Antimicrobial peptides: pharmacodynamics, combinatorial effects and resistance evolution

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Chapter 1

Summary

Antimicrobial peptides (AMPs) are ancient and conserved across the tree of life. They are the most important components in immune system due to their distinct mechanisms of killing bacteria. In this thesis, a pharmacodynamic approach was taken to investigate why bacteria are less likely to develop resistance to the nature immune system, especially to one of its components AMPs.

In this thesis, the combination effects of AMPs were firstly investigated. Six different AMPs from different organisms were selected to test their individual and combined effects *in vitro*. With an approach based on pharmacodynamics and Loewe additivity, the interactions of AMPs were found mostly synergistic. Three-AMP combinations displayed stronger synergism than two-AMP combinations. Additionally, AMPs displayed a sharp increase in killing within a narrow dose range contrasting with those of antibiotics.

Followed by a theoretical study, the combination effect between AMPs was explored using mathematical model that captures the dynamics of attachment and detachment between AMPs and cell membrane. In this multi-hit model, bacteria are killed when a certain number of targets are hit by antimicrobials. This bottom-up approach revealed that Bliss independence should be the model of choice if no interaction between antimicrobial molecules is expected; Loewe additivity, on the other hand, describes scenarios in which antimicrobials affect the same components of the cell, i.e. are not acting independently. The choice of the additivity term is essential to determine synergy or antagonism of antimicrobials.

The AMPs were found fundamentally different from antibiotics in their pharmacodynamic characteristics. This difference was further implemented within a theoretical framework to predict the evolution of resistance. The comparative analysis of resistance evolution demonstrated that pharmacodynamic differences all combine to produce a much lower probability that resistance will evolve against antimicrobial peptides. The finding can be generalized to all drugs with pharmacodynamics similar

to AMPs. Pharmacodynamic concepts are familiar to most practitioners of medical microbiology, and data can be easily obtained for any drug or drug combination. The theoretical and conceptual framework is therefore widely applicable and can help avoid resistance evolution if implemented in antibiotic stewardship schemes or the rational choice of new drug candidates.

Next, A model multiple-step mutations which can describe more complicated situation was used to simulate the resistance evolution in the treatment of antimicrobials. In this model, each mutant was captured by a set of pharmacodynamics. By monitoring the time of resistance emergence, simulations showed that mutants with medium increment of MIC will emerge earlier. Mutation with fitness cost will slow down the resistance evolution. The fitness cost in resistant mutants is likely to be compensated as lately as possible, otherwise will hinder the emergence of later fitter mutant and thus slows down the resistance evolution. For a given mutants, the shape of dose-response and maximal killing rate that can be achieved by antimicrobials nearly have no influence on the time of their emergence. Because of the emergence and selection of fitter mutant always happens in the subMIC of this mutant. It also showed that treatment strategy and pharmacokinetics do not affect the rage of concentration that select resistance.

Taken together, the thesis highlights that pharmacodynamic parameters of antimicrobials plays a decisive role in resistance selection. This can be applied in screening for resistance-proof drugs. In addition, it also explains the evolution of innate immune system which usually produces a mixture of AMPs to fight against infections. For example, mixtures of AMPs show strong synergism and steeper dose response curves in their pharmacodynamics.

Zusammenfassung

Antimikrobielle Peptide (AMPs) sind ein ursprüngliches Merkmal, welches im Stammbaum des Lebens konserviert ist. Aufgrund ihrer besonderen Mechanismen,

mit denen sie Bakterien abtöten, sind sie die wichtigsten Komponenten im Immunsystem. In dieser Arbeit wurde mit einem pharmakodynamischen Ansatz untersucht, warum Bakterien mit geringerer Wahrscheinlichkeit Resistenzen gegen das angeborene Immunsystem, insbesondere gegen AMPs, entwickeln.

Zuerst wurde der Effekt der Kombination von AMPs getestet. Sechs verschiedene AMPs von verschiedenen Organismen wurden auserwählt um ihre individuellen und kombinatorischen Auswirkungen *in vitro* zu ermitteln. Mit einem Ansatz, welcher auf Pharmakodynamik und der Loewe Additivität basiert, zeigten sich größtenteils synergistische Interaktionen der AMPs. Kombinationen von drei AMPs zeigten stärkere Synergien als solche mit nur zwei Komponenten. Weiterhin wurde, im Gegensatz zu Antibiotika, ein deutlicher Anstieg im Abtöten von Bakterien innerhalb eines engen Dosierungsbereichs beobachtet.

Im Folgenden wurde theoretisch der kombinatorische Effekt von AMPs mit einem mathematischen Modell untersucht, welches die Dynamiken von Bindung und Ablösen zwischen AMPs und Zellmembran berücksichtigt. In diesem Modell werden Bakterien als getötet betrachtet, wenn eine bestimmte Anzahl von Zielen von antimikrobiellen Peptiden angegriffen wurde. Dieser *Bottom-up* Ansatz zeigte, dass die Unabhängigkeit nach Bliss als Modell verwendet werden sollte, wenn keine Interaktion zwischen den antimikrobiellen Molekülen zu erwarten ist. Die Loewe Additivität hingegen beschreibt Szenarien in denen die AMPs dieselben Komponenten der Zelle angreifen und demnach nicht unabhängig voneinander agieren. Die Auswahl der additiven Bedingungen im Modell ist essentiell um Synergien oder Antagonismen von antimikrobiellen Peptiden zu bestimmen.

Die phamakodynamischen Merkmale der AMPs erwiesen sich als fundamental unterschiedlich gegenüber denen von Antibiotika. Dieser Unterschied wurde weiterhin in einem theoretischen Rahmen zur Vorhersage der Evolution von Resistenzen angewendet. Die vergleichende Analyse der Resistenzevolution machte

deutlich, dass die kombinierten pharmakodynamische Eigenschaften für eine geringere Wahrscheinlichkeit der Entwicklung von Resistenzen gegenüber AMPs sorgen.

Das Ergebnis kann hinsichtlich aller Wirkstoffe mit ähnlichen Pharmakodynamiken wie AMPs verallgemeinert werden. Pharmakodynamische Konzepte sind den meisten Fachleuten in der medizinischen Mikrobiologie bekannt und Daten können einfach sowohl für beliebige einzelne, als auch für Kombinationen von Wirkstoffen ermittelt werden. Das theoretische Konzept ist demnach im breiten Rahmen anwendbar und kann helfen, Evolution von Resistenzen zu vermeiden, wenn es in Verwaltung von Antibiotika oder der Auswahl neuer potentieller Wirkstoffe berücksichtigt wird.

Darüber hinaus wurde ein Modell mit mehrstufigen Mutationen verwendet, welches kompliziertere Situationen hinsichtlich der Resistenzevolution bei der Behandlung mit antimikrobiellen Peptiden simulieren kann. In diesem Modell wurde jeder Mutant mit einer Auswahl von Pharmakodynamiken erfasst. Indem der Zeitraum in dem sich Resistenzen entwickelt hatten, ermittelt wurde zeigten Simulationen dass Mutanten mit einem mittleren Zuwachs in der MIC früher hervortraten. Mutationen mit Fitnesskosten verlangsamen die Resistenzevolution.

Die Fitnesskosten in resistenten Mutanten werden höchstwahrscheinlich so spät wie möglich kompensiert, da sie ansonsten das Auftreten der späteren, fitteren Mutanten verhindern und die Evolution von Resistenzen verlangsamen würden. Für gegebene Mutanten haben die Reaktionen auf die Dosierung und die maximale Tötungsrate, welche mit antimikrobiellen Peptiden erreicht werden können, fast keinen Einfluss auf den Zeitpunkt ihres Auftretens. Dies kann dadurch erklärt werden, dass das Auftreten und die Selektion von fitteren Mutanten immer in im subMIC-Bereich des jeweiligen Mutanten passiert. Auch wurde verdeutlicht, dass Behandlungsstrategie und Pharmakokinetik keinen Einfluss auf das Konzentrationsspektrum, in dem auf Resistenzen selektiert wird, haben.

Zusammengenommen betont die vorliegende Arbeit, dass pharmakodynamische Parameter von antimikrobiellen Peptiden eine entschiedene Rolle in der Selektion von Resistenzen spielen. Die Ergebnisse können in der Auswahl von resistenzsicheren Wirkstoffen Anwendung finden. Weiterhin liefern sie Erklärungen für die Evolution von angeborenen Immunsystemen, welche normalerweise einen Mix an AMPs im Kampf gegen Infektionen produzieren. Beispielsweise zeigen Mixe von AMPs starke Synergien und steilere Dosis-Reaktions-Kurven in ihrer Pharmakodynamik.

Chapter 2

General introduction

Resistant bacteria are rapidly selected under intensive antibiotic treatment. On average it takes two years for a given pathogenic bacterium to cause resistance problems for the newly introduced drug [1]. To overcome this pressing resistance problem, two main strategies could be deployed: exploring new treatment regimens with existing antibiotics and developing new drugs.

One of the frequently proposed treatment strategies is combination therapy. Combination of synergistic drugs is commonly applied to maximize the antimicrobial effect and minimize the resistance evolution [2, 3]. These drugs are used either as a combination or in a fashion of "antibiotic cycling" [4]. Synergistic drug pairs can substantially enhance the effect of treatment. This practice has been widely adopted in treating various diseases including cancer, infectious disease caused by bacteria, fungi and virus, and many other diseases [5-10]. Such combination effects are largely determined by specific cellular metabolic networks on which the drugs can target. However, recent quantitative studies allow one to determine the combination effects of multiple drugs without knowing those mechanistic details [11-14]. Moreover, predicting resistance under multiple drug treatment is context–dependent and sometimes rather difficult. Although synergistic pairs are more effect on eliminate bacteria, it also more likely to select resistance [15]. Recent quantitative study showed that the speed of evolution is depended on the ratios of drugs in the pairs [16].

Besides, One need to develop new drugs to relive the rapid evolution of antibiotic resistance. These new drugs could be screened from the natural products or synthetic compounds. Recently, several new antimicrobial agents were identified from uncultured bacteria and resident bacteria on human body [17, 18]. They showed potential antimicrobial effects ether on gram-negative or gram-positive bacteria with varied mechanism. According to the authors' selection experiments, these compounds do not select bacterial resistance within a period constant selection [17]. However, systematical evaluation of a screened drug requires long time with many steps involved. Predictive method could lower the risks and advance the process of drug

evaluation. Most of preclinical antibiotic evaluations rely on some oversimplified indicators, such as minimal inhibitory concentration (MIC) [19]. Experimental evolution is also a widely used approach in determining the ability of resistance selection. But range of antibiotic concentration and variation in bacterial density might get inconsistent or even controversial results. For example, in the case of evolution of resistance to antimicrobial peptides (AMPs), some of the studies demonstrate that bacteria are less likely to develop resistance to AMPs [17, 18]. But some evolution experiments showed different bacterial species can develop considerable resistance to different AMPs [20-22].

Therefore, I will review recent development on determination of combination effects with a focus on AMPs. Then I will discuss the possibility that whether some key parameters can be used to both determine the combination effect and predict the evolution of antibiotic resistance. Based those information, a pipeline that is able to accomplish multiple tasks at the same time could be developed. It can determine the antibiotic effect, characterize combination and predict the resistance of evolution.

Antimicrobial peptides: a distinct class of antimicrobials

AMPs are evolutionarily conserved across the tree of life [23]. For example, bacteria secret AMPs to eradicate close residing individuals in resource-depleted environment [24]. In the multicellular organism, however, AMPs act as the most important components of innate immune system [25]. Insects which have no adaptive immune system, synthesize several AMPs when their immune system was challenged by bacteria [26]. Amphibians constantly release a layer of AMP-cocktails on their skin to prevent infectious bacteria and fungi [27, 28]. Moreover, antimicrobial peptides are also important antibacterial and antifungal substance on the human skin and in the mucosal secretions. Additionally, plants also use AMPs to fight against infectious disease [29, 30].

Active AMPs are usually consisting of 10-60 amino acid, they are usually cleavage of larger protein molecules. Mature AMPs have particular secondary structure like alpha-helix and beta-sheet. Some of their structures are underpinned by specific di-sulfate bridges [31, 32]. Some peptide chains contain large percentage of specific amino acids, such as proline, tryptophan and arginine. Such diversity in structure further relates other physical properties which depends on the spatial organization of amino acids residuals. AMPs therefore can be classified into hydrophilic, hydrophobic and cationic, but all these AMPs can form amphiphilic structures [33].

