A Hybrid Drug Limits Resistance by Evading the Action of the Multiple Antibiotic Resistance Pathway

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Abstract

Hybrid drugs are a promising strategy to address the growing problem of drug resistance, but the mechanism by which they modulate the evolution of resistance is poorly understood. Integrating high-throughput resistance measurements and genomic sequencing, we compared *Escherichia coli* populations evolved in a hybrid antibiotic that links ciprofloxacin and neomycin B with populations evolved in combinations of the component drugs. We find that populations evolved in the hybrid gain less resistance than those evolved in an equimolar mixture of the hybrid's components, in part because the hybrid evades resistance mediated by the multiple antibiotic resistance (*mar*) operon. Furthermore, we find that the ciprofloxacin moiety of the hybrid inhibits bacterial growth whereas the neomycin B moiety diminishes the effectiveness of *mar* activation. More generally, comparing the phenotypic and genotypic paths to resistance across different drug treatments can pinpoint unique properties of new compounds that limit the emergence of resistance.

Key words: **antibiotic resistance, experimental evolution, hybrid drug, multidrug resistance.**

Introduction

Drug resistance is a growing problem in cancer and infectious disease (Ramos and Bentires-Alj 2014; World Health Organization 2014). Prolonged use of chemotherapeutic agents inevitably selects for resistance in the target organisms, reducing drug efficacy. Most anticancer and antimicrobial drugs target a single essential process, allowing resistance to rapidly emerge by spontaneous mutations or horizontal transfer of resistance genes (Walsh 2003; Ramos and Bentires-Alj 2014).

Drug combinations have long been employed to slow down the evolution of resistance. Drugs with different mechanisms of action often require different resistance mutations, such that the probability of any cell simultaneously gaining resistance to both drugs is extremely low (Zhanel et al. 2006; Michel et al. 2008). Furthermore, when combinations are used, synergistic or antagonistic drug interactions can alter the evolution of resistance (Hegreness et al. 2008; Michel et al. 2008). In addition, the evolution of resistance to drug combinations can be slowed if a mutation that confers resistance to one drug increases sensitivity to another drug (Szybalski and Bryson 1952; Pluchino et al. 2012; Imamovic and Sommer 2013; Lázár et al. 2013; Kim et al. 2014).

Another intriguing possibility is that chemically linking two drugs into a single hybrid molecule can incorporate the properties of both compounds and attack the target cell through two modes of action (Barbachyn 2008; Pokrovskaya and Baasov 2010; Fortin and Bérubé 2013). This dual-action strategy could be used to ensure simultaneous delivery of two drugs to the target cells, improve pharmacokinetic and pharmacodynamic properties (Albrecht et al. 1990), avoid toxicity (Grapsas et al. 2001), lead to better uptake (Alovero et al. 1998), and increase retention (Bremner 2007; Robertson et al. 2008a). Furthermore, these hybrid compounds often surpass their component drugs, either alone or in combination, by maintaining their activity against cells that are resistant to one or both of the component drugs (Jones et al. 1989; Gu and Neu 1990; Hubschwerlen et al. 2003; Bremner et al. 2007; Robertson et al. 2008a; Pokrovskaya et al. 2009), and can even decrease the frequency of resistant mutants (Robertson et al. 2008a; Pokrovskaya et al. 2009).

However, it is unclear how hybrid compounds affect resistance acquisition (Pokrovskaya and Baasov 2010). Indeed, some hybrid antibiotics select for high levels of resistance (Gu and Neu 1990; Robertson et al. 2008b). In cases where resistance is impeded, the hybrid's ability to stave off resistance may result from a range of mechanisms, including increased target affinity, drug interactions or collateral sensitivity between its components, a new mode of action, or the ability to escape active resistance mechanisms such as efflux or degradation.

Here, we focus on hybrid compounds that we generated by linking two commonly used antibiotics, ciprofloxacin (Cpr) and neomycin B (NeoB) (Pokrovskaya et al. 2009). Cpr is a fluoroquinolone that inhibits DNA replication by binding to DNA gyrase and topoisomerase IV (Wolfson and Hooper 1985; Khodursky et al. 1995). NeoB is an aminoglycoside that inhibits protein translation by binding to the 30S

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ribosomal subunit (Fourmy et al. 1998). The Cpr–NeoB hybrids may take advantage of a dual mode of action; they were shown to have increased in vitro affinity to Cpr targets, with slightly reduced in vitro activity on the NeoB target (Pokrovskaya et al. 2009). In addition to showing activity against a wide range of wild-type Gram-negative and Gram-positive bacteria, the Cpr–NeoB hybrids were also able to overcome resistance by both horizontally transferred mechanisms, such as modification by plasmid-born NeoB resistance genes, and vertical evolution (Pokrovskaya et al. 2009).

To understand the mechanism by which these Cpr–NeoB hybrid compounds limit the evolution of resistance, we followed the phenotypic and genotypic evolution of resistance in *Escherichia coli* to one such hybrid, compound **1m** (fig. 1*A*), and to combinations of its component antibiotics. We measured the cross-resistance of experimentally evolved strains and used genomic sequencing to identify adaptive mutations in each condition. Comparing these data across different drug conditions, we identified genotypic constraints that limit resistance to the hybrid antibiotic.

