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Exploring Synergy between Classic Mutagens and Antibiotics

to Examine Mechanisms

of Synergy and of Antibiotic Action

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Microbiology, Immunology and Molecular Genetics

by

Yun Song

2016

ABSTRACT OF THE THESIS

Exploring Synergy Between Classic Mutagens and Antibiotics

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Yun Song

Master of Science

in Microbiology, Immunology and Molecular Genetics

U8niversity of California, Los Angeles, 2016

Professor Jeffrey H. Miller, Chair

I used classical mutagens in Escherichia coli to study synergies with different classes of antibiotics, test models of antibiotic mechanisms of action, and examine the basis of synergy. The strongest synergies were detected with 4-nitroquinoline 1-oxide (4NQO), which oxidizes guanines and ultimately results in double-strand breaks when paired with the bactericidal antibiotics vancomycin (VAN), ciprofloxacin (CPR), trimethoprim (TMP), gentamicin (GEN), but no synergies with the bacteriostatic antibiotics tetracycline (TET), erythromycin (ERY), and chloramphenicol (CHL). Other mutagens tested display synergies with the bactericidal antibiotics to various degrees,

also with some of the other mutagens. The results support models showing that bactericidal antibiotics kill bacteria by generating more double-strand breaks than can be repaired. The synergies represent dose effects of not the proximal target damage but rather the ultimate resulting double-strand breaks. I also used pairwise tests to place the mutagens into functional antibacterial categories within a previously defined drug interaction network.

The thesis of Yun Song is approved.

Arnold J. Berk

Pamela J. Yeh

Jeffrey H. Miller, Committee Chair

University of California, Los Angeles

2016

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INTRODUCTION

New strategies are needed to combat the rise of multidrug resistant pathogens (1,2). One avenue of research takes advantage of synergy between antibiotics in combination (*e.g.* 3; see review, 4). Previously, one has used the synergy between different antibiotics to potentiate the small concentration of vancomycin that is able to enter Gram-negative cells (5). The outer membrane of these cells normally acts as a barrier to vancomycin and many other drugs (6,7). A more comprehensive understanding of the basis of synergy between certain pairwise combinations of antibiotics would be important for developing this approach more thoroughly. Recently, Kohanski and coworkers have proposed that bactericidal antibiotics kill cells in part by generating hydroxyl radicals (8), and cause DNA damage that leads to double-strand breaks (9). Recent works by Dwyer et al. (10) and Belenky et al. (11) strongly support this idea. Here, I use a new strategy to examine both the mechanism of synergy and the mechanism of action of antibiotics by quantifying interactions between classic mutagens and different classes of commonly used antibiotics. In particular, I focus on mutagens that are strongly bactericidal via known mechanisms, generating double-strand breaks. I used 4-nitroquinoline 1-oxide (4NQO), zebularine (ZEB), 5-azacytidine (5AZ), 2-aminopurine (2AP), and 5-bromodeoxyuridine (5BrdU), whose bactericidal potencies are, in decreasing order, (4NQO>ZEB>5AZ>2AP>5BrdU). Using synergy relationships allows one to look at the mechanism of antibiotic killing through a different lens, and shows that strongly bactericidal mutagens, and particularly 4-nitroquinoline-1-oxide (4NQO), are highly synergistic with bactericidal antibiotics. The results suggest that this

synergy may be due to what would be equivalent to a dose effect of the ultimate lethal lesion resulting from both types of agents, namely double-strand breaks. Thus, this study provides support for the idea that double-strand breaks may play a significant role in some antibiotics' mechanism of killing. Moreover, one can place the mutagens into the drug interaction network defined by Yeh and coworkers (3) based on their pairwise interactions with traditional antibiotics. The network approach aids in the understanding of the cellular targets of drugs and their mechanism of action.

MATERIALS AND METHODS

***E. coli* strains**. The RecA, and RecB deficient strains used here are from the Keio collection, described in Baba *et al*. (12), made from the starting strain BW25113 (13). This starting strain (*lacI^q^ rrnB_{T14} ΔlacZ_{WJ16} hsdR514 ΔaraBAD_{AH33} ΔrhaBAD_{LD78}*) is used as WT in the experiments reported here, unless otherwise stated. Each mutant carries a complete deletion of the respective gene, with a *kan* insert in place of the gene.

Media. The following medium (14) was used. LB (10 g tryptone, 5 g yeast extract,10 gm NaCl per liter).

Growth conditions. Unless otherwise stated, all genetic methods are as described by Miller (14). Overnight cultures containing different concentrations of a given antibiotic were seeded with approximately 1×10^3 cells by inoculating 2 ml cultures with 50 μl of a 10^{-4} dilution of an over-day culture. After 18 hours incubation at 37°C on a rotor at 50 rpm, the OD_{600} was measured. Graphs of these data display percent growth versus that in LB.

Determination of single drug concentrations. Overnight cultures containing a range of concentrations of a given antibiotic (usually from the reported MIC in two-fold intervals) were prepared using the methods described above. Sub-inhibitory concentrations were typically chosen to be those that yielded 50-95% growth compared to that of cultures without any antibiotics.