Distinct structures and physical properties make AMPs excellent scavenger of microbes, fungi and virus. For example, classical mechanistic models predict that positively charged cationic AMPs can be attracted to negatively charged bacterial membrane. Secondary structures of these peptides further allow them to form polymers on the membrane and finally lyse the bacterial cell. Moreover, recent report shows short peptides with new functioning patterns do not lyse bacterial cell, they instead translocate the proteins on the membranes and interrupt energy production [34]. Some AMPs, like Apidaecin, reveal complex pattern of antimicrobial effect. When the concentration is low, it can be transported into plasma and attached on ribosomes to inhibit synthesis of protein [35]. While in higher concentrations, it functions like typical cationic peptides and kill bacteria by lysing the membrane.

AMPs are able to eliminate bacteria efficiently with above distinct properties. With sufficient high concentration, AMPs are able to kill bacteria within one minute [36]. As cationic antimicrobial peptides kills bacteria by forming pores on the membrane, the subsequent leakage of cytoplasm will change the morphology of the surface of bacteria. Cellular scanning using Atomic Force Microscope (AFM) showed that the change of surface happens 50-200 seconds after incubation with AMPs [36]. Similar study using staining method also showed similar results of fast killing [37, 38]. The killing speed of AMPs is rather quicker than those of antibiotics, which usually varies

from minutes to hours [39, 40].

Combination effect of AMPs

An evolutionary origin of combination

Organisms living in a complex environment are at high risk of being infected with multiple pathogens. Expressing multiple anti-pathogen agents as a long-time fashion of combination is an advantage to overcome the potential danger from different aspects. This is, however, also the consequence of co-evolution between host and pathogen. Genes encoding several different AMPs have been identified in evolutionarily ancient insect [41]. Drosophila secrets cocktails of AMPs to fight against pathogens [42]. Among these AMPs, cecropins mainly target on the gram-negative bacteria, while defensins only target on gram-positive bacteria. And thanatin are able to kill both. Fungal infection could be eliminated by drosomycin, a special class of AMP in Drosophila.

Combinations of AMPs which target the same pathogen have advantage in maintaining the immune system as well. Organisms have evolved a sophisticated regulatory system to manage the cost of immune response. The system manifests itself as using specific pattern recognition models to identify pathogens, and then initiating corresponding response. However, the immune system also constantly maintains instant antimicrobial activity to cope with those rapid-propagating pathogens. Such functions are commonly supported by a combination of synergistic AMPs, which can substantially reduce the total cost of immune response of the host. Co-expressing AMPs targeting gram-negative bacteria in bumblebees showed strong synergistic effect [43]. When managing to achieve the same effect, combination could reduce several times of total cost of AMPs in terms of absolute quantity. In addition, The reduction in absolute quantity could significantly reduce the side effects in the immune reaction, such as the cytotoxicity and self-immunity.

Combination of AMPs *in vitro* experiments and preclinical tests

Since we are facing more serious problems of drug resistance, AMPs are also joining the campaign of fighting super bugs, while usually combined with other drugs. Most of the studies revealed combinations of/with AMPs are synergistic regardless of physical and chemical properties of AMPs [44-55]. Additionally, the synergistic effect is not restricted on specific targeting organisms [46, 56, 57]. Early study showed mammal AMPs demonstrated broad synergistic effects on several important pathogenic bacteria [55]. Besides, similar synergism was also found between AMPs and conventional antibiotics [52, 53]. Meanwhile, antagonistic effects between AMPs are less frequently reported. Like their evolutionary significance in the immune systems, synergistic combination of AMPs could be explored to reduce the toxicity and the cost of treatment.

Quantification of combinations

Due to the anticipated synergism between drugs, quantitative methods are needed to characterize the combination effects. Several classic methods, such as Loewe additivity [58, 59], Bliss independence [60] and mass-action models [61], have been widely used to determine the combination effects in pharmacology and clinical treatments. These methods usually require effects of both drug combinations and single drugs to calculate the combination index, which is mainly relied on to determine the combination effects, such as synergistic and antagonistic. Those are non-predictive methods. Recently, some predictive methods are developed to predict the combination effects. Such methods can directly obtain the effect of drug combinations based on the effect of individual drugs or their pairwise interactions [11-13, 62].

Non-predictive methods for quantifying combinations

For the non-predictive quantitative methods, it is necessary to collect the information of dosage and corresponding effects of single drugs and combinations. Non-predictive methods have two categories: the effects-based and dose-effect based [58]. Bliss

independence is one of the effect-based methods. It is built on the assumption that drugs in the combination do not interact with each others, and the combination effect is purely the probabilistic outcome of each drugs' effects. The effect of Bliss independence describing two-drug combination could be captured by following equation:

$$E_{bliss} = E1 + E2 - E1 \times E2$$

The above equation describe that the combination effect in the framework of bliss independence is the difference sum of individual drug's effect and the sum of combination effect of any drug. The bliss independence has its limits when the drugs do not have an exponential dose-response curve [10]. Besides, unknown interaction between drugs also constraints it's wide application [10].

Meanwhile, Loewe additivity is another wildly applied framework which is based on the dose-effect relation. The method looks into the concentration rather than the effect achieved by the concentration. In other word, it requires one to find out the equivalent concentration of drugs which can reach the same effect when determining the combination effect. This can be formulized as,

$$1 = \frac{C_{1,iso}}{C_1} + \frac{C_{2,iso}}{C_2}$$

Where, C_1, C_2 are the concentration of a given drug applied alone, $C_{1,iso}$, $C_{2,iso}$ are the concentration of a given drug in the combination.

The criteria of combination effects is defined by the combination index. The combination index of Bliss independence is between 0 and 1. The significance of a combination effect is decided by the statistical difference between the effect of combination and the effects of single drugs. The combination index of Loewe additivity is above 0. However, it is usually considered as synergistic when CI < 1, additive when CI =1, and antagonistic when CI > 1. Similar as Bliss independence, the difference of combination effected is also determined by the statistics.

Inspired by the concept of Bliss independence and Loewe additivity, similar method was invented to test the combination effect, for example the graph based isobologram effect [5, 10]. Due to the limited range of concentrations, the pharmacodynamic curves combined with the framework Loewe or Bliss were also introduced to determine the combination effect [58, 63]. However, most of these methods can only determine the combination effect of two drugs. High-order drug combinations are constrained by the intensity of laboratory work and unpredictable variations, thus new methods are urgently needed.

Predictive methods for quantifying combinations

Recently, new methods have been developed to predict combination effect of drugs with higher orders [11-14, 62, 64]. These methods are able to predict the combination effect based on single drug effect or drug-pair interaction, and sometimes do not necessarily rely on the Bliss or Loewe frame work. Only using growth data of bacteria, Wood and colleagues showed that high order of drug interaction obeys the statistical laws rather chemical law. Their striking method is implemented with a maximum entropy method and Isserlis theorem, in which the three- and four-drug interaction can be predicted by the pairwise interaction. Its power of prediction is completely independent of any specific mechanisms. Similar methods using fixed concentrations combined with the frame work of Bliss independence determined antagonism-biased combination effect in higher order of drug combinations [14]. Zimmer *et al*. extended the Bliss formula which embedded in the Mechaelis-Menton-like and Hill-like dose response curve. This method accurately predicted high order of interactions based on pairwise interaction [13]. Moreover, The methods are able to predict full-dose-range of high order interaction based on limited number of dosages [13, 65]. When compared with other previous models, their model showed significantly improved accuracy in different drug combinations. Collectively, recent advance in methods of determining the combination effect of drug interaction especially in high order drug combination could largely reduce the labor intensity in identifying the effective drug

combinations and also advance the clinical treatment strategies.

Resistance evolution

Bacteria inevitably gain resistance to any antimicrobials through selection and evolution. The resistance evolution and it's rate are determined by factors from various aspects. Genomic mutation, horizontal gene transfer and phenotypic changes all together confer the resistance to antimicrobials. However, the dynamics of resistance evolution is closely related to viable population size and the concentration of antimicrobials.

General cause of resistance by mutation

Genetic mutation plays an utterly important role in evolution of resistance. Most if not all of antimicrobials kill bacteria through targeting specific position of molecule and thus interrupting the whole metabolism of the bacterial cells. Most of these positions are consist of residuals of amino acids. Resistance can arise from any replacement and loss of these residuals. Short life cycle and relatively small genome makes bacteria more likely to mutate to gain resistance to any antimicrobials. Many genetic mutations are deleterious or neutral mutations, which either drastically reduce the bacterial fitness or confer no resistance at all. Those mutations confer to general resistance could largely increase the fitness of bacteria under drug pressure. Mutants modify the target on which the drug attaches are also high likely to confer resistance. For example, the rRNA mutation C2534U in presence of mutations of L3 and L4 results rather high resistance to linezolid in *Staphylococcus aureus* [66]. Moreover, mutation modifying membrane will also result resistance to certain drugs. Over-expression and under-expression of a group of transporter will generally lead to multi-drug resistance, these mutations either make the cell pumps drug out stop the drugs entering bacterial cells [67, 68]. Over-expression of drug target or target protection by other proteins will also lead to resistance [69, 70]. Notably, Mutation under stress of antimicrobials is depended on other conditions, such as the categories

of antimicrobials and their gradients of concentrations. Experiments show that antibiotics induce higher mutation rate than antimicrobial peptides [71]. While exposed in long term sub-lethal concentration of norfloxacin, the genome-wide mutation rate of E. coli significantly increase with concentration [72].

Horizontal gene transfer

Horizontal gene transfer (HGT) is another important factor that causes resistance to different drugs. Antimicrobial resistant genes are highly conserved in the phylogeny tree of bacteria [73]. Antimicrobials are ancient weapons used bay bacteria to conquer the enemies in the resource competition, therefore bacteria has already evolved counter strategy [74, 75]. These genes are not only heritable but also transferrable from one population to another of the same species, even from one species to another [76, 77]. These genes can be transferred on the different site of genome by transposon, moved on plasmids then transferred to other individuals or species. Resistance to the last resort polymyxin antimicrobials are largely caused by plasmids-carried resistant genes, for example the *mcr-1* [78].

Phenotypic resistance

Phenotypic resistance contributes to the drug resistance in a condition-dependent way. Many antimicrobials exert their functions relying on the fast growth of bacterial cells which constantly provide the candidate binding target for drugs. Bacterium is able to instantly shut down the metabolism and turn into a dominate cell at the presence of drug while restore the metabolism at the absence of the drug. This phenomenon is called bet-hedging [79]. This is the typical strategy adapted by bacteria when facing pressure of beat-lactam antimicrobials [80, 81]. The switch to the dominate state can be enhanced by the gene *hip7A* in *E. coli* [80]. This phenomenon is not quite clear in other class of antimicrobial. But a recent study showed that a toggle-switch network in bacterial could also contribute to the phenotypic resistance by slowing down the growth in the presence of a ribosomal targeting antibiotic [82].

The mutant-selection-widow theory of resistance evolution

Once the bacteria acquired resistance trough mutation, they will have higher fitness and be selected under antibiotic treatment, thus the resistance strain will soon take over the population. However, this dynamics is largely depended on the concentration and the corresponding effects of a given drug. A theoretical framework, which is termed as mutant selection window (MSW), incorporated with pharmacokinetics and pharmacodynamics has been used to determine the range of concentration (window) in which resistance can be selected [83-85]. Originally, the MSW is defined as the range of concentration which is higher than that can kill sensitive strains and lower than that can kill resistant strains. Latter, the lower bounder of MSW is extended to the intersection of pharmacodynamic curve of sensitive strain and resistant strain, as the experiment showed concentration below MIC of sensitive strain still select resistance [86]. This theoretical framework requires clear definition of the pharmacodynamics of both sensitive and resistant strains.

A modified Hill equation has been developed to characterize the pharmacodynamics of antimicrobials [87]. This model includes four parameters that could be easily determined experimentally in the lab. The parameters include maximal growth rate of bacterial without drug, MIC, maximal killing rate of the drug and a shape parameter. This method allows one to obtain the pharmacodynamics of any drug targeting any bacteria species. Due to the difference in parameters of the pharmacodynamics, a more clearly framework is defined to characterize the evolution of resistance [88]. Fitness cost of different mutants and concentrations antimicrobial interact to generate a complex picture of mutant selection. If mutant suffers fitness cost, it is less likely been selected in low concentration of antimicrobials. As drug concentration increase, the total bacterial population will decrease, but the population of resistant strain remains or increase at the same time, both increase the resistance-sensitive ratio [88].