Results

Escherichia coli Evolve Less Resistance to the Hybrid Antibiotic than to an Equimolar Mixture of Its Components

We measured how *E. coli* K-12 MG1655 evolve resistance to the **1m** hybrid (fig. 1*A*) (Pokrovskaya et al. 2009) compared with four combinations of its parent antibiotics, Cpr and NeoB (table 1). *Escherichia coli* were evolved in Cpr and NeoB individually and in two different mixtures. The equimolar (EqM) mixture contains Cpr and NeoB in the same ratio as the hybrid: One molecule of Cpr per molecule of NeoB (table 1). The equipotent (EqP) mixture compensates for the different potencies of Cpr and NeoB by mixing them in a ratio proportional to their minimal inhibitory concentrations (MICs), thus applying selective pressure on both drugs' targets simultaneously (table 1). The EqP ratio is based on the MICs of the ancestral strain and is kept constant for the duration of the experiment (supplementary fig. S1, Supplementary Material online). To account for the

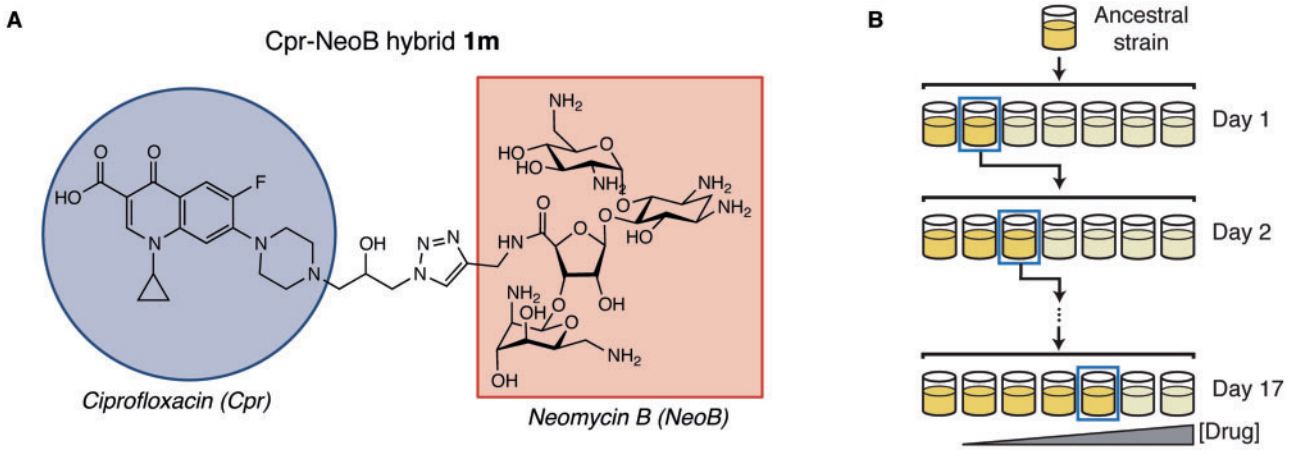

Fig. 1. Assessing resistance to antibiotic hybrid **1 m**. (*A*) Hybrid compound **1 m** chemically links two antibiotics, Cpr and NeoB. (*B*) Schematic of the serial passage selection procedure for a single population. Darker yellow culture color indicates higher bacterial density. In total, 50 parallel populations were evolved in five drug selection conditions, and four parallel populations were evolved in no drug, over 17 days. The populations were evolved in 2-fold dilution series of compound(s). For each population, the well growing at the highest concentration of drug was propagated daily into fresh drug. On each day of passage, the drug concentration range was adjusted such that the highest concentration was at least 8× the greatest MIC across all populations in that drug.

Table 1. Initial and Final MICs in Each of Five Antibiotic Treatments.

	Antibiotic(s)	Day 1 MIC (μM)	Day 17 MIC (μM)	Fold MIC Increase (day 17/day 1)
	Ciprofloxacin (Cpr)	0.1 ± 0.0	4.5 ± 1.4	45.3 ± 14.2
	Neomycin B (NeoB)	21.4 ± 1.5	98.5 ± 20.5	4.6 ± 1.0
1 1	Hybrid 1 m	6.2 ± 0.7	30.3 ± 3.4	4.9 ± 0.7
1 1	Cpr: NeoB = 1:1 Molar (EqM)	0.11 ± 0.02	4.2 ± 0.48	36.8 ± 5.1
1 200	Cpr:NeoB = MIC_{Cpr}:MIC_{NeoB} (EqP)	0.05:10 ± 0.0:0.0	0.19:37.3 ± 0.01:2.6	3.7 ± 0.3