Drug interaction assay. Cell cultures were prepared with the same method as above, using LB media supplemented with no drugs, each drug individually, and both drugs together at sub-inhibitory concentrations. Each set of single antibiotics and combinations were carried out together at the same time. Experiments were done multiple times on different days. Bar graphs were used to compare the effects of the paired drugs with those of the corresponding single drugs at the same dose, and with the control grown in LB only.

Classification of Drug Interactions. Drug interactions are defined using the same classifications detailed in previous work (3). Additivity is defined as $W_{xy} = W_x W_y$ where W_x is the proportion of growth relative to the control with no drug, with drug X, W_y is the proportion of growth with drug Y, and W_{xy} is the proportion of growth with the drugs combined. For example, if drug X has a residual growth of 0.6 of the control with no drug, and drug Y has a residual growth of 0.7, the additive expectation of the two drugs together would be 0.42. There is a range around 0.42 that would still be considered additive, for example 0.43. We discuss how to calculate this range below. Anything above this range would be antagonistic and anything below this range would be synergistic. More formally, deviation from additivity is defined by $\tilde{\varepsilon}$, which is calculated from the formulas below. When $\tilde{\varepsilon}$ falls within the range of -1 to -0.5, we classify it as synergistic; when $\tilde{\varepsilon}$ is between -0.5 and 0.5, we classify it as additive; when $\tilde{\varepsilon}$ falls between 0.5 and 2, we classify it as antagonistic.

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$$\tilde{\varepsilon} = \frac{W_{xy} - W_x W_y}{\left| \widetilde{W}_{xy} - W_x W_y \right|}$$

$\widetilde{W}_{xy}$ = min $[W_x, W_y]$ for $W_{xy} > W_x W_y$, and is 0 otherwise

If W_{xy} > min $[W_x, W_y]$, then

$$\tilde{\varepsilon} = \left[\frac{(W_{xy} - \text{min } [W_x, W_y])}{(1 - \text{min } [W_x, W_y])} \right] + 1$$

A pair was labeled inconclusive if the results from multiple experiments were inconsistent, and thus inconclusive.

Antibiotics. Tetracycline (TET), erythromycin (ERY), chloramphenicol (CHL), ciprofloxacin (CPR), gentamicin (GEN), trimethoprim (TMP), vancomycin (VAN), 4-nitroquinoline-1-oxide (4NQO), 2-aminopurine (2AP), 5-azacytidine (5AZ), and 5-bromodeoxyuridine (5BrdU) were purchased from Sigma (St. Louis, MO). Zebularine was a gift from Victor Marquez.

RESULTS

We hypothesize that if a principal mode of killing were indeed due to the generation of double-strand breaks, then one would expect the following: synergy between bactericidal drugs of different functional classes can result from each drug contributing damage that results in double-strand breaks. We can test this by using compounds that cause double-strand breaks via known mechanisms, and thus act as antimicrobials. These compounds, shown in Figures 1 and 2, include commonly used mutagens. Compound 2-5 are base analogs. Note that even though each of these mutagens has a primary target or effect that results in base mispairing, there are secondary effects that can play a role in their action, such as saturating out repair systems (e.g. the mismatch repair system), inducing or partially inducing stress response systems, or altering the pools of dNTPs.

Mutagen:antibiotic pairs: The following mutagens are used in this study.

1. 4-nitroquinoline 1-oxide (4NQO; See Figure 1), a compound that forms adducts to guanine that oxidize to 8-oxodeoxyguanosine (8-oxodG), and that also can be metabolized to generate anion radicals (15,16).

2. zebularine (ZEB; Figure 2), a cytidine analog lacking the amino group that when incorporated into DNA forms covalent complexes with deoxynucleotide methyltransferases (17). However, its toxicity emanates from mismatch repair correction that leads to strand breaks (18).

3. 5-azacytidine (5AZ; Figure 2), another cytidine analog, blocks replication after making complexes with deoxynucleotide methyltransferases, leading to double-strand breaks and cell death (19-22).

4. 2-aminopurine (2AP; Figure 2) is significantly less toxic than ZEB and 5AZ, but ultimately leads to excessive mismatch repair excision and some resulting double-strand breaks. It is very toxic, however, in *dam* strain backgrounds (23).

5. 5-bromodeoxyuridine (5BrdU; Figure 2) is barely toxic, even in *recA* strains (24), but is a potent base analog mutagen (*e.g.* 25). The presumption is that a very small number of double-strand breaks are generated.

Figure 3 shows the increased sensitivity of *recA* and *recB* strains to the first four of these compounds by sequential spotting, together with results for the bactericidal antibiotics used here. RecA is involved in both the repair of both single and double-strand breaks, while RecB is involved only in recombination and recombinational repair of double-strand breaks. Increased sensitivity of *recB* strains is an indication of failure to repair double-strand breaks.

We examined pairwise combinations of the five mutagens with seven different antibiotics for synergistic effects on cellular growth inhibition, using assays that we have described previously (8; see also Materials and Methods). Three of these antibiotics are bactericidal (VAN, CPR, and GEN), three are bacteriostatic (ERY, CHL, and TET), and one, TMP, is sometimes bacteriostatic and sometimes bactericidal. We aimed for concentrations that would allow partial growth, ideally 60%-90% of bacterial growth in

no-drug environments. Table 1 shows the concentration ranges for each antibiotic and mutagen.