MSW also can be determined using pharmacokinetics [83, 89]. Once a drug is

injected into the body, it is quickly absorbed and reaches the maximal concentration. Then the concentration slowly drops down. Like the pharmacodynamics, the MSW also between the concentration that kills sensitive strain and concentration kills resistant strain. The pharmacokinetics of an injected drug can be easily monitored through sampling on different time points. However, the pharmacokinetics of naturally occurred antimicrobials, such as antimicrobial peptides, have rarely been determined since it requires determining of the quantity in real time which is possibly limited by techniques. Studies in beetles quantified the transcriptions in real time. Quantification of the expression of AMP genes in real time when the host is challenged by pathogens showed the change of gene expressions resembles the typical pharmacokinetics of antibiotics in body. The level of expression is also associated with types of pathogens [90, 91]. However, the mutant selection window of AMPs is even more difficult to determine *in vivo [92]*.

The work in this thesis

Combination effect of antimicrobial peptides

In the first manuscript, the combination effects of AMPs from different organism are studied with a pharmacodynamic method built on the frame work of Loewe Additivity. The results showed there is broad synergism between two-AMPs and three-AMPs combinations. The synergism is more pronounced in 3-AMPs combinations as showed high percentage of synergistic combinations and higher degree of synergism. This reveals the synergistic combinations do not only exist between AMPs in the same organisms as previously explored but also exist between AMPs in different organisms. In addition, the combination effects of AMPs are also reflected in the parameters of the pharmacodynamics. For example, the three-AMPs combinations with more synergism generally have lower MICs and higher kappa values, an indicator of dose-response sensitivity, than those of two-drug combinations. When compared with those of antibiotics, AMPs have steeper dose-response curves.

Mechanistic model explaining combination effect of antimicrobial peptides

In the second manuscript, a mechanistic model was built to explain the synergism between different AMPs. As most of cationic AMPs function through attaching on the bacterial membrane, a mechanistic multi-hit model illustrates that AMPs randomly attaching and detaching membrane. When the critical amount of AMPs on the membrane is reached, bacterial membrane will lose its integrity which ultimately leads to cellular death. In particular, the model predicts a "zombie" stage in which bacterial cell membrane was occupied by critical number of AMPs. In this stage, cells are doomed to die but are still temporarily alive and not able to grow any more. Incorporated into the frameworks of Bilss independence and Loewe additivity, the multi-hit model explains the synergism between AMPs from a perspective of molecular interaction.

Predicting antimicrobial resistance

In the third manuscripts, a model was built to predict the evolution of antibiotic resistance. In the first manuscript, we found a significant different in a pharmacodynamic parameter, kappa, between antibiotics and antimicrobial peptides. These information was incorporated into a simple population model based on pharmacodynamics, and predicted that any antimicrobials with its phamacodynamic properties analogous to AMPs will less likely to select resistance. These properties are indicated by higher kappa value, higher killing rate and lower mutation rate in pharmacodynamics. The model is also consistent with the previous conceptual framework of mutant selection window.

The path leading to resistance under different treatment regime.

In the fourth manuscript, the previous model was extended to a model with multi-step mutations which allows us to investigate more details in the process of resistance evolution. In this model, each mutant was described with a set of pharmacodynamics parameters. With this model, several important questions were studied, which

includes how MIC will increase during the evolution, how fitness cost influences the emergence of resistance. In addition, pharmacokinetics and treatment strategy are also taken into consideration in this model. The results showed that these two factors do not influence the range of drug concentration that select resistance.

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Chapter 3

Combination effects of antimicrobial peptides

manuscript 1

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Combination Effects of Antimicrobial Peptides

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Antimicrobial peptides (AMPs) are ancient and conserved across the tree of life. Their efficacy over evolutionary time has been largely attributed to their mechanisms of killing. Yet, the understanding of their pharmacodynamics both *in vivo* and *in vitro* is very limited. This is, however, crucial for applications of AMPs as drugs and also informs the understanding of the action of AMPs in natural immune systems. Here, we selected six different AMPs from different organisms to test their individual and combined effects *in vitro*. We analyzed their pharmacodynamics based on the Hill function and evaluated the interaction of combinations of two and three AMPs. Interactions of AMPs in our study were mostly synergistic, and three-AMP combinations displayed stronger synergism than two-AMP combinations. This suggests synergism to be a common phenomenon in AMP interaction. Additionally, AMPs displayed a sharp increase in killing within a narrow dose range, contrasting with those of antibiotics. We suggest that our results could lead a way toward better evaluation of AMP application in practice and shed some light on the evolutionary consequences of antimicrobial peptide interactions within the immune system of organisms.

Combinations of drugs can result in three different forms of interactions: synergism, additivity, and antagonism (1–4); i.e., the effect of two drugs combined is stronger, equal, and weaker than that of the individual drug in the equivalent dose, respectively. Combination treatment is supposed to potentially eliminate resistant strains, delay the evolution of drug resistance, reduce the dosage of individual drugs, and hence, diminish side effects (3, 5, 6). A few recent studies, however, report that the success of combination therapy is context dependent, particularly when targeting both sensitive and resistant strains with a combination of drugs of unknown interaction (7–9). These results demonstrated that synergistic drug pairs can efficiently eradicate bacteria but exacerbate selection of resistance, while antagonistic drug pairs showed the reverse trends.

Various methods have been developed to address the efficacy of mostly two-way drug combinations (1, 2). One of the most commonly used approaches in both theoretical and applied research is Loewe additivity (2, 9–11). Here, the effect of two drugs in combination is determined by the sum of ratios of concentrations of drugs in combination divided by concentration of drugs used individually. Note that both the individual drug concentrations and the combined concentrations have the same effect on bacterial growth; we call these concentrations isoeffective concentrations. Theoretically, if the isoeffective concentrations of equivalent effect level achieved in a matrix of gradients of concentrations were connected by line, a concave line represents synergism, while a convex line represents antagonism (2, 12, 13). Recently, a mechanism-free approach was used successfully to predict the outcome of three antibiotics on the interaction between all three possible two-way combinations (14, 15), but the results do not particularly address the question about the nature of interaction (synergism, additivity, or antagonism). How these approaches can be used for a new class of antimicrobials, antimicrobial peptides (AMPs), is basically unknown. Studies on combinatorial effects of antimicrobial peptides, especially within a pharmacodynamics framework, are scarce (16, 17).

Antimicrobial peptides (AMPs), which form an important component of immune defenses in multicellular organisms (18, 19), have been proposed and are being used as new antibiotic drugs. Some AMPs are already commercially available and ready to be applied in clinical practice to replace or accompany conventional antibiotics (20). Additionally, they are supposed to be less likely to induce resistance and mutagenesis in the natural environment, although resistant strains can be obtained under intensive selection in the laboratory (21–23). When AMPs are used in medical applications, they necessarily interact with the patient's own AMPs. Some experimental studies have addressed the effect of individual pairs of AMPs within the context of innate immunity. Coexpressed AMPs on frog skin, PGLa and magainin-2, are synergistic when applied to both *Escherichia coli* and tumor cells (16). Moreover, AMPs from mammals (17) and insects (24, 25) were shown to synergize. Hence, understanding general principles of AMP interaction will also contribute to our understanding of interactions of AMPs as immune effectors.

Here, we take a pharmacodynamic approach to study the combination effects of AMPs and with combinations of two and three AMPs. Pharmacodynamics capture the functional relationship between drug dosage and bacterial growth or death. We use a modeling approach based on the Hill function (26–28). This model estimates four parameters: MIC, κ, ψ_{min}, and ψ_{max} (Fig. 1A). The minimal concentration at which antibiotic substances can inhibit growth of bacteria is MIC; κ depicts the steepness of the curve relating bacterial growth to drug concentration (Fig. 1B); ψ_{min} and ψ_{max} represent the minimum and maximum growth rates of bacteria, respectively. We studied the pairwise and three-

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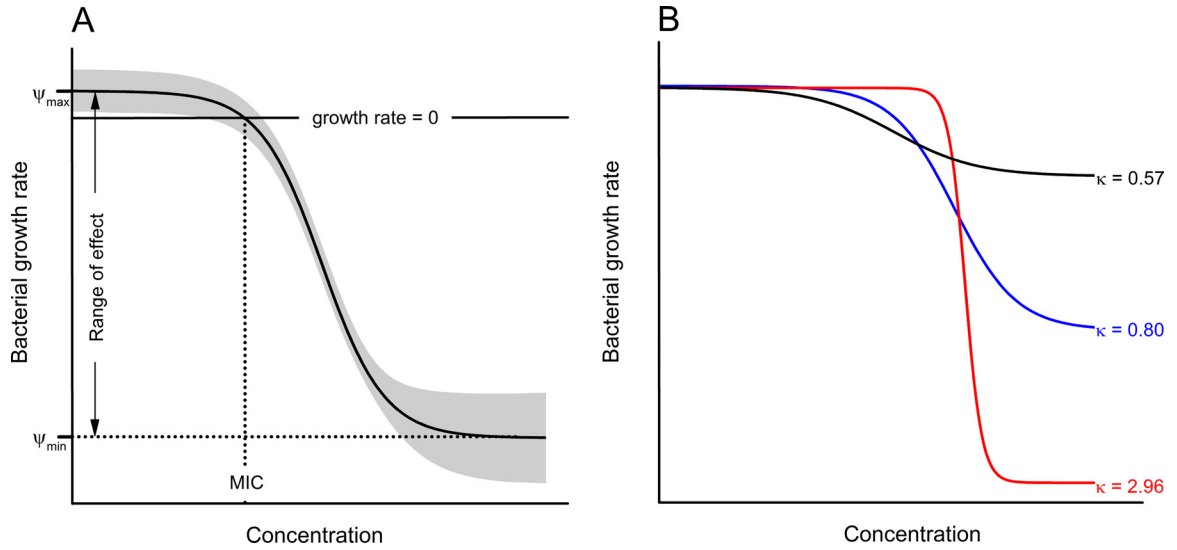

FIG 1 Schematic illustration of four parameters, MIC, ψ_{max}, ψ_{min}, and κ, predicted by the Hill function. The MIC is estimated by the lowest concentration that inhibits the growth of the whole treated bacterium population. ψ_{max} and ψ_{min} represent the maximal and minimal growth rates of bacteria under gradients of drug treatment, respectively. κ predicts the shape and slope of the pharmacodynamic curve; the higher the κ value, the steeper the pharmacodynamic curve.

way interactions of AMPs using this pharmacodynamic approach embedded in a Loewe additivity framework. According to the work of Loewe, this was achieved by using either one-half (pairwise) or one-third (three-way) of the concentration of each individual drug (see Materials and Methods) (10). We examined the nature of the interactions, synergism or antagonism, and the concentration dependency of the killing. Using a derivative of the human AMP LL-37 enabled us to study interactions between AMPs expressed by patients' innate immunity and AMPs employed as drugs.

MATERIALS AND METHODS

Bacteria and media. *Escherichia coli* MG1655 was grown in Luria-Bertani (LB) broth at 37°C with aeration at 220 rpm in 50-ml tubes. Two hundred microliters of overnight culture was resuspended into 15 ml fresh LB broth, cultured under the same conditions for an additional 2 h, and then used for subsequent assays. Mueller-Hinton (MH) broth was used for the assay of MICs and time-killing curves.

AMPs and antibiotics. We used six different AMPs from different classes of organisms that are commercially available (AnaSpec): cecropin A (Cec) (insect), LL 19-27 (LL) (mammal), melittin (Mel) (insect), pexiganan (Pex) (synthesized AMP, an analog of magainin II; a kind gift of Michael Zasloff), indolicidin (Ind) (mammal), and apidaecin (Api) (insect) (see Table S1 in the supplemental material). These AMPs are effective on either Gram-positive or Gram-negative bacteria (see reviews in references 29 and 30). However, some of these AMPs, e.g., melittin and pexiganan, have anticancer effects (16, 31, 32), which means that they are potentially toxic to human cells, such as erythrocytes. Recently, some low-cell-toxic and serum-stable AMPs have also been under development (33, 34). All these AMPs were dissolved in distilled water with an initial concentration of 1 mg/ml, 5 mg/ml, 10 mg/ml, 1 mg/ml, 1 mg/ml, and 25 mg/ml, respectively, as stock solutions. All antibiotics—ampicillin, ciprofloxacin, gentamicin, kanamycin, neomycin, rifabutin, spectinomycin, and tetracycline—were also dissolved in distilled water and made into 10-mg/ml stock solutions. All the solutions of AMPs and antibiotics were stored at −20°C in a dark environment.