Note.—The table lists, for each antibiotic selection condition, the average MIC values of the ancestral strain (day 1 MIC) and evolved strains (day 17 MIC), and the fold increase in MIC ± standard error of the mean. Day 1 and 17 MICs are averages of the ten populations evolved in parallel. The five drug selection conditions are ciprofloxacin only (Cpr, blue circle), neomycin B only (NeoB, red square), the hybrid compound **1 m**, Cpr and NeoB mixed in an equimolar ratio (EqM), and Cpr and NeoB mixed in a ratio proportional to their initial MICs (EqP). The EqP mixture is intended to compensate for the dissimilar MICs of Cpr and NeoB and maintain selective pressure on bacteria to develop resistance to both compounds.

stochasticity of the evolutionary process, ten replicate, parallel populations of *E. coli* were evolved in each of the five drug selection conditions. All populations were evolved through 17 days of serial selection in 2-fold dilution series of drug, passaging from the highest drug concentration allowing growth (fig. 1*B* and supplementary fig. S2, Supplementary Material online, Materials and Methods).

Assuming that chemically linking Cpr and NeoB does not change their individual activities and the spectrum of resistance mutations, resistance to the hybrid and to the EqM mixture should evolve similarly because both conditions contain one molecule of Cpr per molecule of NeoB. Furthermore, under this assumption, evolution in the hybrid and the EqM should be similar to evolution in Cpr alone. The initial MIC of Cpr is approximately 200 times less than that of NeoB (table 1 and supplementary fig. S1, Supplementary Material online), making it the vastly more potent of the two components and the main evolutionary pressure.

Despite this expectation, strains evolved in the hybrid gained much less fold-increase in resistance than strains evolved in the EqM mixture and strains evolved in Cpr. On average, populations evolved in Cpr or the EqM mixture developed a 40-fold increase in resistance (fig. 2*A*). However, strains evolved in the hybrid averaged only a 4.6-fold increase in resistance (table 1 and fig. 2*A*). In fact, selection in the hybrid resulted in a rate and level of MIC increase resembling selection in NeoB and the EqP mixture (fig. 2). This could be explained by the hybrid unexpectedly acting through its NeoB moiety, or acting through its Cpr moiety but avoiding a specific resistance mechanism.

Phenotypic Cross-Resistances of Evolved Strains Suggest that the Hybrid Primarily Inhibits Cpr Targets

To understand the basis of this diminished fold-resistance in the hybrid, we tested the cross-resistance phenotypes of all evolved strains to all selection conditions (supplementary fig. S3, Supplementary Material online) and found evidence that the hybrid restricts growth primarily through its Cpr moiety. First, all strains evolved in the hybrid gained Cpr resistance (2.2-10 fold), suggesting that the hybrid's Cpr moiety drives the evolution of hybrid resistance (fig. 3*A*). Second, strains evolved in the hybrid are not NeoB resistant, but actually show increased sensitivity to NeoB (fig. 3*A*). This could imply that the NeoB moiety does not place sufficient pressure on the cell to select for resistance, or it could mean that NeoB resistance mutations are incompatible with Cpr resistance mutations. Contrary to the latter hypothesis, the populations evolved in the EqP mixture gain both Cpr and NeoB resistance (fig. 3*A*), showing that it is possible to evolve resistance to both drugs. Therefore, the Cpr moiety appears to be responsible for most of the hybrid's inhibitory effect, whereas the antibacterial contribution of its NeoB moiety is negligible. When NeoB and Cpr are present at equivalently inhibitory levels, as in the EqP mixture, NeoB restricts bacterial growth and the fold-resistance; thus, the fold increase in resistance to Cpr is similar to the increase in resistance to NeoB in the EqP mixture (fig. 3*A*).

Although the hybrid acts primarily through its Cpr moiety, the hybrid-evolved strains do not gain as much Cpr resistance as the Cpr-evolved strains (fig. 3*B*). Cpr-evolved strains developed a 7.1-fold higher increase in Cpr resistance than hybrid-evolved strains, suggesting that strains evolved in the hybrid do not access all Cpr resistance mutations (fig. 3*B*). Furthermore, high Cpr resistance does not translate into high hybrid resistance; strains evolved in Cpr gain only as much hybrid resistance as strains evolved in the hybrid (fig. 3*B* and supplementary fig. S3C, Supplementary Material online), suggesting that only a subset of the Cpr resistance mutations provides resistance to the hybrid. The EqM-evolved strains match the phenotype of the Cpr-evolved