The antibiotics were chosen as representatives of the main groups of antibiotics as differentiated through their mechanism of action, used in two prior studies (3,5). Figure 4 displays the results in the format employed by Yeh and coworkers (3). Here, percent residual growth versus growth in LB without antibiotic is plotted for each single antibiotic, and for the pair of antibiotics. This latter study characterized the interactions as additive, suppressive, antagonistic, and synergistic (see Materials and Methods for a fuller explanation; *e.g.* synergistic effects are those that are significantly greater than simple additive effects). The background color of each graph designates the form of epistasis where red is strong synergy ($\tilde{\epsilon}_{max} < -0.5$); pink is weak synergy ($-0.5 < \tilde{\epsilon}_{max} < -0.25$); white is additive ($-0.25 < \tilde{\epsilon}_{max} < 0.5$, and $-0.5 < \tilde{\epsilon}_{min} < 0.25$); green is strong antagonistic buffering ($0.5 < \tilde{\epsilon}_{min} < 1.15$); light green is weak antagonistic buffering ($0.25 < \tilde{\epsilon}_{min} < 1.15$); blue is antagonistic suppression ($\tilde{\epsilon}_{min} > 1.15$); and grey is an inconclusive result.

It is evident from Figure 4 that the strongest bactericidal agent among the classical mutagens, 4NQO, separates the antibiotics based on their bactericidal properties. Namely, strong synergies are found with VAN, CPR, GEN, and TMP, but not with CHL, ERY, and TET. In fact, various levels of suppression or antagonism are seen with the pairing of 4NQO and CHL, ERY, or TET. 4NQO causes DNA damage that leads to double-strand breaks (15,16), and therefore this result implies that VAN, CPR, GEN, and TMP lead to double-strand breaks that combine with the 4NQO-caused breaks to

yield too large a load for the cellular repair systems (see Discussion). The mutagen ZEB is strongly synergistic with two of the bactericidal antibiotics, weakly synergistic with a third, and additive with a fourth (GEN), but also, shows weak synergy with CHL and ERY. An unexpected result is the strong synergy of ZEB with TET, an antibiotic that fails to show strong synergy with any of 21 antibiotics tested with it in pairs (3, 5). The three remaining mutagens 5AZ, 2AP, and 5BrdU, all of which are weaker bactericidal agents than ZEB or 4NQO, give mixed responses when paired with this set of antibiotics. With respect to displaying synergies against these antibiotics, they are clearly much weaker than 4NQO and ZEB.

Mutagen-mutagen pairs. From the right side of Figure 4 we can see the results of pairwise combinations of mutagens with each other. Interestingly, each agent, like other antibiotics, has a distinct pattern of interaction. Again, 4NQO has the most synergistic interactions in pairings with other mutagens. However, a striking exception is the antagonistic buffering exhibited by the 4NQO-5AZ pair! Given the strong synergies 4NQO displays with the bactericidal antibiotics and the other three mutagens, including 5BrdU (the weakest agent with regard to killing), the suppression seen with 5AZ is remarkable.

Classification of mutagens based on pairwise interactions. We applied the methodology of Yeh and coworkers (3) to place the mutagenic antibacterial agents within the context of groupings based on their mechanism of action using the data from pairwise tests with representative antibiotics (Figure 4). This resulted in the interaction

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network shown in Figure 5. However, as one adds more data this picture changes and comes into better focus, as shown in Figure 6. Here the expanded interaction network is shown using the results in this study together with the data of Yeh and coworkers (3), and our recent results with VAN (8). Note that ZEB and VAN fit perfectly into the same grouping, and 4NQO fits into the aminoglycoside grouping. The red lines correspond only to strong synergies ($\tilde{\varepsilon}_{max} < -0.5$) but the green lines include the suppressive ($\tilde{\varepsilon}_{min} > 1.15$) and buffering interactions ($0.25 < \tilde{\varepsilon}_{min} < 1.15$). Additive interactions ($-0.25 < \tilde{\varepsilon}_{max} < 0.5$, and $-0.5 < \tilde{\varepsilon}_{min} < 0.25$) are not included in this figure, as they represent no interaction between antibiotics.

Discussion

Yeh and coworkers examined pairwise interactions of 21 antibiotics, and generated a drug interaction network (3), classifying antibiotics based on whether they demonstrated synergy, antagonism, or suppression of other antibiotics (3; see also 5). Synergy between drug pairs allows one to design multidrug therapies (*e.g.* review, 4). What is the mechanism of synergy? An examination of the synergies displayed by 22 antibiotics (3, 5) reveals synergies between the vast majority of pairs of bactericidal antibiotics that are within the same class (*e.g.* aminoglycosides). These synergies likely are due to a straightforward dose effect (26). Thus, adding the doses of two different aminoglycosides, for example, is equivalent to doubling the dose of either drug alone. Sometimes, synergy can be explained by two drugs operating on a different step in the same pathway, such as trimethoprim and sulfanilamide (27). However, there are strong synergies evident among drug pairs involving antibiotics of different categories (3, 5). The majority of these involve antibiotics that are bactericidal (*e.g.* aminoglycosides, β-lactams, fluoroquinolines, vancomycin). The classical explanation for the synergy between β-lactams and aminoglycosides is that the inhibition of cell wall synthesis by β-lactams increases the permeability and thus the efficacy of aminoglycosides (*e.g.* 4). However, Kohanski and coworkers have shown that bactericidal antibiotics can also act via a pathway that generates hydroxyl radicals (8) that lead to double-strand breaks (9), even though their initial (proximal) target may be different. More recent results from kohanski and coworkers provide extensive experimental data that support this and that also corroborate the work presented here (10,11). I hypothesize that these inter-class