MIC determination. According to a standard protocol (35), stock solutions of AMPs were diluted in MH broth and then diluted in 96-well plates with a 2-fold gradient, that is, from 0.25 μg/ml to 128 μg/ml. All the gradients of antibiotics were from 0.02 μg/ml to 50 μg/ml, except that the gradient of ciprofloxacin was from 0.002 μg/ml to 1 μg/ml. Approximately 5×10^5 log-phase bacteria were added to each well. A positive control containing MH broth and bacteria and a negative control containing only MH broth were included in each plate, and plates were incubated at 37°C overnight.

Measuring killing curves. To estimate killing curves of each AMP and all possible combinations of AMPs, 100× MICs of AMPs were combined as a volume ratio of 1:1 and 1:1:1 in two-AMP combinations and three-AMP combinations; hence, the concentrations of individual drugs are halved or reduced by two-thirds. Thus, Loewe additivity would result in a MIC of any one combination equal to the MIC of the individual drugs (see equations 5 and 6). Thus, 21 two-AMP combinations and 20 three-AMP combinations were generated. An AMP(s) was diluted, starting with 100× MIC, in a 96-well plate to form a 2-fold gradient of concentrations, and 2×10^6 log-phase bacteria were added to a total volume of 100 μl. The plates were incubated at 37°C. Killing was assessed within 1 h, as killing by AMPs is very fast (36, 37). Ten microliters of a mixture of AMPs and bacteria was taken out every 20 min and then immediately diluted in saline solution and plated on the solid agar plates. These solid agar plates were transferred into a 37°C incubator and cultured overnight for CFU determination. The limit of detection in our system is 100 CFUs.

Modeling killing curves. To model the killing curve, the relationship between the concentration of AMP(s) and the killing and/or growth rate of exposed bacteria, we used a Hill function (26):

$$\mu(a) = E_{max} \frac{(a/EC_{50})^{\kappa}}{1 + (a/EC_{50})^{\kappa}} \quad (1)$$

Here, $\mu(a)$ is the killing rate at a given concentration of AMP(s); a is a given concentration; E_{max} is the maximal killing rate of the given AMP(s). κ is the Hill coefficient. We then defined growth rate $\psi(a)$ as follows:

$$\psi(a) = \psi_{max} - \mu(a) \quad (2)$$

Here, ψ_{max} is the maximal growth rate of bacteria without AMP(s). The maximum effect of AMP(s) is defined by

$$E_{max} = \psi_{max} - \psi_{min} \quad (3)$$

Thus, the effect of AMP(s) in a given concentration, $\mu(a)$, can be rewritten as

$$\mu(a) = \frac{(\psi_{max} - \psi_{min})(a/zMIC)^{\kappa}}{(a/zMIC)^{\kappa} - \psi_{min}/\psi_{max}} \quad (4)$$

zMIC is the estimated MIC. Growth rate and killing rate of bacteria are estimated from the time-kill curves as the change of CFU over time by using generalized linear regression. The data for CFU were all log transformed. The start point of linear regression was the first measurement. We then fitted the growth rate and killing rate with equation 4 based on the Markov chain Monte Carlo (MCMC) method using *rjags* (38) in R (39) and generated the pharmacodynamic curves.

Determining the effect of combination. Based on the Hill function and isobologram analysis, we obtained the isoeffective concentrations of single drugs and of combinations which achieved a given percentage of their maximal effects or fraction level. The Loewe additivity model defines the additive effect of isoeffective combinations of drugs that result in a certain effect. For example, the combination of drug A and drug B in the isoeffective concentrations, which are C_{isoA} and C_{isoB}, can achieve a level of effect which can also be achieved individually by drug A or drug B with a concentration of C_A or C_B, respectively. Mathematically, the combination effect of drug A and drug B is defined as follows:

$$CI = \frac{C_{isoA}}{C_A} + \frac{C_{isoB}}{C_B} \quad (5)$$

For three-drug combinations

$$CI = \frac{C_{isoA}}{C_A} + \frac{C_{isoB}}{C_B} + \frac{C_{isoC}}{C_C} \quad (6)$$

Additive combination effects were then defined by a combination index (CI) equal to 1, antagonism was defined as a CI greater than 1, and synergism was defined as a CI lower than 1.

RESULTS

Killing and pharmacodynamic curves. We tested the *in vitro* effects of single AMPs, two-AMP combinations, and three-AMP combinations on *E. coli*. All killing curves were obtained by counting viable CFU after treatment (see Fig. S1 in the supplemental material). In most cases, the number of surviving bacteria drastically decreased as a function of time at higher concentrations while slightly increasing at lower concentrations. Killing occurred very quickly at higher concentrations in our system (i.e., bacterial densities below the limit of detection).

The four pharmacodynamic parameters, MIC, κ, ψ_{max}, and ψ_{min}, were estimated by the MCMC method using the generalized linear regression fitted killing rate as a function of concentrations of AMP(s) (Fig. 2 and 3; also see Table S2 in the supplemental material). Notably, all the single AMPs and two- and three-AMP combinations had almost the same ψ_{min} (analysis of variance [ANOVA], ψ_{min}, $F_{1,39} = 1.855$, $P = 0.181$) (Fig. 3; see also Table S2 in the supplemental material). ψ_{max} values were also identical in different treatments as the growth rate of bacteria in low concentrations of AMP(s) was presumably close to the natural growth rate. Two pharmacodynamic parameters, MIC and κ, varied among different treatments, with three-AMP combinations having the lowest MICs and the highest κ values (ANOVA, MIC, $F_{1,39} = 6.647$, $P = 0.0138$; κ, $F_{1,39} = 7.447$, $P = 0.00935$) (Fig. 3; see also Table S2 in the supplemental material). All the treatments showed nearly the same pharmacodynamic trend: a sharp decrease of net bacterial growth with an increasing concentration of AMP(s) as depicted by κ.

Most AMP combinations are synergistic, but synergy is stronger in three-AMP combinations. To determine the interaction of AMPs, we used Loewe additivity (see equations 5 and 6). The combination index was calculated for concentrations between 5% and 95% of the maximal effect (equation 3). For two-AMP combinations, we found that most of the two-AMP combinations (67%) were completely synergistic (combination indexes were lower than 1) within the effect range, except for the combination of apidaecin and LL 19-27 (ApiLL), which was antagonistic across the whole range; the combinations PexApi and IndApi were antagonistic in low-concentration combinations but synergistic in high-concentration combinations (Fig. 4; also see Fig. S2 in the supplemental material). However, the combinations CecApi and MelApi had a reverse trend, as they were synergistic in lower-concentration combinations and antagonistic in higher-concentration combinations (Fig. 4; see also Fig. S2 in the supplemental material). Eighty-five percent of three-AMP combinations were completely synergistic within the effect range while the combination LLPexApi was completely antagonistic; LLIndApi showed synergistic effects in lower-concentration combinations and antagonistic effects in higher-concentration combinations, but MelIndApi had the reverse trend (Fig. 4; see also Fig. S2 in the supplemental material).

Another interesting finding is that three-AMP combinations generally have stronger effects than do two-AMP combinations at a given fraction level within the effect range. The average combination indexes of three-AMP combinations were 30% lower than those of two-AMP combinations (Student's *t* test, $t = 8.2016$, df = 606.57, $P = 1.42e{-}15$) (Fig. 5). We observed no differences between effects of different fractions for the three-way interactions (ANOVA, $F_{1,661} = 1.332$, $P = 0.2488$) (Fig. 5).

Relationship between κ values and selection. We compared the κ values of different combinations of AMPs and between AMPs and antibiotics. κ values are higher the more AMPs that are combined. We also found that κ values of AMPs are significantly higher (ANOVA, $F_{1,77} = 150.5$, $P < 0.001$) (Fig. 6) than those of antibiotics, for data obtained in both our laboratory and other laboratories (ANOVA, $F_{1,36} = 1.591$, $P = 0.215$) (Fig. 6).

DISCUSSION

Pharmacodynamic approaches have been frequently applied to conventional antibiotics (2, 11, 26, 40). A good understanding of how antimicrobial peptides eradicate bacteria in complex systems not only relies on the molecular mechanisms of killing but, importantly, necessitates investigation of pharmacodynamics *in vitro*, as done here and *in vivo* (C. Zanchi, P. R. Johnston, and J. Rolff, unpublished data). Generally, the maximal killing values were almost identical in treatments with all the AMPs and their two- and three-way combinations, which means that high concentrations of an AMP(s) may eradicate bacteria with similar efficiencies. Due to fast killing of AMPs and the limit of detection in our system, the real maximal killing rate might be masked at higher concentrations, e.g., concentrations in which the limit of detection is reached within 20 min. However, the MIC and κ significantly varied among single AMPs and two- and three-AMP combinations. As numbers of AMPs increased in combination, the MIC of that combination decreased, with the lowest value seen in three-AMP combinations, and κ was much higher in three-AMP combinations (Fig. 3). More AMPs combined with lower MICs demonstrate that the absolute quantity of AMP needs to be decreased to achieve the same killing. Higher κ values in combined AMPs result in a drastic decrease in bacterial killing rate within a narrow range of concentrations of the AMP(s). The combination of AMPs might improve the efficiency of bacterial killing.

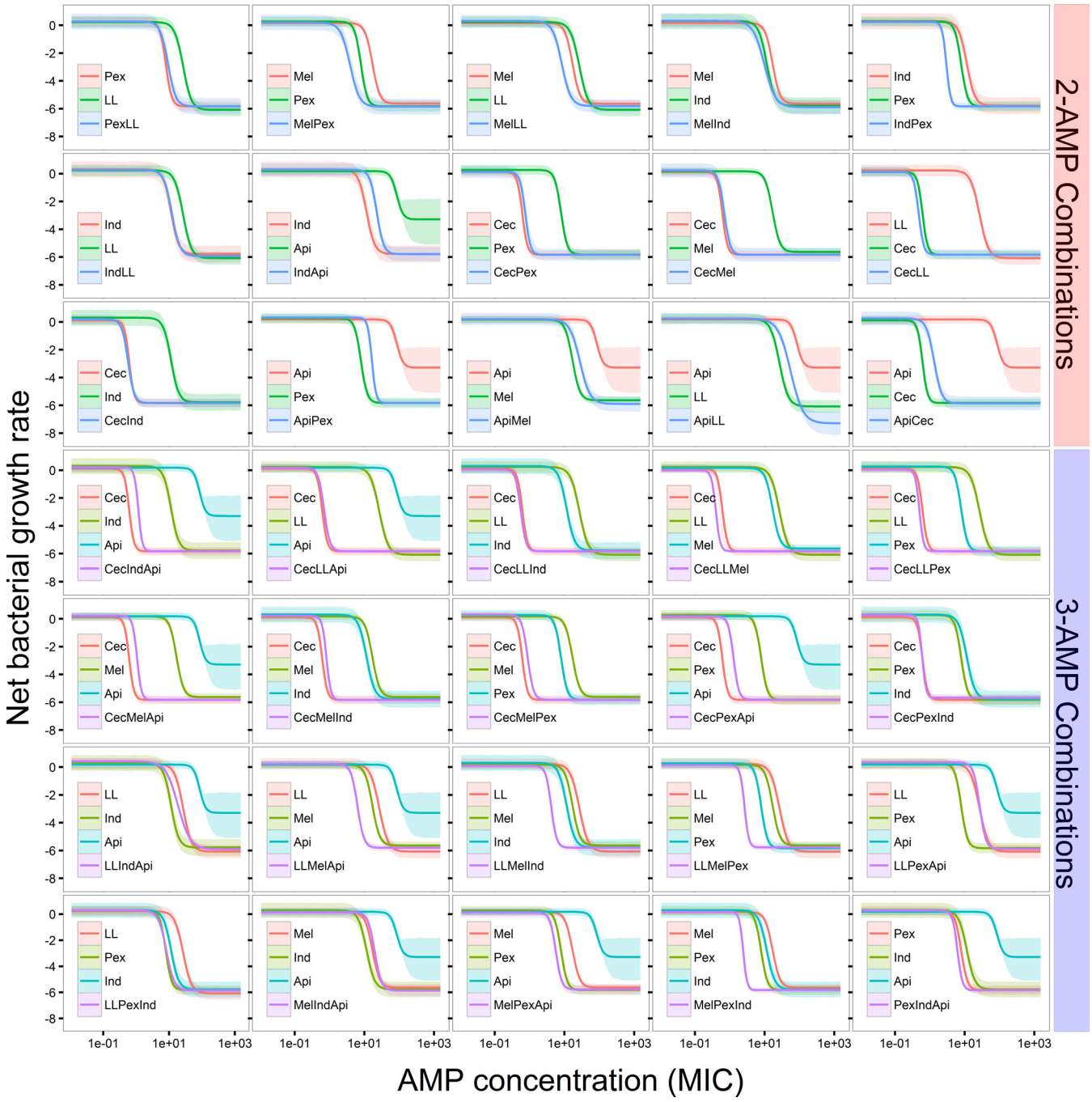

FIG 2 Pharmacodynamic curves of AMPs. The pharmacodynamic curves of AMPs were obtained by fitting killing curves to the Hill function (see equation 4). Combinations of two or three AMPs were differentiated. The curves illustrate the effects (reflected as net bacterial growth rate) of increasing the concentrations of AMP(s). The ribbon represents the 95% confidence interval.