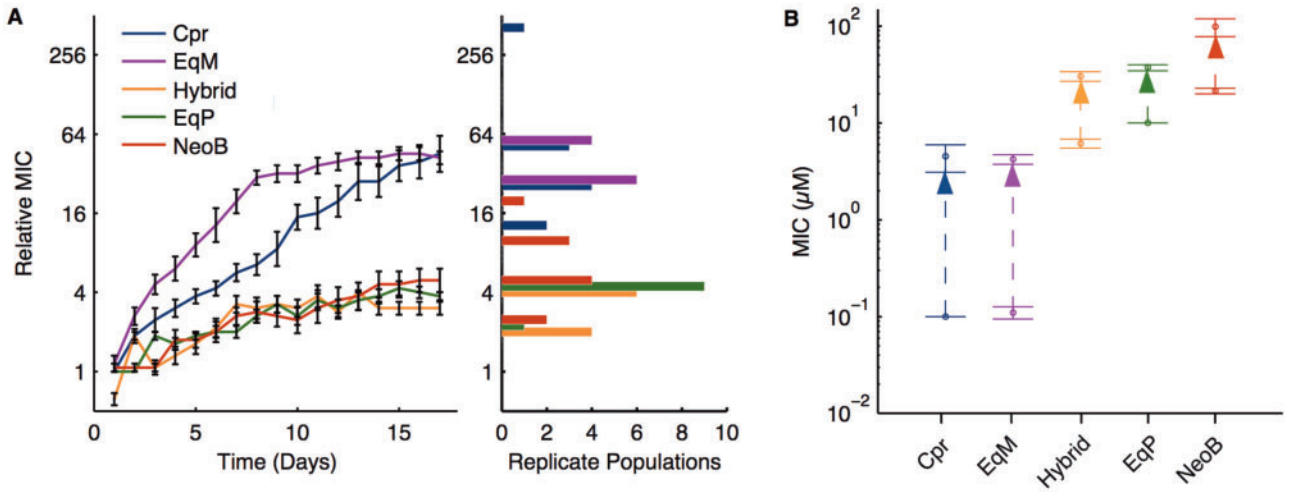

Fig. 2. Evolution of resistance to hybrid compound follows a different trajectory than an EqM mixture of components. (*A*) Mean resistance trajectories. Populations were evolved in each of five drug conditions and serially passaged daily for 17 days. Each line indicates average MIC (highest drug concentration where $OD_{600} < 0.2$) of ten replicate populations relative to the ancestral control strain on the same plate. Error bars indicate standard error. The histogram at right shows the distribution of day 17 MICs relative to the ancestral strain. (*B*) Increase (arrow) in absolute MIC between day 1 (start point) and day 17 (end point) of the evolution experiment. Error bars indicate standard error.

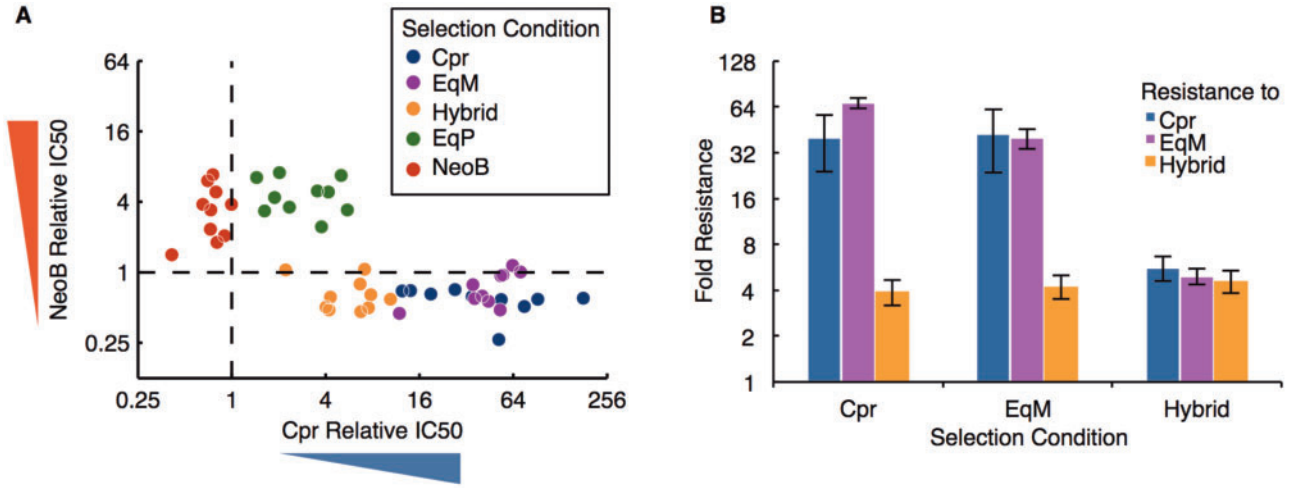

Fig. 3. Selection for resistance to hybrid **1 m** and molar mixture is driven by the Cpr component. (*A*) Log_2 Cpr and NeoB IC50s of day 17 isolates relative to the ancestral strain. Each point represents a single isolate from one evolved population. Isolates were evolved in Cpr (blue), NeoB (red), EqM mixture (purple), EqP mixture (green), hybrid compound (orange). (*B*) Mean resistances to Cpr, EqM, and hybrid (IC50 relative to the ancestral strain) of isolates selected in Cpr, EqM, and the hybrid. Bars represent averages of ten single colonies, one picked from each final evolved population. Error bars indicate standard error.

strains: An 8.1-fold higher increase in resistance to the EqM mixture arose in EqM-evolved strains compared with hybrid-evolved strains (fig. 3*B* and supplementary fig. S3*D*, Supplementary Material online). This shows that the mere presence of NeoB does not limit Cpr resistance. Only when NeoB is linked to Cpr, as in the hybrid, is an EqM ratio of Cpr and NeoB able to limit resistance.