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synergies (*e.g.* fluoroquinollone – β-lactams pairs; see 3) result from a dose effect of the ultimate double-strand breaks that finally exceeds the repair capacity of the cell. To test this, we have used antibacterial compounds that are highly mutagenic and thus are not used for clinical treatment. However, they cause lethality to varying degrees by generating double-strand breaks via different known mechanisms (15-22). One can see the increased sensitivity of *recA* and *recB* strains to the most bactericidal of these mutagens (4NQO, 5AZ, ZEB; see Figure 3). The strongest lethal effects are exerted by 4NQO, which oxidizes DNA to 8-oxodG and causes the generation of anionic radicals, and leads to double-strand breaks (15, 16). If a buildup of double-strand breaks that exceeds the cellular repair capacity is the cause of synergy for bactericidal antibiotics, then 4NQO should be synergistic with bactericidal drugs but not bacteriostatic antibiotics. This is exactly what was found, as shown in Figure 3. On the other hand, 4NQO displays antagonistic buffering with CHL, and displays strong suppression with TET.

A systems analysis of pairwise interactions places 4NQO in the same group as aminoglycosides in the drug interaction network (Figure 6). This is fascinating, because it shows that the bactericidal properties of a mutagenic agent allow us to classify it as we would any typical antimicrobial drug. At this point, it is worth noting that a number of compounds that are used as mutagens in basic research are also employed as chemotherapeutic agents. Thus, bleomycin and cisplatin are used as mutagens in studies in bacteria and higher cells (*e.g.* references 28 and 29), yet are also used as effective agents against testicular cancer (30, 31). Both 5AZ and ZEB that are employed in this study (*e.g.* Figure 4) are used in chemotherapy as demethylating agents to

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reactivate silenced tumor suppressor genes (32, 33). Interestingly, ZEB fits into the same drug class as VAN in the drug interaction network (Figure 6), while 5AZ constitutes its own group at this stage. 2AP also defines a new group, but 5BrdU groups with the quinolones and fluoroquinolones.

With regard to drug interactions, ZEB shows strong suppression with two of the four bactericidal drugs, weak suppression with a third, and additivity with a fourth. However, what makes ZEB unique, is that it not only shows weak synergy with ERY and CHL, but strong suppression with TET. This is extraordinary, since none of the 21 antibiotics previously paired with TET showed strong suppression with TET. It remains for future studies to unravel the mechanism of this interaction. Because ZEB toxicity is reduced in a mismatch repair deficient strain (18) we tested whether strong synergies would also be reduced. This is indeed the case for the tested pair of ZEB + VAN, which displays a strong synergy in the wild-type strain that is completely eliminated in a mismatch repair deficient background (figure 7). The weaker bactericidal agents show differing patterns with the antibiotics that allow us to place all of them in different groups (Figure 6). 5BrdU, although a strong mutagen, is the weakest bactericidal of the mutagens used here, with 1000 μg/ml concentrations barely affecting viability (24). That it shows even weak synergy with any of the drugs (VAN) is extraordinary.

We can also look at the effects of pairing the five mutagens with each other. These results (Figure 4) again show that 4NQO is the strongest with respect to displaying synergy for bactericidal effects. Even the pairing of 4NQO and 5BrdU displays strong synergy, in accord with the idea that the number of double-strand breaks resulting from 4NQO is sufficient to combine with the very small number of such

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breaks generated by 5BrdU to result in synergistic effects. An unexpected result is that the pairing of 5AZ with 4NQO results in weak antagonistic buffering. Future studies will be aimed at deciphering the nature of this effect.

The exact nature of the DNA damage from the generated hydroxyl radical pathway of conventional antibiotics (8, 9) that causes double-strand breaks is an interesting question. The results with 4NQO shown here (Figure 4) might appear to support the idea that the main lesion responsible is 8-oxodGuanine, and particularly from oxidized dGTP precursors, with the combined action the MutY and MutM proteins generating double-strand breaks (9). This is based in part on increased resistance to bactericidal antibiotics in strains overproducing the MutT protein that hydrolyzes oxidized dGTP, and in strains lacking both MutY and MutM (9). However, the picture appears more complicated, as MutT deficient strains are not more sensitive to antibiotics (our unpublished results), and even MutY MutM double deficient strains still show 99% killing by bactericidal antibiotics (compared with 99.9% killing of the wild-type; 9). It remains for additional experiments to clarify the exact cause of DNA strand breaks.