Taken together, the decreasing MIC and increasing κ values in combinations with increasing numbers of AMPs suggest that synergism is common in AMP combinations (17, 24, 41).

We observed broad synergistic effects in almost all the two- and three-way combinations. Although some AMPs, like apidaecin, had a relatively weak effect with a high MIC, the killing could still be enhanced by adding one or more AMPs with stronger individual effects. Synergism, albeit not within a pharmacodynamics framework, has been reported for 2-way combinations of antibiotics (8, 42), AMPs (16, 17, 24), antimicrobial peptoids (43), antibiotics and AMPs (44, 45), and AMPs and antimicrobial peptoids (43). The AMPs, originating from different species in our experiment, showed robust synergism, which suggests a general effect.

The molecular mechanisms of interaction, especially antagonisms, of AMPs are largely unknown. As most AMPs target the

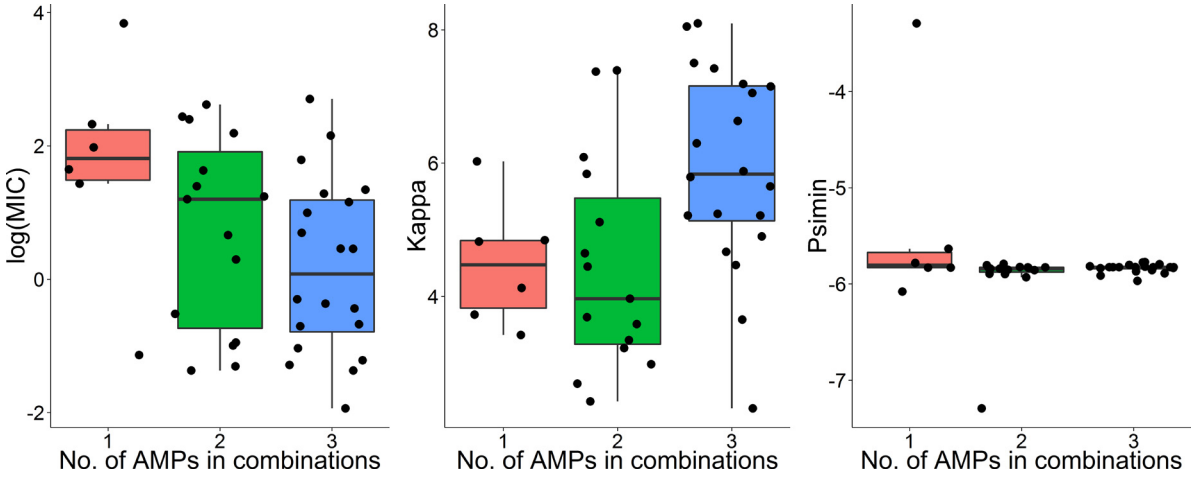

FIG 3 Variations of MIC, κ, and ψ_{min} in the Hill function predicted by the MCMC method. Results showed that these parameters vary among combinations with different numbers of AMPs. MICs declined with increasing numbers of AMPs in combination (ANOVA, $F_{1,39} = 6.647$, $P = 0.0138$); combinations with three AMPs had the highest κ values (ANOVA, $F_{1,39} = 7.447$, $P = 0.00935$). ψ_{min} (Psimin) did not show significant differences among single AMPs and two- and three-AMP combinations (ANOVA, $F_{1,39} = 1.855$, $P = 0.181$).

membrane of pathogens, their interactions are unlikely to directly disrupt the metabolic network in the cell like certain antibiotics. A recent study suggested that synergism was caused by the conjugation of coapplied AMPs, which form a supermolecule and better-stabilized pores (41). This is also confirmed by chemically conjoined synthesized peptides (46). Furthermore, pore-forming peptides can also assist other coapplied transmembrane AMPs to quickly invade bacterial cells and substantially interrupt the metabolism (47).

In our pharmacodynamic model, the important parameter κ depicts the steepness of the pharmacodynamic curve and is a measure of the sensitivity of the response of the bacteria to changes in the concentrations of the antimicrobial substances. A steeper pharmacodynamic curve with higher κ values illustrates that bacteria are very sensitive to the change of concentrations of AMPs and antibiotics, which means that the given antibiotic substance (e.g., combinations of AMPs) has a narrower range of concentrations exerting selection on bacteria.

Additionally, κ value could be an important indicator of resistance selection of given antibiotic agents. Traditionally, the presence of antimicrobial substances above the MIC is thought to favor resistant strains. The mutant selection window (MSW) is defined as the difference in the MICs of a resistant and a susceptible strain (48, 49). Thus, the MSW can be specifically defined as the range between the concentration killing all the sensitive strains and the concentration killing all the resistant strains. Additionally, MSW also can be defined as a range of concentrations which can *de novo* select mutant strains from a completely sensitive population (50–52). Higher κ values in combinations of AMPs denote a steeper pharmacodynamic curve, which means that the range of concentrations selecting resistance—the MSW—can be narrowed. Especially, the sub-MIC part of the MSW is predicted to be very small for high κ values. A previous theoretical study also demonstrated that the synergistic contribution of the immune system can potentially narrow the mutant selection window of antibiotics (53). We observed a synergistic interaction in combinations of AMPs that mirrors, in the case of LL 19-27, interactions between the immune system and drugs. Higher κ values of AMPs than of antibiotics might partially explain the fact that bacteria are unlikely to develop resistance to AMPs in nature, although resistant strains can emerge under intensive selection in the laboratory (21, 54).

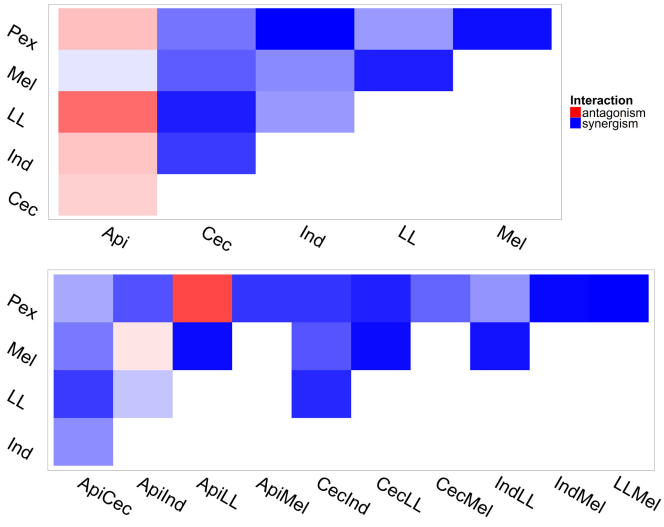

FIG 4 Combination index of AMPs applied at concentrations which can achieve 50% of their maximal effect (E_{50}). At E_{50}, all the combinations with Api (except the combination of Api and Mel) showed antagonistic effects in two-AMP combinations, but only two combinations, ApiIndMel and ApiLLPex, showed antagonistic effects in three-AMP combinations. The gradient of colors represents different levels of each interaction.

Conclusion. Our study suggests that the synergistic effect between AMPs may be a common phenomenon, as we observed strong synergistic interactions in two-AMP and three-AMP combinations. Interestingly, these three-AMP combinations are even more synergistic than two-AMP combinations. If synergistic interactions of AMPs are ubiquitous, than two practical implications arise: (i) AMPs that strongly synergize with host AMPs should be utilized and (ii) combinations provide the opportunity to reduce side effects, as they lead to an overall reduction in dos-

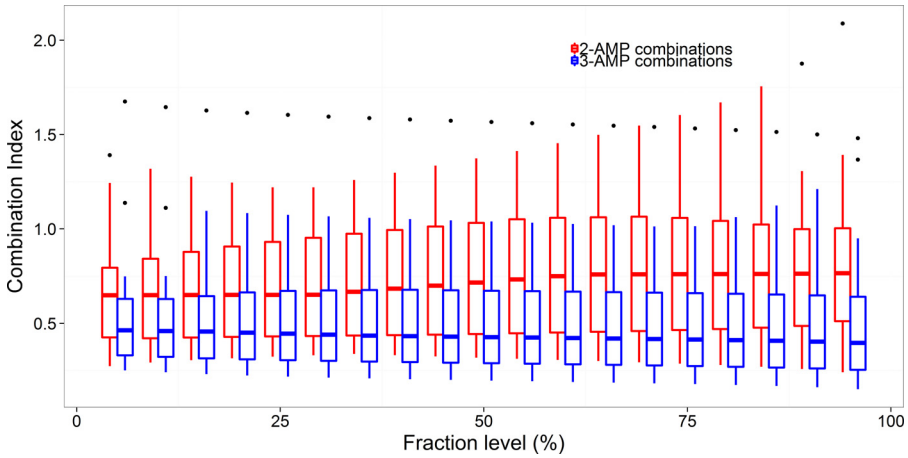

FIG 5 Combination index of fraction level within the effect range. Three-AMP combinations are more significantly synergistic than two-AMP combinations (Student's *t* test, $t = 8.2016$, df $= 606.57$, $P = 1.42e{-}15$). The combination index did not vary within different effect ranges in both two-AMP and three-AMP combinations (ANOVA, $F_{1,661} = 1.332$, $P = 0.2488$). Black dots denote outliers.

age. In the context of innate immunity, selection should favor organisms producing AMP cocktails. This can be considered a cost-efficient way of reducing bacterial loads in a host (19, 55).

Long-lasting coexpression of combinations of AMPs has been recorded in *Xenopus laevis* (56) and *Tenebrio molitor* (19), where it is correlated with metabolic suppression. Thus, evolving a more efficient killing system based on a relatively energy-constrained system, which expresses only a limited number of AMPs, is necessary and practical. A function of synergism among AMPs is one of the possible ways to mitigate the costs.

Our results have some implications for the applied use of AMPs as drugs. The production of AMPs is currently expensive (20). The broad synergism observed in our experiment means that combined applications of AMPs could also reduce the consumption of total AMPs just as in the immune system, which could eventually save costs of treatment and reduce toxicity. As humans express AMPs such as LL-37 in their innate immune system, synergisms between these AMPs and AMPs applied as drugs should be taken into account. In our study, the human AMP derivative LL 17-29 synergized with almost all combinations of AMPs. Though resistance to single AMPs evolves readily *in vitro*, it is might be less likely under combinations (54). It is possible that in some situations combinations delay the development of resistance in medical practice, as pathogens could pay a higher cost to evolve resistance to multidrug treatment (57–60).

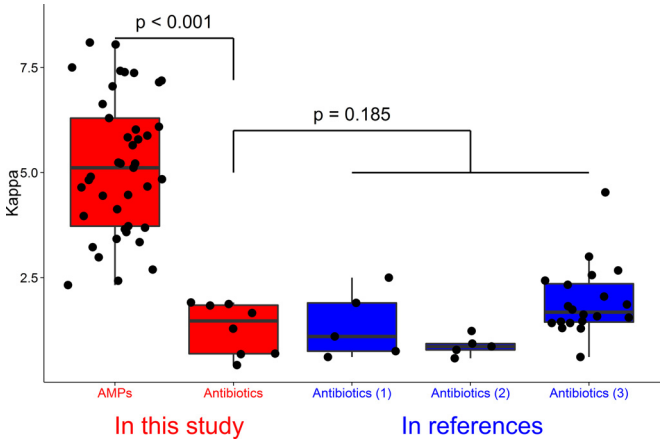

FIG 6 Comparison of κ values of AMPs and antibiotics in our experiment and other, similar experiments. With similar experimental methods, conditions of measurements, and mathematical models, κ values of antibiotics in our experiment are similar to those of antibiotics in other experiments (ANOVA, $F_{1,36} = 1.591$, $P = 0.215$). However, κ values of AMPs are significantly higher than those of antibiotics both in our experiment and in other experiments (ANOVA, $F_{1,77} = 150.5$, $P < 0.001$). Data in boxes "Antibiotics (1)," "Antibiotics (2)," and "Antibiotics (3)" are from references 26, 40, and 11, respectively.