Genotypic Data Reveal Constraints on the Evolution of Resistance to the Hybrid

To identify the resistance mutations and understand the constraints on evolution to the hybrid, we sequenced the whole genomes of isolates from all 50 evolved populations, as well as the ancestral strain and four isolates from populations passaged without drug selection. We identified single nucleotide polymorphisms (SNPs) and small insertions and deletions (indels) that differed from the wild-type *E. coli* MG1655 genome and were not present in the no-drug controls (Materials and Methods). A total of 219 mutations were identified in 104 unique genes (supplementary table S1, Supplementary Material online). Mutations displayed strong parallelism, with 51% of mutations present in genes that were mutated in at least three different isolates (fig. 4*A* and supplementary table S2, Supplementary Material online).

The mutational profiles mimicked the phenotypic results, with isolates evolved in the hybrid having mutation profiles similar to those evolved in Cpr and different from those evolved in NeoB. The gene *gyrA*, which encodes the Cpr target DNA gyrase, was mutated in nine of ten parallel isolates evolved in Cpr, in all ten isolates evolved in the EqM mixture, and in nine of ten isolates evolved in the hybrid (fig. 4*A*). In contrast, *fusA*, which encodes the ribosomal translocase elongation factor G, was mutated in seven of ten parallel NeoB-evolved isolates, but was not mutated in any of the hybrid-evolved isolates (fig. 4*A*). In line with these results, group analysis across all mutated genes showed that hybrid-selected isolates are similar to Cpr-selected isolates and distinct from NeoB-selected isolates (fig. 4*B*). In total, these data suggest that both the hybrid compound and the EqM mixture primarily inhibit cell growth through the Cpr component.

Absence of *marR* Mutations Limits Resistance to the Hybrid Antibiotic

Surprisingly, mutations in one gene, *marR*, arise from selection in the EqM mixture and Cpr, but not in the hybrid antibiotic. The marR transcriptional repressor is the negative regulator of the *marRAB* operon, which controls the multiple antibiotic resistance (*mar*) phenotype (Alekshun and Levy 1999) (fig. 4*C*). Mutations in *marR* arose in eight of ten isolates evolved in Cpr and in nine of ten isolates evolved in the EqM mixture (fig. 4*A*). However, no *marR* mutations arose in any hybrid-evolved strains (fig. 4*A*). Given this mutational pattern, we hypothesized that *marR* is responsible for the disparity in phenotypic cross-resistance between the hybrid and Cpr.

To investigate whether *marR* mutations confer resistance to the hybrid, we compared the activities of the hybrid, Cpr, the EqM mixture, and NeoB against a *marR* knockout and wild-type *E. coli*. As expected from the genomic data, *mar* activation confers resistance specifically to Cpr and the EqM mixture but not to the hybrid. The *marR* knockout was 1.7-fold more resistant to Cpr and 2.2-fold more resistant to the EqM mixture, but was not resistant to the hybrid (fig. 4*C*). Overexpression of *marA*, transcriptional activator of the *mar* pathway (Alekshun and Levy 1999), also conferred resistance to Cpr and the EqM mixture in a dose-dependent manner, up to 3.0-fold resistance to Cpr and 2.0-fold resistance to the EqM mixture, but did not confer resistance to the hybrid (fig. 4*D*). Differences in resistance conferred by *mar* activation account for 24–39% of the discrepancy in resistance between hybrid-evolved strains and Cpr-evolved strains (figs. 3*B*, 4*C*,

