a selection procedure that facilitates the rapid identification of target-encoding clones among suppressors. Proof of principle for this system has been achieved with the isolation of genes encoding the targets of known antibiotics and of novel antibacterial leads. The isolation of multicopy suppressors harboring *folA* encoding DHFR with two antibacterial molecules **1a** and **2a** highlights the feasibility of the approach. Derived from a screen for growth inhibition of just 8640 small molecules from a diverse commercial library, these molecules and their analogs represent novel leads with well-behaved permeability characteristics that inhibit a validated antibacterial target.

Surprising to us, nevertheless, was the frequency with which we selected clones using multicopy suppression that contained *acrB* encoding a multidrug efflux pump. Multidrug efflux pumps are understood to have a role in protecting most living cells from action of cell permeable and toxic molecules. The AcrB protein is a proton-energized drug efflux pump of broad and poorly understood substrate specificity that is well known as a menacing clinical problem in bacterial infections [6, 36]. In Gram-negative pathogens, in particular, increased expression

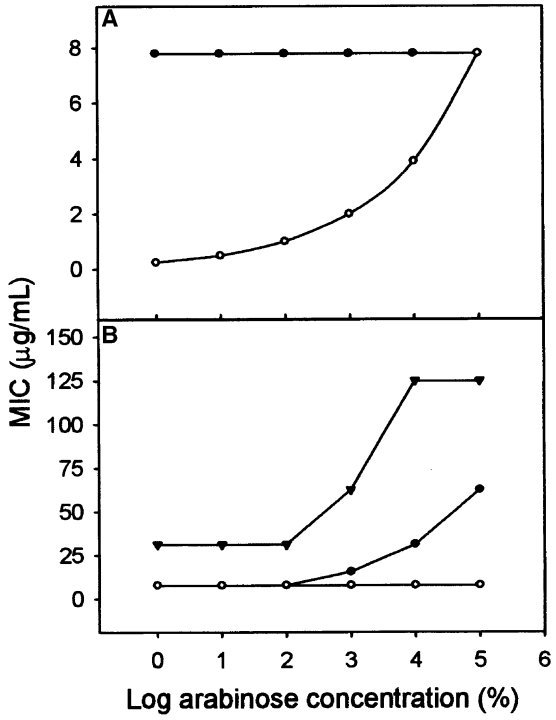


Figure 4. Regulated Suppression of Growth Inhibition through Controlled Expression

Shown is the influence of MIC on the controlled expression of DHFR from an arabinose inducible promoter system pBAD18-*folA* in *E. coli* CW2553 containing pAK01.

(A) The arabinose dependence of the MIC in LB-Amp-Cmp broth of the positive control trimethoprim (open circles) compared to that for the negative control tetracycline (closed circles).

(B) Analogous data for compounds **1a** (closed circles), **2a** (closed triangles), and tetracycline (open circles). The constuct pBAD18-*folA* was made as follows. Gene *folA* was amplified by PCR from genomic DNA of *E. coli* MG1655 using the primers 5′-C GCT CTA GAT TTT TTT TAT CGG GAA ATC TCA ATG -3′ and 5′- CTA AAG CTT TTA CCG CCG CTC CAG AAT C-3′, containing XbaI and HindIII restriction sites (underlined), respectively. The resulting product was cloned into the XbaI and HindIII site of pBAD18-Ap^r to create pBAD18-folA that puts the expression of gene folA under the control of the arabinose promoter [25]. Controlled arabinose-inducible expression from plasmid pBAD18-folA was accomplished by transformation into *E. coli* strain CW2553 containing pAK01 [26].

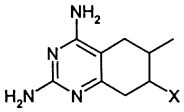
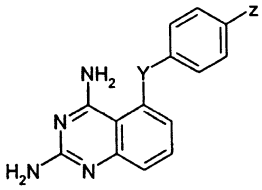
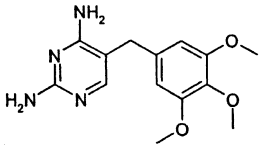
of AcrB orthologs is often associated with antibiotic resistance. Furthermore, basal levels of expression are increasingly thought to mediate intrinsic resistance to antibacterial molecules. It is therefore tempting to speculate that the lion's share of growth inhibitory molecules, for which we were unable to isolate suppressors other than *acrB* (31 of the 33 molecules that yielded supressors), might be structures that have inherently inferior permeability characteristics. In that regard, it is noteworthy that compounds **1a** and **2b** were apparently the poorest substrates for efflux and therefore may provide the most intriguing leads among the diaminopyrimidines or diaminoquinazolines examined in this study. Furthermore, the finding in this work that propensity for suppression by AcrB correlates by and large with calculated LogP, a gross molecular descriptor of hydrophobicity, is consistent with the conventional view that hydrophilicity is an important permeability characteristic.