Table 1 List of mutagens and antibiotics used in the study with dosage and primary targets

Mutagen and Antibiotic	Abbreviation	Dose Range (μg/ml)	Primary Target
4-nitroquinoline 1-oxide	4NQO	0.38 – 1.47	Guanine residues
Zebularine	ZEB	5	Cytidine analog
2-aminopurine	2AP	500 – 700	Adenine analog
5-azacytidine	5AZ	20	Cytidine analog
5-bromodeoxyuridine	5BrdU	150 – 300	Deoxyuridine analog
Chloramphenicol	CHL	0.5	Protein synthesis, 50S
Ciprofloxacin	CPR	0.012 – 0.013	DNA gyrase
Erythromycin	ERY	50 – 150	Protein synthesis, 50S
Gentamicin	GEN	0.4	DNA
Tetracycline	TET	0.2 – 0.25	Protein synthesis, 30S
Trimethoprim	TMP	0.115 – 0.15	Folic acid biosynthesis
Vancomycin	VAN	12.5 - 150	Cell wall synthesis
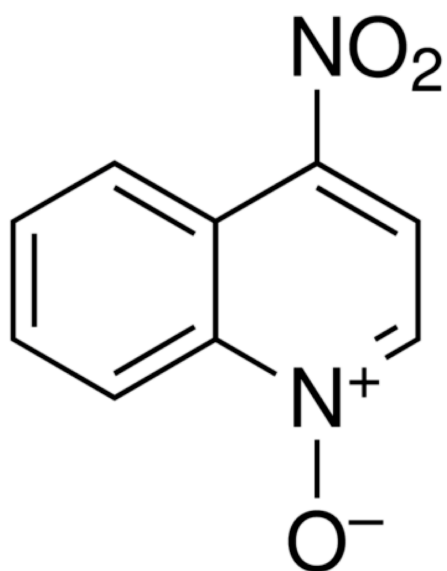
4-Nitroquinoline 1-oxide

Figure 1. Molecular structure of 4-nitroquinoline 1-oxide.