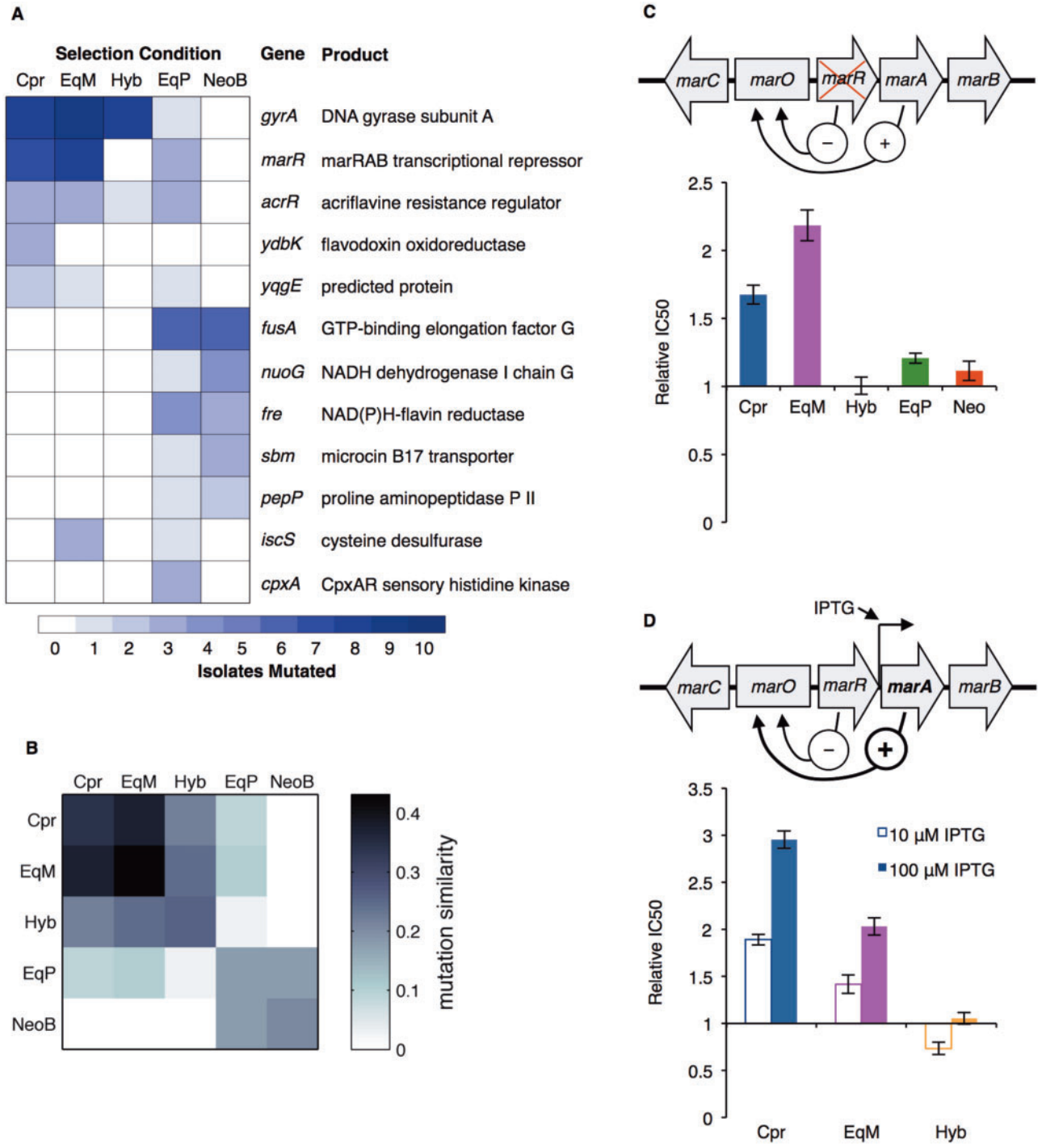

FIG. 4. The hybrid antibiotic limits evolution of resistance by restricting the effect of *marR* mutations. (*A*) Resistance mutations found by genomic sequencing of single colonies, grouped by each antibiotic selection condition. Genes mutated in at least three independent isolates are included. Colors indicate the total number of mutated isolates in each selection condition. Selection conditions are Cpr, EqM mixture, hybrid **1 m** (Hyb), EqP mixture, and NeoB. (*B*) Similarity of mutated genes, assessed by mean fraction of total mutated genes shared between any 2 of 50 random mutants from either the same or different selection condition. (*C*) Relative IC50 of *Escherichia coli* BW25113 Δ*marR* compared with wild-type BW25113. Schematic represents regulation of *marRAB* operon, with *marR* deletion indicated. (*D*) Relative IC50s of *E. coli* BW25113 transformed with ASKA *marA* overexpression vector compared with *E. coli* BW25113 transformed with control vector. Expression was induced with10 or 100 μM ITPG. Error bars indicate standard error.

and 4*D*), and 24–27% of the discrepancy between hybrid- and EqM-evolved strains (figs. 3*C*, 4*C*, and 4*D*). Thus, the inability of the *mar* phenotype to confer hybrid resistance explains, in part, why the hybrid selected for less resistance in the evolution experiment, compared with Cpr and the EqM mixture. We hypothesize that the hybrid drug evades *marRAB*-regulated drug efflux, a major mode of multidrug resistance, thereby greatly restricting the paths to resistance.

Discussion

Using a combination of experimental evolution, cross-resistance profiling, and genomic sequencing, we determine a mechanism by which a Cpr–NeoB hybrid drug limits the evolution of resistance. The **1 m** hybrid inhibits bacterial growth through its Cpr moiety and limits resistance through its NeoB moiety. Our results suggest that the hybrid drug binds effectively to the well-known Cpr target *gyrA*, as bacteria evolved resistance to the hybrid primarily through mutations in this gene. The absence of *marR* mutations in hybrid- and NeoB-evolved strains suggests that the NeoB component is limiting efflux-mediated resistance to the hybrid.