In conclusion, we suggest that multicopy suppression is a workable forward genetic method to isolate targets for small molecules identified in phenotype-based screening. The approach is clearly generalizable to a wide variety of experimental systems, including the discovery of novel antifungal, antiparasitic, and anticancer molecules and their targets. In the context of the antibacterial screening campaign described, we predict that on-going screening and suppression studies will continue to identify both targets and resistance genes for novel antibacterial molecules. It is further suggested that the capacity to select for suppressors with bona fide targets in high copy, and not trivial resistance genes such as multidrug efflux pumps, has implications for the potential of any such molecule as a lead for new antibacterial drugs.

Significance

Microbial genetic studies have frequently exploited gene dosage to select for genetic interactions between a particular mutant and clones from a random genomic library at high copy. This approach, termed multicopy suppression, has likewise been used to identify resistance genes involved in efflux or modification of chemotherapeutic agents and, less frequently, to identify the cellular targets of agents of unknown mechanism. We report here the first use of multicopy suppression as a forward chemical genetic method to determine cellular targets and potential resistance mechanisms, for novel antibacterial compounds identified with high throughput screening. A high-throughput screen of 8640 small molecules for growth inhibitory activity toward a hyperpermeable strain of *Escherichia coli* resulted in the selection of 49 lead molecules for analysis using multicopy suppression. Selection from a pool of clones harboring a random genomic library of *E. coli* revealed suppressors of the antimicrobial activity of 33 of the compounds. The majority of these clones were found to contain a gene encoding the inner membrane component of the multidrug efflux pump AcrB, indicating that these compounds were substrates for efflux. Two compounds, one a 2,4-diaminopyrimidine and the other a 2,4-diaminoquinazoline, gave rise to clones containing the gene *folA*, encoding dihydrofolate reductase. Controlled expression of FolA in *E. coli* confirmed that suppression of the activity of 2,4-diaminopyrimidines and 2,4-diaminoquinazolines was dependent on dihydrofolate reductase levels, and steady-state enzyme kinetics revealed that these compounds were competitive inhibitors of *E. coli* DHFR. The work illustrates the power of multicopy suppression as a forward chemical genetic method to reveal the mechanism of action and efflux susceptibility of novel antibacterial leads. Finally, we believe that this approach is generalizable to a wide variety of systems including the discovery of novel antifungal, antiparasitic, and anticancer molecules and their targets.

Table 2. Analogs of **1a** and **2a** and Their Respective Antibacterial and Anti-DHFR Potencies

		x	y	z	MIC (μg/ml)	K_i (nM)
	**1a**	CH_3	—	—	16	1000
	1b	H	—	—	64	1100
	**2a**	—	O	CH_3	4.0	230
	2b	—	O	F	16	180
	2c	—	S	CH_3	8.0	150
	2d	—	S	Cl	4.0	38
	Tmp	—	—	—	0.03	3.0

Experimental Procedures

Primary Screening

The screen of *Escherichia coli* MC1061 (*hsdR mcrB araD*139 D(*araABC-leu*)7679 Δ*lac*X74 *galU galK rpsL thi*) against 8640 small molecules was fully automated with the use of a SAGIAN Core System (Beckman Coulter, Inc. Fullerton, CA) equipped with an ORCA arm for labware transportation, a Biomek FX with a 96-channel head for liquid handling, and a Spectromax absorbance plate reader (Molecular Devices Corp., Sunnyvale, CA); the entire system was integrated through SAMI software (v. 3.5, Beckman Coulter, Inc.). Incubations were done in duplicate and contained 50 μM library compounds sourced from Maybridge plc (Cornwall, UK). *E. coli* MC1061 was grown overnight in Luria-Bertani (LB) broth containing 50 μg/ml streptomycin (Str), diluted 10^5-fold in LB-Str broth, and deposited into 96-well microwell plate (200 μl/well). To each test well, 10 μl of screening compound (1 mM in DMSO) was added and the plate incubated for 16 hr at 37°C with shaking (150 rpm) before reading the optical density (600 nm). High control wells contained 10 μl of DMSO while the low control wells contained 50 μg/ml Ampicillin (Amp). Primary hits were defined as those compounds that reduced growth by 25% compared to the high controls. Activity Base (v. 5.0.5, ID Business Solutions Limited, Emeryville, CA), SARgen (v. 1.0, ID Business Solutions Limited), and Spotfire DecisionSite (v. 7.1.1, Spotfire Inc., Somerville, MA) were used for data analysis.