Zebularine   5-Azacytidine   2-Aminopurine   5-Bromodeoxyuridine
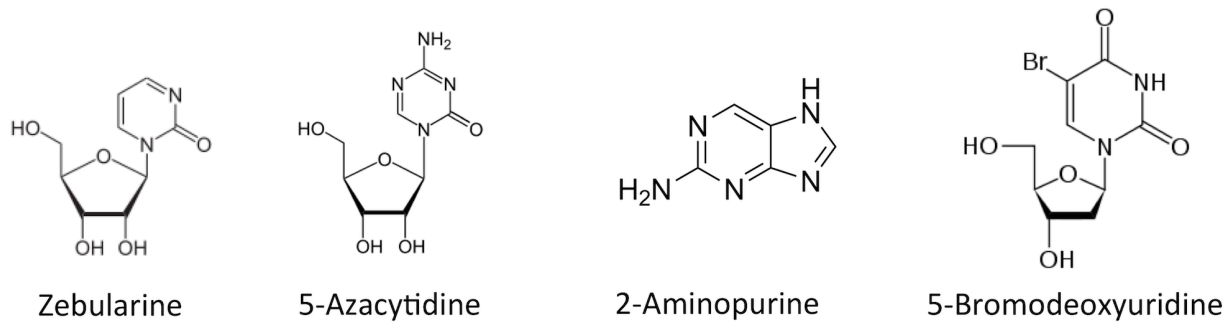
Figure 2. Molecular structures of zebularine, 5-azacytidine, 2-aminopurine, and 5-bromodeoxyuridine.
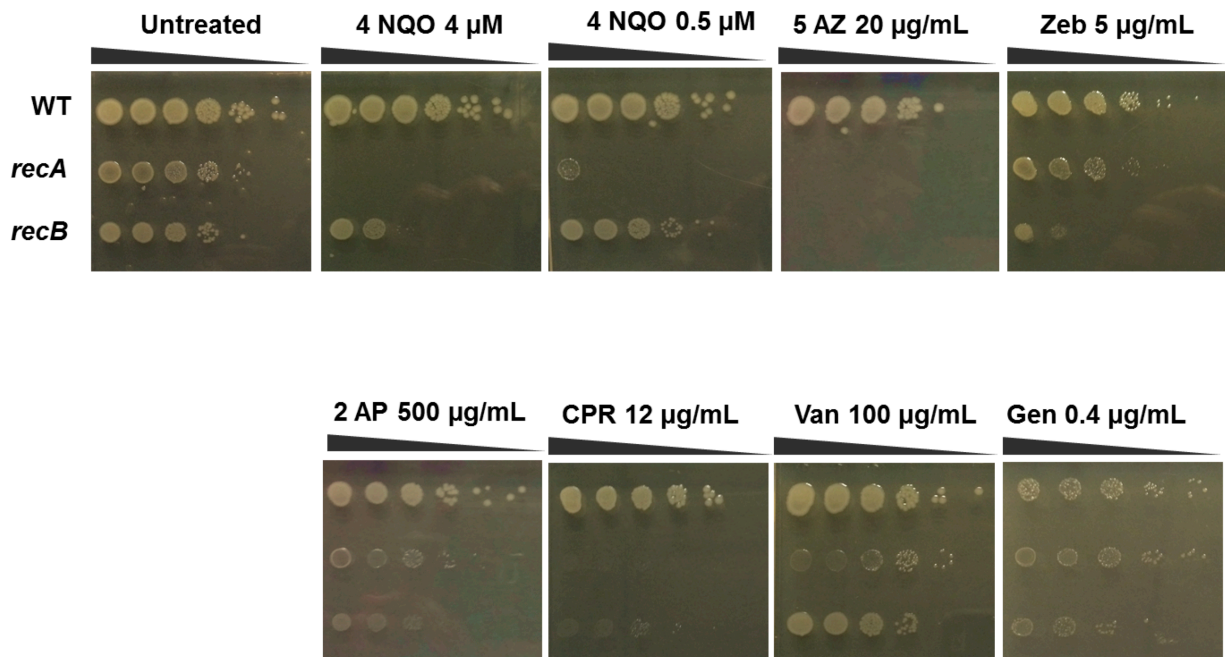
Figure 3. Sequential spotting tests for sensitivities to different mutagens and antibiotics for wild-type, *recA*, and *recB* mutants. Serial dilutions of cells were printed onto plates containing each agent, and incubated at 37°C for 18 hr. The number of cells spotted ranged from 0.5×10^6 to 0.5×10^1.
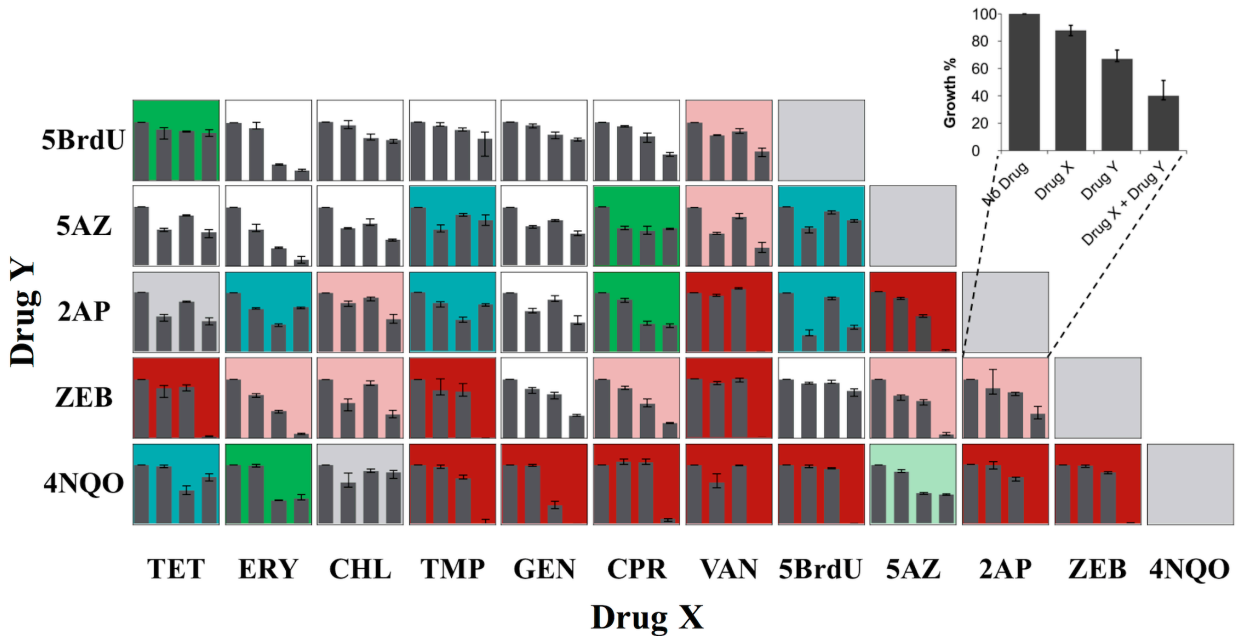
Figure 4. Systematic measurements of pairwise interactions between all combinations of agents X and Y. Each bar graph represents one experiment performed on one day for that drug pair. Within each panel, the bars, left to right, represent median growth rates of four replicates for cultures with: no drugs, drug X only, drug Y only, and the combination of the two drugs X and Y. Growth rates are represented as percentages of the no drug control. Error bars represent the range of replicate measurements for each experiment. The background color of each graph designates the form of epistasis where red is strong synergy ($\tilde{\varepsilon}_{max} < -0.5$); pink is weak synergy ($-0.5 < \tilde{\varepsilon}_{max} < -0.25$); white is additive ($-0.25 < \tilde{\varepsilon}_{max} < 0.5$, and $-0.5 < \tilde{\varepsilon}_{min} < 0.25$); light green is weak antagonistic buffering ($0.25 < \tilde{\varepsilon}_{min} < 0.5$); green is strong antagonistic buffering ($0.5 < \tilde{\varepsilon}_{min} < 1.15$); blue is antagonistic suppression ($\tilde{\varepsilon}_{min} > 1.15$); and grey is an inconclusive result. See Table 1 for list of antibiotics used, their abbreviations, and the range of concentrations tested for each antibiotic.