MarR is a repressor of the *mar* operon that regulates the AcrAB-TolC efflux system, outer membrane porins such as OmpF, and other genes that modulate susceptibility to multiple antibiotics (Cohen et al. 1988; Okusu et al. 1996; Alekshun and Levy 1999). Mutations in *marR* are known to increase resistance to a broad spectrum of antibiotics, including fluoroquinolones (such as Cpr), β-lactams, tetracyclines, and chloramphenicol (Maneewannakul and Levy 1996; Alekshun and Levy 1997). However, aminoglycosides such as NeoB are highly hydrophilic and cannot be exported through the AcrAB-TolC efflux system upregulated by *marR* mutations (Elkins and Nikaido 2002; Aires and Nikaido 2005). Therefore, the NeoB component could limit *marR*-mediated efflux of the hybrid, thereby removing *marR* as a path to hybrid resistance. Although these experiments were performed in Gram-negative *E. coli*, the NeoB component may similarly enable the hybrid to evade multidrug resistance pumps in Gram-positive bacteria, which also extrude Cpr, but not NeoB (Neyfakh 1992), thus accounting for lower hybrid resistance levels previously observed in *Bacillus subtilis* (Pokrovskaya et al. 2009).

To the best of our knowledge, the **1 m** Cpr–NeoB hybrid antibiotic studied here is the first quinolone that can evade resistance mediated by the *mar* operon. Furthermore, this Cpr–NeoB hybrid series has higher in vitro affinity to the quinolone targets, DNA gyrase and topoisomerase IV, than Cpr itself (Pokrovskaya et al. 2009). However, the **1 m** Cpr–NeoB hybrid has inferior whole-cell activity compared with Cpr (table 1), likely due to poor permeability. The size and charge of the NeoB moiety may be responsible for both the hybrid's poor penetration and its ability to evade the *mar* pathway. As the hybrid's antibacterial activity does not rely on the NeoB moiety binding to the ribosome, it is likely that NeoB may be replaced with chemically similar groups or substructures of NeoB to find a compromise between permeability and evasion of the *mar* pathway.

Although *mar* activation confers significantly more resistance to Cpr and the EqM mixture than to the hybrid, this accounts for only part of the discrepancy between Cpr, EqM, and hybrid resistance in the final evolved strains. The remaining difference could be explained by several mechanisms. First, mutations in genes other than *marR* could confer additional differential effects on resistance between the drug treatments. Second, a nonlinear relationship may exist between extra- and intracellular drug concentrations, leading to differential MICs. Third, mutations can have nonadditive effects on resistance in different genetic backgrounds. Thus, the differential effect of *mar* activation on resistance in the final evolved strains could be greater than observed from studies of the single mutant strains.

These results can inform the future design of hybrid compounds that aim to prevent the emergence of multidrug resistance. Hybrids can have properties beyond those of their individual components, and important functions other than binding canonical drug targets. Therefore, even when the two component drugs of a hybrid have dissimilar molar MICs, combining the drugs can have a beneficial effect. In this case, a potent antibiotic whose efficacy is eventually limited by *mar*-mediated resistance is linked to a less potent antibiotic that evades this pathway, resulting in a hybrid compound that combines the benefits of the first component's mechanism and the second component's restriction on resistance. Future hybrid compounds could be developed according to this principle to limit the emergence of multidrug resistance in cancer and infectious disease.

The approach developed here represents a general way to evaluate new hybrid compounds for function and resistance evolution potential. By comparing the mutational pathways between a hybrid compound and its components, we can tease apart each moiety's contribution to efficacy and resistance. We anticipate this approach to be beneficial not only for the design of novel hybrid drugs but also for designing and evaluating analogs of the same chemical scaffold. Assessing the mutational paths that a compound prevents—not just those taken—is a powerful tool for understanding, predicting, and manipulating the evolution of resistance.

Materials and Methods

Strains

Escherichia coli K-12 MG1655, a Gram-negative laboratory strain with minimal genetic manipulation and no plasmids, was used as the ancestral strain during experimental evolution (Blattner et al. 1997). A single colony of *E. coli* MG1655 was picked and grown overnight to saturation at 37 °C with shaking at 225 rpm. Aliquots of the ancestral strain were stored at −80 °C in 16.7% glycerol and were used to initiate the evolution experiment and to serve as ancestral controls (1/500 dilution). The Δ*marR* strain was generated by using pCP20-delivered FLP recombinase (Datsenko and Wanner 2000) to remove *kanR* from the Δ*marR*::*kanR* strain (JW5248) of the *E. coli* BW25113 Keio collection (Baba et al. 2006). The *marA* overexpression strain was generated by transformation of the ASKA ORF library *marA* vector into *E. coli* BW25113 (Kitagawa et al. 2006). A vector control strain was generated by transforming pCA24N-Δpromoter-yfp into *E. coli* BW25113 (Kitagawa et al. 2006).

Drug Conditions

Stocks of antibiotics Cpr only, NeoB only, the EqM mixture, and the EqP MIC_{Cpr}:MIC_{NeoB} mixture were prepared in LB media from powder stocks, by 2-fold dilution in 25 ml of LB,

starting at approximately 512 times their MICs. Stocks were stored at 4 °C. Conditions with hybrid were prepared fresh each day, from 2 mg/ml stocks in dH_2O stored at −20 °C, due to limited quantities of the drug. The lowest drug concentration where OD_{600} <0.2 was considered the MIC.