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Appendix

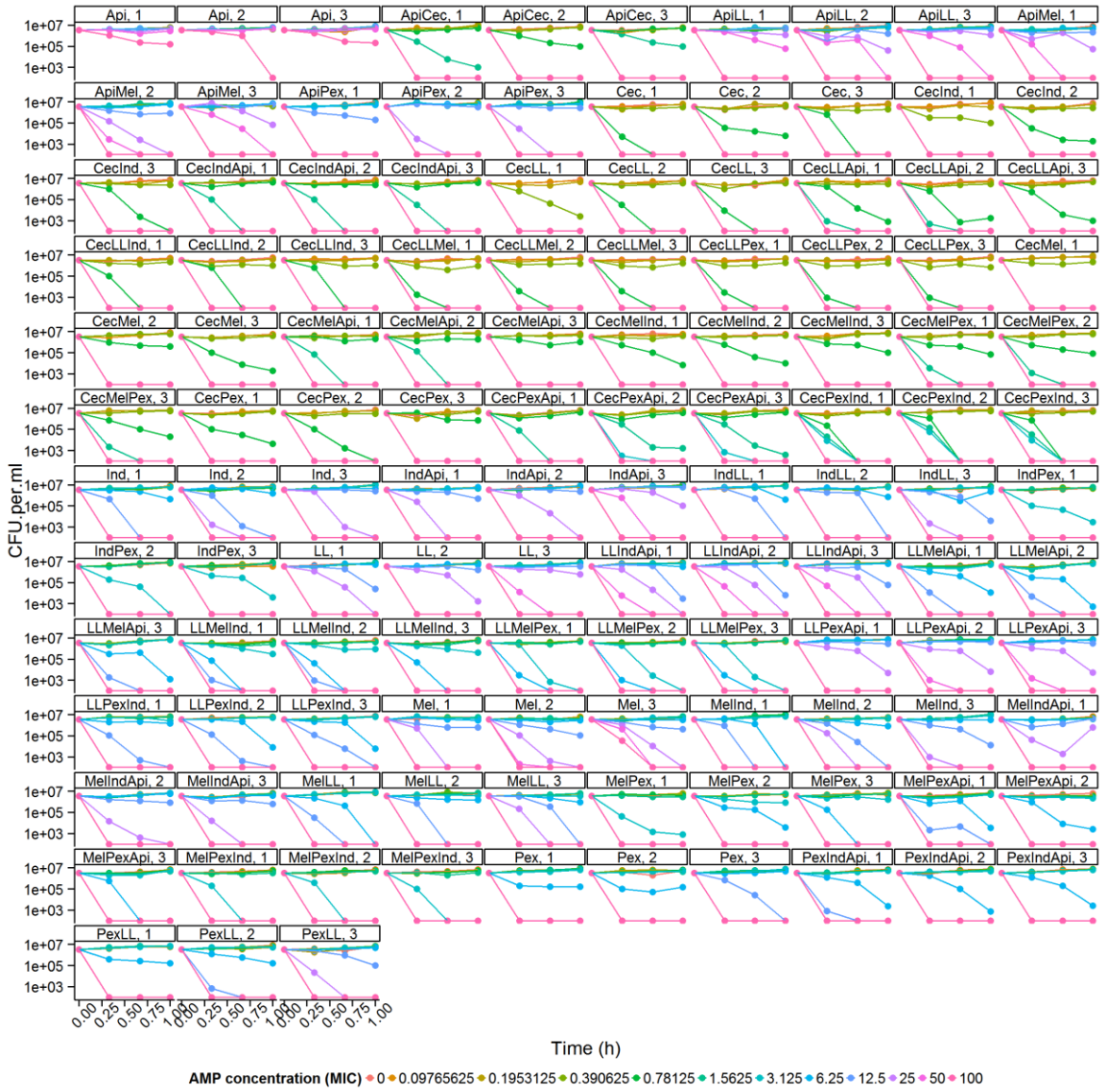

Figure S1

The killing curve of *E.coli* under treatment of different AMPs and their combinations in different concentrations. The AMPs and their combinations are marked on each panel. Numbers behind represent different biological replicates.

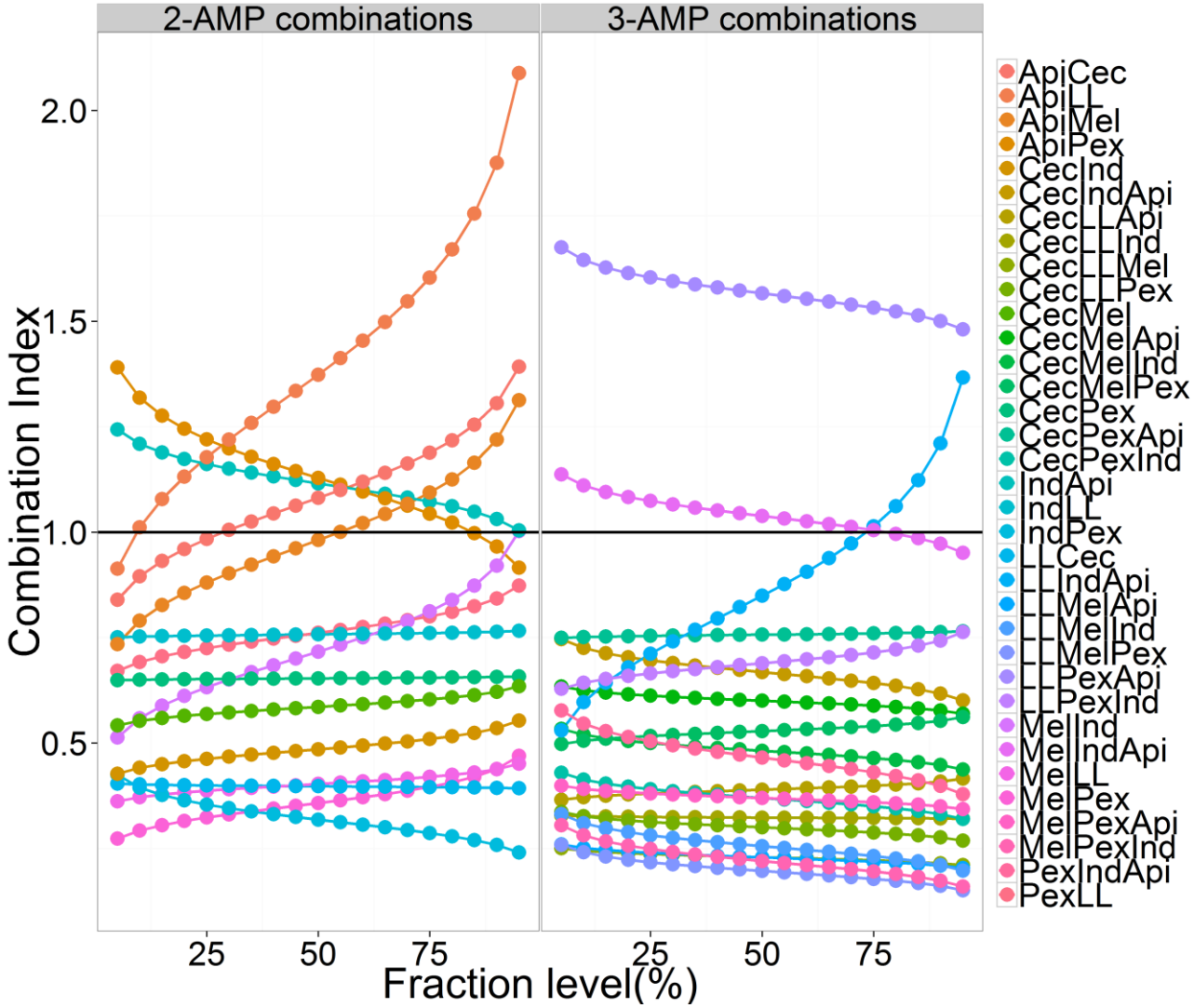

Figure S2

Combination Index of different fraction level within the range of effect (see Figure 1). Values above, below and on one represent synergism, antagonism and additivity in a given combination, respectively.

Table S1.

The details of AMPs used in current study.

Name	Abbreviations	Sequence	Molecular Weight	Source	MIC (μg/ml)
Cecropin A	Cec	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQI AK-NH_2	4004.8	AnaSpec	1
LL 17-29	LL	FKRIVQRIKDFLR	1719.1	AnaSpec	16
Melttin	Mel	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	2846.5	AnaSpec	4
Pexiganan	Pex	GIGKFLKKAKKFGKAFVKILKK-NH_2	2477.2	Michael Zasloff	2
Indolicidin	Ind	ILPWKWPWWPWRR-NH_2	1906.3	AnaSpec	4
Apidaecin IB	Api	GNNRPVYIPQPRPPHPRL	2108.4	AnaSpec	4

Table S2. The four parameters zMIC, κ, ψ_{max} and ψ_{min} which are extracted from the fitting to the Hill function. Numbers in brackets represent lower and upper of 95% confidence intervals.

AMP(s)	zMIC[a] (lower,upper)	κ (lower,upper)	ψ_{max} (lower,upper)	ψ_{min} (lower,upper)
Api	46.40(39.1,52.94)	4.82(3.00,7.90)	0.18(0.13,0.23)	-3.29(-5.04,-1.91)
ApiCec	0.59(0.49,0.70)	3.97(3.29,4.80)	0.25(0.10,0.38)	-5.86(-5.97,-5.74)
ApiLL	13.73(11.63,16)	2.42(2.06,2.82)	0.25(0.18,0.31)	-7.29(-8.05,-6.55)
ApiMel	8.94(7.19,10.59)	2.98(2.47,3.53)	0.20(0.11,0.29)	-5.90(-6.23,-5.60)
ApiPex	11.00(10.58,11.44)	7.37(6.39,8.46)	0.32(0.26,0.36)	-5.82(-5.92,-5.73)
Cec	0.32(0.28,0.37)	6.02(5.17,6.84)	0.12(0.03,0.22)	-5.83(-5.89,-5.77)
CecInd	0.27(0.24,0.31)	4.65(4.19,5.15)	0.19(0.09,0.30)	-5.83(-5.90,-5.77)
CecLL	0.25(0.20,0.30)	6.09(5.07,7.27)	0.13(0.01,0.24)	-5.83(-5.91,-5.75)
CecMel	0.37(0.27,0.46)	5.12(3.69,7.06)	0.23(0.03,0.38)	-5.84(-5.95,-5.73)
CecPex	0.39(0.17,0.50)	5.84(4.06,7.79)	0.13(0.00,0.25)	-5.84(-5.94,-5.75)
Ind	5.20(4.14,6.28)	3.73(2.85,4.79)	0.30(0.17,0.42)	-5.78(-6.01,-5.56)
IndApi	11.44(9.56,13.34)	4.45(3.47,5.60)	0.28(0.18,0.37)	-5.79(-6.00,-5.58)
IndLL	5.11(4.68,5.56)	3.58(3.24,3.94)	0.27(0.22,0.32)	-5.93(-6.03,-5.84)
IndPex	1.94(1.67,2.12)	7.39(5.39,8.93)	0.24(0.18,0.30)	-5.83(-5.89,-5.77)
LL	10.23(8.70,11.82)	3.42(2.84,4.05)	0.23(0.14,0.31)	-6.08(-6.32,-5.86)
Mel	7.22(6.57,7.85)	4.13(3.79,4.51)	0.17(0.12,0.22)	-5.64(-5.74,-5.52)
MelInd	3.32(2.78,3.86)	2.69(2.30,3.05)	0.32(0.22,0.43)	-5.90(-6.08,-5.71)
MelLL	3.46(3.04,3.92)	3.34(2.91,3.81)	0.31(0.21,0.40)	-5.81(-5.96,-5.65)
MelPex	1.34(1.09,1.73)	3.23(2.67,3.75)	0.20(0.09,0.35)	-5.85(-6.01,-5.69)
Pex	4.19(3.86,4.54)	4.84(4.23,5.51)	0.27(0.20,0.35)	-5.83(-5.94,-5.71)
PexLL	4.03(3.38,4.68)	3.69(3.11,4.36)	0.27(0.14,0.38)	-5.83(-6.02,-5.64)
CecIndApi	0.74(0.70,0.78)	7.42(6.74,8.09)	0.21(0.15,0.26)	-5.82(-5.87,-5.78)
CecLLApi	0.35(0.32,0.38)	5.24(4.55,5.79)	0.17(0.10,0.23)	-5.82(-5.87,-5.78)
CecLLInd	0.25(0.06,0.28)	5.88(5.55,6.40)	0.06(0.00,0.11)	-5.83(-5.87,-5.79)
CecLLMel	0.14(0.07,0.30)	7.05(6.44,7.71)	0.00(0.00,0.10)	-5.83(-5.87,-5.78)
CecLLPex	0.28(0.12,0.31)	7.50(6.75,8.27)	0.07(0.00,0.12)	-5.83(-5.87,-5.79)
CecMelApi	0.65(0.61,0.68)	6.63(6.06,7.20)	0.22(0.16,0.28)	-5.83(-5.87,-5.78)
CecMelInd	0.51(0.45,0.57)	7.15(5.19,8.60)	0.20(0.14,0.27)	-5.84(-5.89,-5.79)
CecMelPex	0.49(0.46,0.52)	5.21(4.65,5.79)	0.30(0.24,0.36)	-5.83(-5.87,-5.78)
CecPexApi	0.69(0.63,0.77)	5.79(5.06,6.67)	0.16(0.10,0.23)	-5.82(-5.87,-5.76)
CecPexInd	0.27(0.25,0.29)	7.97(6.80,8.89)	0.32(0.20,0.43)	-5.97(-6.07,-5.88)
LLIndApi	5.99(5.59,6.44)	2.32(2.16,2.49)	0.42(0.38,0.47)	-5.91(-6.05,-5.77)
LLMelApi	3.18(2.94,3.50)	4.67(4.08,5.42)	0.24(0.19,0.29)	-5.80(-5.87,-5.73)
LLMelInd	2.00(1.83,2.17)	5.65(5.22,6.03)	0.09(0.06,0.14)	-5.79(-5.85,-5.74)
LLMelPex	1.58(1.42,1.80)	7.19(6.13,8.99)	0.15(0.10,0.20)	-5.77(-5.82,-5.72)
LLPexApi	14.94(13.89,16.15)	4.90(4.22,5.68)	0.34(0.30,0.38)	-5.89(-5.99,-5.78)
LLPexInd	3.61(3.35,3.87)	3.65(3.36,3.96)	0.29(0.23,0.34)	-5.86(-5.95,-5.77)
MelIndApi	8.62(7.41,9.98)	4.47(3.89,5.15)	0.15(0.06,0.22)	-5.87(-6.03,-5.70)
MelPexApi	2.71(2.18,3.18)	5.21(4.02,6.60)	0.13(0.05,0.21)	-5.78(-5.89,-5.68)
MelPexInd	1.58(1.46,1.69)	8.05(7.01,8.92)	0.19(0.14,0.24)	-5.83(-5.87,-5.78)
PexIndApi	3.82(3.42,4.15)	6.30(4.97,7.39)	0.28(0.24,0.32)	-5.83(-5.89,-5.77)