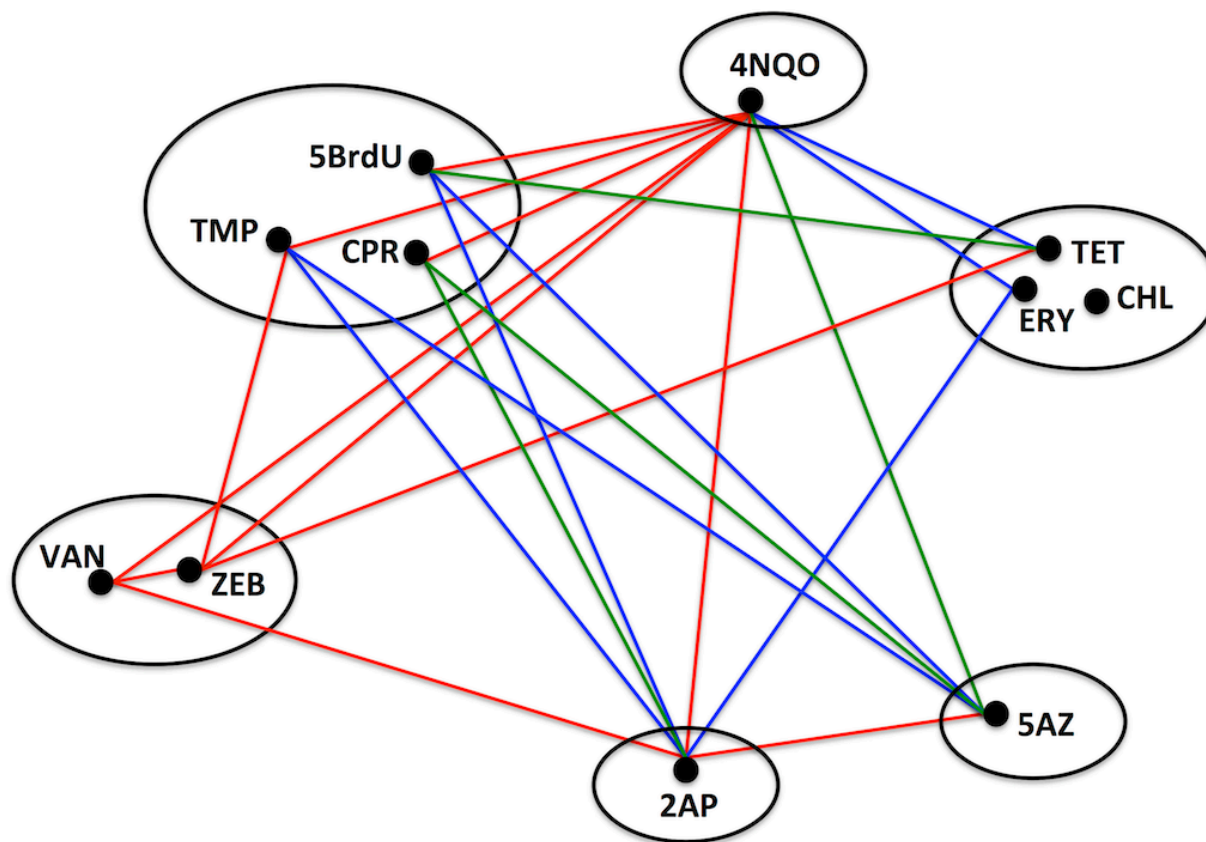
Figure 5. Classification of the antibiotic network into monochromatically interacting classes of drugs into mechanisms of action. Red lines represent strong synergistic interactions between groups ($\tilde{\varepsilon}_{max} < -0.5$) green lines represent antagonistic buffering between groups ($0.5 < \tilde{\varepsilon}_{min} < 1.15$), and blue lines represent antagonistic suppression between groups ($\tilde{\varepsilon}_{min} > 1.15$).
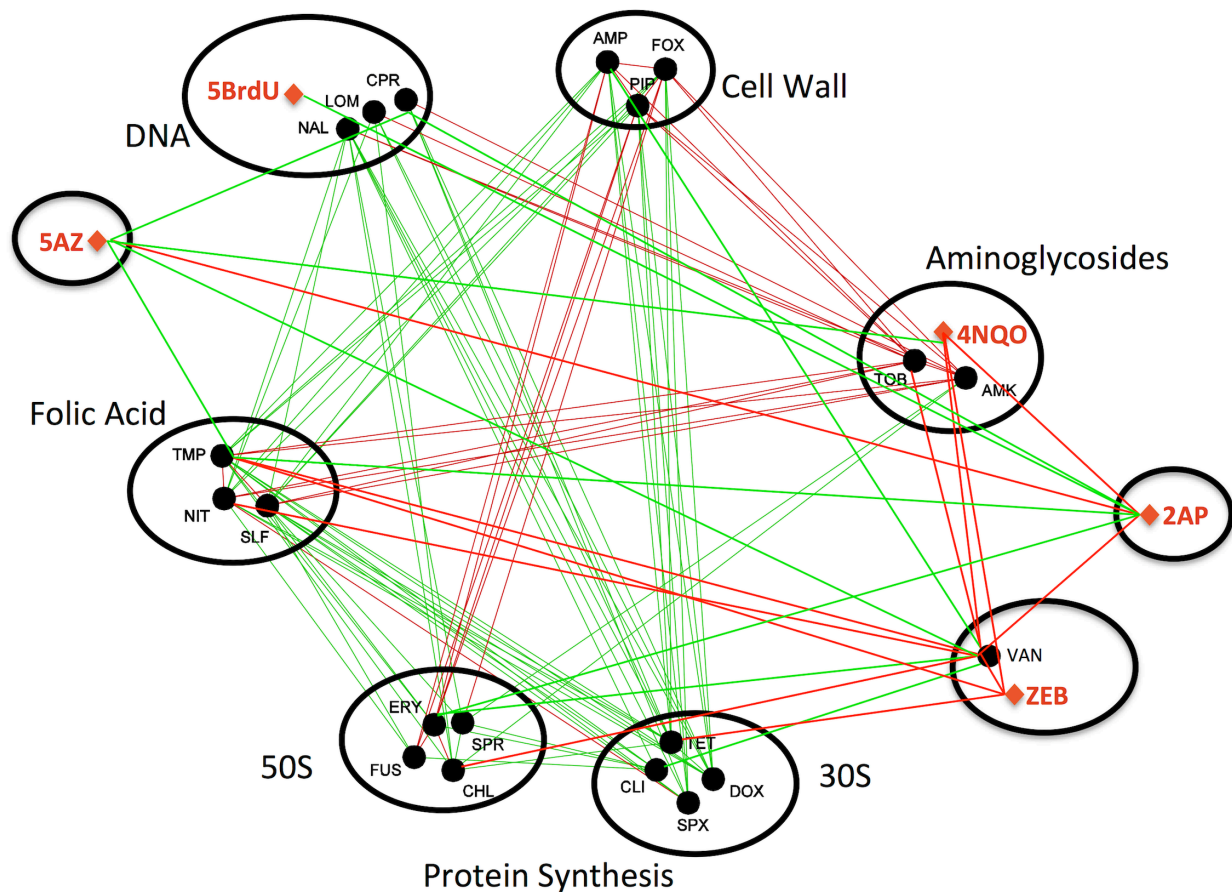
Figure 6. Expanded interaction network using the results in this study together with the data of Yeh and coworkers (3), and our recent results with VAN (5). Note that while 5AZ and 2AP still cluster by themselves in two separate groups, and ZEB clusters with VAN, as seen in the previous interaction network (Figure 5) with fewer data points, we now see 4NQO clustering with the aminoglycosides, and 5BrdU which previously clustered with both the folic acid biosynthetic inhibitor group (TMP) and the DNA gyrase inhibitor group now clusters only with DNA gyrase inhibitors (LOM, CPR, NAL). The comparison of these two figures shows us both the power and limitations of interactive networks, in terms of how data is needed to yield the most accurate picture of mechanisms of action. Amikacin (AMK), Ampicillin (AMP), Cefoxitin (FOX), Clindamycin