Experimental Evolution

Evolution experiments were conducted in 96-well Corning microtiter plates with a final volume of 150 µl per well. Each of the 12 columns contained a 2-fold dilution series of one drug condition. The drug concentration range for the hybrid was adjusted daily such that the highest concentration was at least eight times the MIC of the most resistant lineage. Each plate included one column for the ancestral control, and one blank column. Each of the remaining ten columns contained replicate populations of bacteria. Each day, the 600 nm absorbance (OD_{600}) of the cultures was measured using the Victor3 Multilabel Plate Counter (PerkinElmer). For each population (each column excluding ancestral controls and blanks), the well with an OD_{600} above 0.4 at the highest concentration of antibiotic was propagated. Populations selected at a lower cutoff (OD_{600} = 0.2) had high variability of recovery, due to slower growth rates, phenotypic resistance, or fitness defects that prevented them from reaching the cutoff OD on subsequent propagation. Therefore, the cutoff of OD_{600} = 0.4 was chosen to correspond with reproducible recovery. An aliquot of each selected well was diluted 1/500 into a new antibiotic gradient plate. Each plate was covered with an AeraSeal (EXCEL) and incubated for 21 h at 37 °C, with shaking at 900 rpm on a Titramax 1000 (Appleton Woods). The remaining population in each selected well was stored at −80 °C, in 5% dimethyl sulfoxide (DMSO). Populations were evolved for 17 days. As a control, four ancestral populations of *E. coli* MG1655 were also propagated in 150 µl of LB without antibiotic selection.

Retrospective Phenotyping of Evolved Strains

At the end of 17 days of selection, samples from all 54 cultures, one from each final evolved population, were streaked onto LB agar plates and grown overnight at 37 °C without antibiotic selection. Single colonies were picked and grown to saturation in 2 ml of LB at 37 °C, and then separated into aliquots for either 1) storage at −80 °C in 5% DMSO, 2) assessment for resistance to all five drug conditions using the same conditions and plate layout as that used in the evolution experiment, or 3) genomic DNA extraction. In the retrospective assessment of drug resistance, the final OD_{600} measurements at each drug concentration were fit to a four-parameter logistic model to determine the concentration of 50% inhibition (IC50).

Genomic Sequencing

A total of 55 single colonies were sequenced: 10 isolates evolved in each of 5 conditions (Cpr only, NeoB only, EqM mixture, EqP mixture, and hybrid), 4 no-drug controls, and 1 ancestral control. Genomic DNA was extracted from 500 µl cultures of each colony using the PureLink Pro 96 Genomic DNA Purification Kit (Invitrogen) according to the supplier's protocol. Purified DNA was quantified using the Quant-iT High-Sensitivity DNA Assay Kit (Life Technologies). Sequencing libraries were prepared using the Nextera XT DNA Sample Preparation Kit (Illumina) with 0.2 ng of input DNA (Baym et al. 2015). Forty-two samples were sequenced at Axeq Technologies, Seoul, South Korea, on an Illumina HiSeq 2000, on one lane of a 100-bp paired-end run. The remaining samples were sequenced at the Harvard Medical School Biopolymers Facility, on an Illumina HiSeq 2500, on one lane of a 100-bp paired-end run. Reads were trimmed using Sickle and aligned to the *E. coli* MG1655 reference genome (GenBank accession number U00096.2) with Bowtie2 (Langmead and Salzberg 2012). Over 99.7% of reads aligned to the reference genome, and average coverage across a sample ranged between 5× and 65× (median 32×). SNPs were identified using SAMtools (Li et al. 2009) and consensus quality (FQ score) cutoff of less than −55 for inclusion. Each variant position that met this cutoff in at least one strain was investigated in remaining strains, and a best call was made based on the aligned reads. Several colocalized false-SNP calls near a large 15-kb deletion were manually discarded. Small indels were called using Dindel (Albers et al. 2011). Candidate indels found in one strain were explicitly tested for in all strains; indels with a quality score greater than 20, with at least 30% of reads in the region supporting the indel, and that were not found in any of the control strains were accepted. Gene names and products not annotated in the GenBank were annotated using UniProt and GeneExpDB.

Growth of Mutant Strains

Growth of the Δ*marR* strain was compared with growth of wild-type *E. coli* BW25113. Growth of the ASKA *marA* overexpression strain was compared with growth of *E. coli* BW25113/pCA24N-Δpromoter-yfp vector control. ASKA vectors were induced with 10 or 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were diluted 1/500 into 150 µl final volume of LB with 2-fold drug dilutions and incubated at 37 °C for 21 h with shaking at 900 rpm. For each strain, OD_{600} measurements at each drug concentration were pooled from three replicates and fit to a four-parameter logistic model to determine the IC50.

Data Deposition

The sequences reported in this article have been deposited in the National Center for Biotechnology Information Sequence Read Archive database, www.ncbi.nlm.nih.gov/sra (accession no. SRP064444, BioProject No. PRJNA297729).

Supplementary Material

Supplementary figures S1–S3 and tables S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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