a, The unit of zMIC is not μg/ml in this table, but the times of original MIC of each AMP(s). We set the initial concentration of AMP(s) to 100 times of original MIC when we were doing the experiment.

Chapter 4

Antimicrobial combinations: Bliss independence and Loewe additivity derived from mechanistic multi-hit models

manuscript 2

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Chapter 5

Predicting drug resistance evolution: insights from antimicrobial peptides and antibiotics

manuscript 3

Submitted

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Abstract

Antibiotic resistance constitutes one of the most pressing public health concerns. Antimicrobial peptides of multicellular organisms are considered part of a solution to this problem, and AMPs produced by bacteria such as colistin are last resort drugs. Importantly, antimicrobial peptides differ from many antibiotics in their pharmacodynamic characteristics. Here we implement these differences within a theoretical framework to predict the evolution of resistance against antimicrobial peptides and compare it to antibiotic resistance. Our analysis of resistance evolution finds that pharmacodynamic differences all combine to produce a much lower probability that resistance will evolve against antimicrobial peptides. The finding can be generalized to all drugs with pharmacodynamics similar to AMPs. Pharmacodynamic concepts are familiar to most practitioners of medical microbiology, and data can be easily obtained for any drug or drug combination. Our theoretical and conceptual framework is therefore widely applicable and can help avoid resistance evolution if implemented in antibiotic stewardship schemes or the rational choice of new drug candidates.

Introduction

Antibiotic resistance is prevalent (1) and evolves quickly. It takes only a few years from the introduction of a new antibiotic to the clinic until resistant strains emerge(2). Prudent use and the introduction and development of novel antibiotics are currently considered to be the most effective ways to tackle resistance evolution(3). The prediction of when and how antibiotic resistance evolves and spreads is notoriously difficult, but would be extremely informative for antibiotic stewardship and the development of new drugs.

Amongst the new drugs under development are antimicrobial peptides (AMPs)(4). AMPs are peptides that have spatially explicit hydrophobic and cationic residues(5). Note that for example polymixins (including colistin) are usually subsumed under antibiotics, also fall into this category as they are AMPs of bacterial origin(6),(7). One of the alleged advantages of AMPs is that bacterial resistance would evolve much more slowly than against antibiotics(5, 8), a highly desirable property(9).

We have recently demonstrated that AMPs from multicellular organisms affect growing bacterial populations differently from antibiotics, i.e. they differ in their pharmacodynamics (or dose-response relationship)(10). A similar observation has been reported for colisitin a last resort drug to treat Pseudomonas infections(11) .Pharmacodynamic characteristics of susceptible and resistant bacterial strains can be used to illustrate the selection of resistance under treatment with range of dosage(12).Such application is based on the concept of the ‘mutant selection window’ (MSW, Fig 1)(13, 14). The MSW has been successfully applied in animal models, demonstrating its value to understand resistance emergence *in vivo*(15). The width of the mutant selection window is partly determined by the steepness of the pharmacodynamic curve (see Fig 1). Importantly the concentration range between no killing and maximal killing is much narrower for AMPs than antibiotics, resulting in a much steeper curve. The maximum killing rate of AMPs is much higher than of

antibiotics, as reflected in quicker killing time(16). Another difference relevant to the evolution of resistance is the finding that many antibiotics increase mutation rates of bacteria(17, 18),(19), but the AMPs tested so far do not show such an effect as they do not elicit bacterial DNA damage responses (17, 18).

Here we use a pharmacodynamics approach that has been widely used to describe sigmoid dose-response relationships (20-23) to study the evolution of resistance of a homogeneous population. Our work uses the formulation of pharmacodynamic function from Regoes et al(20). We particularly explored how the steepness of the pharmacodynamic curve (described by the the Hill coefficient κ), together with other pharmacodynamic parameters determine the probability of resistance evolution(20). The potential importance of the Hill coefficient κ is often overlooked in many pharmacodynamic models, where it simply set to 1 for all drugs(24). Recent work includes the Hill coefficient (25, 26), indicating the importance of this pharmacodynamic parameter.

We use this approach with different parameter values for κ, derived from empirical data, as this allows us to calculate the size of the mutant selection window that generalizes over all possible resistant strains. Gullberg *et al.* demonstrated(14) that resistant mutants are already under positive selection below the MIC (minimum inhibitory concentration) of the susceptible strain. We therefore use the mutant selection concentration (MSC, Fig 1A) as the lower boundary, not the MIC of the sensitive strain that was used previously(12, 13). Using empirical parameter estimates for AMPs and antibiotics, we show that the probability of resistance evolution against AMPs (or any drug with similar pharmacodynamics properties) is much lower than for antibiotics. We therefore provide a robust and generalizable predictive framework for studying the evolution of drug resistance. This is particularly useful to apply when new drugs are introduced, i.e. before resistance has evolved.

Results

The mutant selection window (Fig 1) shows the concentration of an antimicrobial under which susceptible strains are suppressed, but resistant strains can still grow(13). We show that the lower bound of the mutant selection window (MSC) can be calculated based solely on the pharmacodynamics of the susceptible strains and the costs of resistance (Fig 1A, Fig 2A, equation 3). The cost is defined here as the reduction of growth rate in a drug free environment.

The pharmacodynamics of AMPs and antibiotics differ significantly(10): the pharmacodynamic curves of AMPs are much steeper as captured by a higher Hill coefficient κ (see Fig 2A); the step from a concentration with no effect to a killing concentration is therefore much smaller. This feature is likely due to a higher number of "hits" that AMPs need to deliver to bacteria to kill them and perhaps cooperative binding of AMPs molecules to the cell membrane(27). This results in a narrower MSW for AMPs than antibiotics The MSW opens at lower concentrations when the costs of resistance are low. Our re-analysis of data on antibiotic resistance against a variety of antibiotics in a number of different bacterial species (data from(28)) shows that the upper bound of the MSW correlates with the cost of resistance (Fig 2B). Taken together we are now in a position to estimate the size of the MSW for any drug, if estimates of pharmacodynamic parameters based on the sensitive strains, including the MIC, the maximum effect and the steepness of the pharmacodynamics curve are available (Fig 1A, Fig 2C).

Next we wanted to explore if the differences between AMPs and antibiotics in the width of the MSW correlated with different probabilities of drug resistance evolution within a host. A further difference between AMPs and antibiotics is that some antibiotics increase mutagenesis but AMPs do not(17, 18). We incorporated this difference in addition to the difference in the steepness of the pharmacodynamics relationship into a stochastic model describing bacterial replication and evolution

under selection pressure from AMPs. We consider two cases here: (a) do resistant mutants emerge (answering this question requires a stochastic model) and (b) do resistant mutants drive the susceptible strains to extinction?

We find that resistance emerges with a much higher probability for the parameter settings of antibiotics (top row Fig 3B) than for AMPs in our simulations (bottom row Fig 3B, Fig 3A). All intermediate cases, where we simulated changes in one or two of the parameters κ mutation rate and maximum effect, also reduce the probability of resistance emergence compared to 'pure' antibiotics.

We also find that resistant mutants are much more likely to drive the susceptible bacterial populations to extinction under antibiotic than under AMP treatment (Fig 3 B). Again, this result also holds when we study intermediate cases. In summary, our results show that the application of drugs with low κ, mutation elevation and low maximum effect, i.e. characteristics found in most common antibiotics, inherently bears a high risk of causing the evolution of resistance.We have shown before(10) that combinations of AMPs have higher κ and lower MICs than individual AMPs. This also results in differences in resistance selection and the extinction of susceptible strains, consistent with the results above.

Day *et al* (29) provided an approach to calculate a resistance hazard: a measure that combines the time of resistance emergence and its selection within a host. We calculated similar resistance hazard for AMPs in comparison to antibiotics. The simulation results show (Fig 3C) that the hazard is much higher and the concentration range much wider under antibiotic treatment than under AMP treatment. Also, when resistance evolves, it emerges earlier in the antibiotic scenario than in the AMP scenario at low concentrations (Fig 3D). In certain concentrations (for example, around MIC in our simulation), resistance emerges earlier in AMP than in antibiotics (Fig 3D). Time of emergence is mostly affected by κ and mutation rate: higher κ and lower mutation rate, the latter more important when population sizes are smaller,

confer delayed resistance emergency (Fig S4).

Discussion

Our predictions suggest that AMPs, or in fact any antimicrobial drug with similar pharmacodynamics, are much less likely to select drug-resistant mutants than antimicrobials with antibiotic-like characteristics. Our theory is blind to the molecular mechanism of action but captures the dynamically relevant aspects of action. We assume that pharmacodynamics and mutagenic properties of AMPs are significantly different from antibiotics. This assumption is based on limited data of AMPs in the literature(10, 17). More experiments with a variety of antimicrobial peptides are needed to determine if AMP like characteristics can be indeed generalized and if these characteristics are significant different from antibiotics.

In the light of our results, increasing κ and/or the maximum effect are desirable for any drug as well as advantageous to hosts managing their microbiota using AMPs. Our model therefore provides useful information for the development of new antimicrobial drugs: higher κ and maximum effect will impose much weaker selection on the bacteria to evolve resistance in lower concentrations, and clear the bacterial population more quickly in higher concentration which will, in turn, reduce the probability of resistance emergence. Currently mostly AMPs display these properties, but it is likely that new antibiotics that target the cell membrane or wall display similar pharmacodynamics.

The smaller MSW under AMPs is a direct consequence of the steeper pharmacodynamic functions(10). It is important to note that this relationship hinges on the realization that the window opens at the concentration at which the resistant strains have a higher growth rate than the sensitive strain, well below the MIC of the sensitive strain(14). Thus, a high Hill coefficient (κ) would constitute a promising characteristic of new antimicrobials. The other characteristics in which AMPs differ

from antibiotics – mutagenesis and maximum effect – affect mostly the time until resistance emerges, but not the size of the MSW. Because this time becomes shorter with higher population sizes, these characteristics may have less significance for clinical infections (30).