(CLI), Doxycycline hyclate (DOX), Fusidic acid (FUS), Lomefloxacin (LOM), Nalidixic

acid (NAL), Nitrofurantoin (NIT), Piperacillin (PIP), Spectinomycin (SPX), Spiramycin

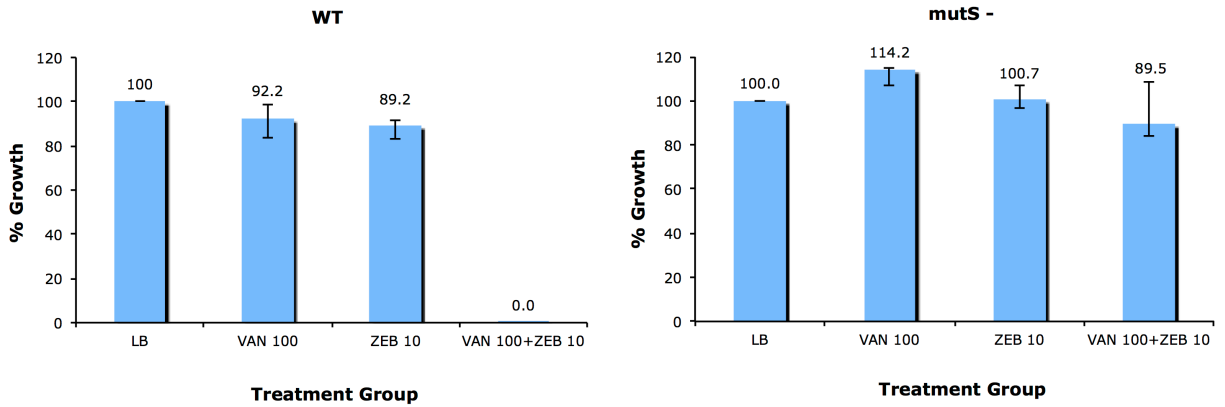(SPR), Sulfamonomethoxine (SLF), Tobramycin (TOB).

Figure 7. Synergy of ZEB and VAN is reduced in mismatch deficient *mutS* background.

Toxicity of ZEB emanates from the mismatch repair system, which results in DNA

strand breaks. Strong synergy of bactericidal antibiotics also depends on the mismatch

repair system.

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