Secondary Screen and Lead Selection

Hits from primary screening in liquid media were further analyzed in duplicate for growth inhibition on solid media. *E. coli* MC1061 were grown overnight in LB-Str broth, diluted 10^5-fold, and 10 μl/well added into 96-well plates containing LB-Str agar (200 μl/well). Test wells contained compounds at 500 μM (10 μl of 5 mM compound in DMSO). High control wells contained 5% DMSO and low control wells, 50 μg/ml Amp. Plates were incubated for 16 hr at 37 °C before reading by visual inspection. Compounds causing complete growth inhibition were clustered by chemical group functionality and representative compounds were selected as leads for selection experiments using multicopy suppression.

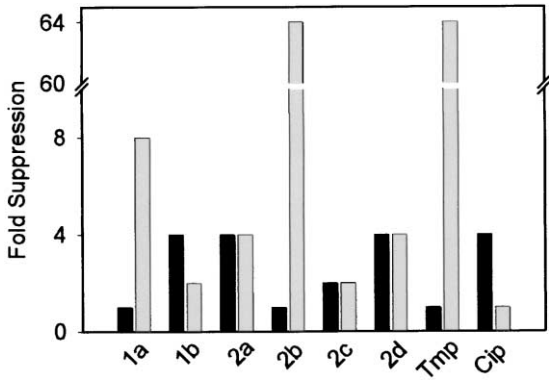


Figure 5. Suppression of Growth Inhibitory Activities of Compounds **1a**, **2a**, and Analogs by Suppressor Clones

The plot shows fold suppression of growth inhibition by compounds, **1a**, **2a**, and analogs (structures are shown in Table 2) as well as controls, Trimethoprim and Ciprofloxacin of *E. coli* MC1061 clones harboring pGEM7-*acrB* (black bars) and pGEM7-*yabF-kefC-folA* (gray bars). Fold suppression for each clone was calculated from MIC determinations (Experimental Procedures) for the compound indicated in LB-Str-Amp and was relative to *E. coli* MC1061 containing pGEM7.

Multicopy Suppression

A random *E. coli* genomic library was a generous gift of Deborah Siegele (Texas A&M University). The library was in the form of a ligation mix, derived from a partial *Sau3AI* digest (3–4 kb gel-purified fragments) of DNA from strain MG1655 cloned into the BamHI site of pGEM7 (Promega, Madison, WI), and was transformed into *E. coli* strain MC1061 and plated on LB-Str-Amp agar. Some 20,000 colonies were picked from these plates after overnight growth (37°C), transferred to a single well in a 96-well plate containing 200 μl LB-Str-Amp broth, and grown overnight with shaking at 37°C. Overnight cultures were pooled, mixed with an equal volume of 30% glycerol in LB broth, and stored in aliquots at −80°C. The latter was referred to as the selection pool. A control pool, *E. coli* strain MC1061 containing pGEM7, was also grown overnight in broth LB-Str and was subsequently mixed with an equal volume of 30% glycerol in LB broth and was stored in aliquots at −80°C.

Suppressor clones capable of growth in the presence of inhibitory concentrations of compounds were selected using the following procedure. Typically, 10^5 bacteria from the control and selection pools were plated on LB-Str agar with increasing concentrations of a given compound. Plasmid DNA was prepared from clones in the selection pool that were capable of growth at concentrations that were inhibitory to the control pool. Because many of the suppressor clones were found to contain *acrB*, encoding the membrane spanning subunit of the acridine efflux transporter [24], PCR was typically used to screen plasmid DNA derived from suppressors prior to sequencing. This PCR screen employed the forward primer (5′-ATG

CTCCTCTAGACTCGAGGAATT-3′) that annealed to the plasmid and a primer designed to anneal to *acrB* (5′-TCAATGATGATCGACAG TATGGCT-3′). Plasmid DNA from PCR-negative clones were sequenced to determine the cloned insert using the pGEM7 forward and reverse (5′-GAATACTCAAGCTATGCATCCAAC-3′) primers. Nucleotide-nucleotide BLAST was used to determine the portion of genomic DNA cloned into pGEM7.

Minimum Inhibitory Concentration Determination

Determinations of minimum inhibitory concentration were made to characterize growth inhibition of test and control compounds as well as to establish the degree of suppression by particular clones selected from the genomic library. Typically, 10^5 *E. coli* MC1061 cells were exposed to 2-fold dilutions of the compound from a stock solution of 6.4 mg/ml in DMSO. Incubations were in LB broth using 96-well microwell plates (200 μl/well) for 16 hr at 37°C with shaking (150 rpm) before determining the optical density (600 nm). Concentrations where the optical density was less than 0.1 absorbance units were deemed MIC.

Determination of Kinetic Inhibition Constants

Recombinant *E. coli* DHFR was prepared and assayed as described previously [27]. NADPH was constant at 80 μM and dihydrofolate was varied from 10 to 300 μM. Data were analyzed using SigmaPlot version 8.0 software and fit to the Michaelis-Menten equation for competitive inhibition: $V = V_{max} \times [S]/(K_M \times (1 + I/K_i) + S)$.

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