We find that time to resistance emergence in AMPs is longer than in antibiotics when the concentration is low (subMIC). Around MIC resistance against AMPs seems to emerge quicker than against antibiotics (FIG D). This counterintuitive result is explained by the fast removal of the sensitive strains caused by the combination of high κ and low psimin and is not related to the mutation rate *per se*. Overall the probability of resistance emergence is lower for AMPs as higher concentrations quickly remove the sensitive population. Chevereau *et al.*(31) reached a different conclusion using a different modeling approach. They modeled the pharmacodynamics only for positive growth and continuously adjusted the drug concentration to maintain the overall growth rate at half of the maximal in the simulation. In this scenario, drugs with sensitive dose-response would facilitate evolution due to the wide distribution of fitness, a scenario that seems unlikely in real antimicrobial treatment.

One recommendation derived from our modeling approach is that drugs that show pharmacodynamics resembling AMPs should be good candidates for slowing the evolution of resistance. Interestingly, combinations of AMPs result in increased κ, which our model predicts to bear lower risks of evolution of resistance(10). It is often argued that combination therapy reduces resistance evolution (but also see (32)), as it is supposedly more difficult to evolve resistance against more than one mechanism at a time. Our approach indicates that combination therapy might even prove effective if there are mutations that confer complete cross-resistance to the drugs in the combination.

It has been proposed that bacterial resistance evolution against AMPs is highly

unlikely (5, 8). Yet, *in vitro* experimental evolution has demonstrated that resistance to AMPs can arise (33–35) and AMP-resistance mechanisms have been characterized (36). Against antibiotics, resistance can increase the MIC by 2-3 orders of magnitude in a relatively small bacterial population(37), a range that has not been observed for AMPs. Though AMPs provide promising leads for drug development (4), their conserved killing mechanisms also argue for caution. In their paper 'arming the enemy', Bell et al.(38) discussed the high likelihood of cross-resistance against, for example, human AMPs. This problem has hardly been studied. Our analysis suggests how one could reap the benefits of AMPs without arming the enemy: we should rely on agents with AMP-like pharmacodynamics. This in principle can be adopted without using AMPs themselves.

Pharmacodynamic estimates can be easily and routinely obtained from time-kill curves. This can also be achieved for drug combinations(10). A report by the *Leopoldina,* the German National Academy of Sciences, recently recommended to use new drugs only in combination to avoid fast resistance evolution(39). The scientific support for this notion is limited and controversial(32, 40, 41). In clinical situations pharmacodynamic approaches can provide a first informed guess. Also, the risk of resistance evolution based on the pharmacodynamics of drug candidates will be a useful additional criterion to develop new drugs. We would also like to note that the concept of the mutant selection window has been applied to understand antiviral resistance evolution(42), and hence our approach has the potential to inform antiviral resistance research and ultimately treatment as well.

In order to generate predictions on resistance evolution based on pharmacodynamics, one of our main goals of the project, we made a number of simplifying assumptions. The pharmacodynamics is based on data of initial killing only. Moreover, we assume homogeneous populations over time and space. Expanding the framework to integrate tolerance and resistance is possible but would require pharmacodynamic estimates and additional functions. Another possible extension of our work would be

to include pharmacodynamic estimates of resistant strains that change over time due to compensatory mutations and to cross resistance or collateral sensitivity when exposed to combinations of antimicrobials. Finally, we assumed the same pharmacokinetics for all cases in our study. As AMPs are currently rarely used (Colistin being the notable exception), future empirical work will inform realistic parameter estimates for pharmacokinetics. In all cases however, the basis of any analysis concerning resistance evolution is the influence of individual pharmacodynamic parameters, for which we provide a framework.

Materials and Methods

For the parameterization of the predictive models, we used two main sources. The pharmacodynamic parameters are taken from one of our own studies that determines pharmacodynamics for AMPs and antibiotics under standardized conditions(10). In short, time kill experiments with different AMP concentrations were conducted and the slopes of the linear regressions were used to calculate the parameters of the pharmacodynamic function. Here, we only took into account the intial kill rates and assumed a homogeneous population structure. The estimates of mutation rates again are from our own comparative study on mutagenesis under AMP and AB treatment(17) .

Calculation of the size of the mutant selection window

The size of the mutant selection window (MSW) depends on the lower and upper bound of the MSW and is calculated as

$$size_{MSW} = \frac{MIC_R}{MSC}. \qquad (1)$$

The lower bound of the MSW is the concentration for which the net growth rate of the resistant strain is equal to the net growth rate sensitive strain and is called the minimal selective concentration (MSC). The upper bound of the MSW is the MIC of the resistant strain (MIC_R) (Fig 1 A). To analytically describe the MSW, we use the pharmacodynamic (PD) function $\psi(a)$, which mathematical describes the net growth rate with a Hill function:

$$\begin{aligned}\psi(a) &= \psi_{max} - d(a) \\ &= \psi_{max} - \frac{(\psi_{max} - \psi_{min})(a/MIC)^{\kappa}}{(a/MIC)^{\kappa} - \psi_{min}/\psi_{max}} \qquad (2)\end{aligned}$$

((10, 20, 21)). Here, a is the antimicrobial drug concentration, $\psi(a = 0) = \psi_{max}$, $d(a)$ is the effect of the antimicrobial with the dose a, and $\psi(a \to \infty) = \psi_{min}$. Therefore, the maximal effect E_{max} is $E_{max} = \psi_{max} - \psi_{min}$. The parameter MIC denotes the

concentration that results in zero net growth (this definition differs from the "official" MIC definition by Mouton et al (43)). The Hill coefficient κ describes the steepness of the curve; functions with higher κ describe steeper curves (Fig 2A). For illustration of the pharamcodynamic parameters see Fig S3). Cost of resistance c is included as a reduction of the maximum growth rate of the resistant strain in absence of antimicrobials with $c = 1\text{-}\psi_{max,R} / \psi_{max,S}$ (Fig 1A, 2A). The pharamcodynamic function can be described for both a drug susceptible strain S and a drug-resistant strain R, with $\psi_S(a)$ *and* $\psi_R(a)$, respectively. The *MSC* is calculated as $\psi_S(a) = \psi_R(a)$. We assume that the net growth rate of the resistant strain below the *MSC* is, for any given concentration a, with $0 < a < MSC$, approximately at the same level as without antimicrobials and therefore we set $\psi_R(a) \approx \psi_{R,approx}$ (illustrated in Fig 2A). With $\psi_{R,approx.} = \psi_{max,R} = \psi_{max,S}(1-c)$, we are able to describe the net growth rate of the resistant strain with the net growth rate of the sensitive strain $\psi_{max,S}$ and the costs of resistance c: $\psi_R(a) \approx \psi_{R,approx=} \psi_{max,S}(1-c)$. This is valid because $MIC_R >> MIC_S$ and assuming $\kappa_R >\approx \kappa_S$. The analytic solution of the *MSC* is

$$MSC = MIC_s \left(\frac{c\psi_{min,S}}{\psi_{max,S}(c-1)+\psi_{min,S}} \right)^{1/\kappa_s} . \quad (3)$$

Analysis of the relationship between cost of resistance c and MIC_R

Data(44) determining relationship between fitness of resistant strains and MIC_R/MIC_S was re-analyzed. The dataset contained information about increase of MIC due to resistance and fitness of the resistant strain. The dataset summarizes cases of bacterial resistance to antibiotics. Similar data for AMPs have been compiled recently(30) but are yet too scarce to include in the following analysis. We therefore assumed similar relationships for both antibiotics and AMPs.

We calculated cost of resistance c as c = 1 – fitness, using n= 128 observations compiled in the mentioned dataset. Fitting a $\log_{10}$ transformed linear regression to the data resulted in the parameterized function $\log_{10}(MIC_R/MIC_S) = 2{,}59 * c + 1{,}65$, ($R^2 = 0.22$). The data was then resampled with using bootstrapping to (i) determine

the 95% confidence interval of log-linear regression of the data as interval, where 95 % of the regression fall into (see fig. 2B) and (ii) to include the variance of the data when determining the size of the mutant selection window (MSW)(see fig. 2C). For the latter, the given dataset was fitted to the mentioned log-linear regression 200 times, resulting in 200 parameter sets for the regression. Each parameter set was then used to calculate the size of the MSW depending on the cost of resistance. The 95% confidence interval was then calculated as the interval, in which 95% of the calculated size of the MSW are in for a given cost.

Model of evolution and prediction of resistance

To study resistance evolution we used a mathematical model that incorporates pharmacodynamics (PD) and pharmacokinetics (PK) and captures population dynamics of bacterial populations under treatment with antimicrobial drugs(20). We ran stochastic simulations to calculate the probability of resistance emergence, the probability of the resistant strain, the time to resistance emergence and the risk of resistance (the resistance hazard(29)).

To simulate treatment, we consider a patient harboring 10^6 susceptible bacteria. Bacterial mutation rates are assumed to depend on the antimicrobial used for treatment (antibiotics or AMPs). When a resistant strain arises it is assumed to have an MIC ten-fold that of susceptible wild-type strain. For simplicity, we only consider one type of mutant. Antimicrobials are administered every day (see Supplement for pharmacokinetics), and treatment lasts one week.

The population dynamics of the susceptible and resistant strains is captured in the following system of differential equations:

$$\frac{dS}{dt} = r_S\left(1-\mu\right)S\left(1-\frac{S+R}{K}\right)-\left[d_S+d_n\right]S$$
$$\frac{dR}{dt} = r_R R\left(1-\frac{S+R}{K}\right)+\mu r_S S\left(1-\frac{S+R}{K}\right)-\left[d_R+d_n\right]R.$$

(4)

Where *S* represents the wild-type strain and *R* represents the resistant strain. The maximum net growth rate ψ_{max} is the difference between the replication rate *r* and the intrinsic death rate d_n: ψ_{max} = r-d_n. μ is the mutation rate.

To include the change of antimicrobial concentrations over time (pharmacokinetics) into our mode, we define the death rate to be dependent on the time-dependent antimicrobial concentration a(t):

$$d_i\left(a\left(t\right)\right)=\frac{\left(\psi_{max}-\psi_{min}\right)\left(a\left(t\right)/MIC\right)^{\kappa}}{\left(a\left(t\right)/MIC\right)^{\kappa}-\psi_{min}/\psi_{max}},\ i=S,R$$

(5)

We assume a time-dependent pharmacokinetic function $a(t)$ of the following form (see also Fig S2):

$$a(t)=\sum_{n}\frac{Dk_a}{k_a-k_e}\left(e^{-k_e[t-(n-1)\tau]}-e^{-k_a[t-(n-1)\tau]}\right),\quad n=1,2,3\ldots$$

(6)

Here, k_a *is* the absorption rate, and k_e is the decay rate. *D* is the dose given each time, *n* is the number of doses, τ is the dosing frequency. We define the treatment dose as the average concentration in the course of treatment:

$$\bar{a}=\frac{1}{t}\int a(t)dt \qquad (7)$$

We implemented the model in Equation 4 stochastically using the Gillespie algorithm(45), which allowed us to monitor how frequently mutants arise. Parameters were selected based on empirical data as stated above. The net growth rate of wild-type in the absence of antimicrobials was set as 1. Mutants suffer fixed or resistant-level related costs (see Fig 2). κ of AMPs and antibiotics were set as 5 and

1.5, respectively (10). $\psi_{\min}$ for AMPs is fixed as -50 hour^{-1}; and for antibiotics is fixed as -5 hour^{-1}. Mutation rates in AMPs are assumed to be three times lower than in antibiotics, in accordance with our empirical estimates (17). All the parameters and their values are listed in Table S1. All the pharmacokinetic parameters are the same in different simulations (see Fig S2). For each set of parameters, cohorts of five hundred infected individuals were simulated. Successful treatment is defined as complete clearance of both sensitive and resistant strains at the end of the one-week treatment. For each cohort, we calculate the probability of treatment success as the proportion of individuals in whom treatment was successful. In each individual, we score the time of emergence of resistance strains, and estimate the resistance hazard based on the average probability of treatment success and the population size of bacteria over time. The hazard function can be written as,

$$H(a,t) = \frac{1}{Kt}\int S(a,t)p_{S\to R}(a)\psi_R(a)dt, \qquad (8)$$

Where K is the capacity, S denotes population size of sensitive strain and $p_{S\to R}$ is probability of a treatment developing resistance, which is calculated from the results of simulations, ψ_R is the growth rate of resistant strain. Our hazard function calculates the average proportion of resistant population under certain treatment dose and duration.

Implementation

The analysis was performed in R (v. 3.1.3&v. 3.2.2) (46) using RSTUDIO (v. 0.98.1103&0.99.903) [35]. The code is available upon request.

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Figures

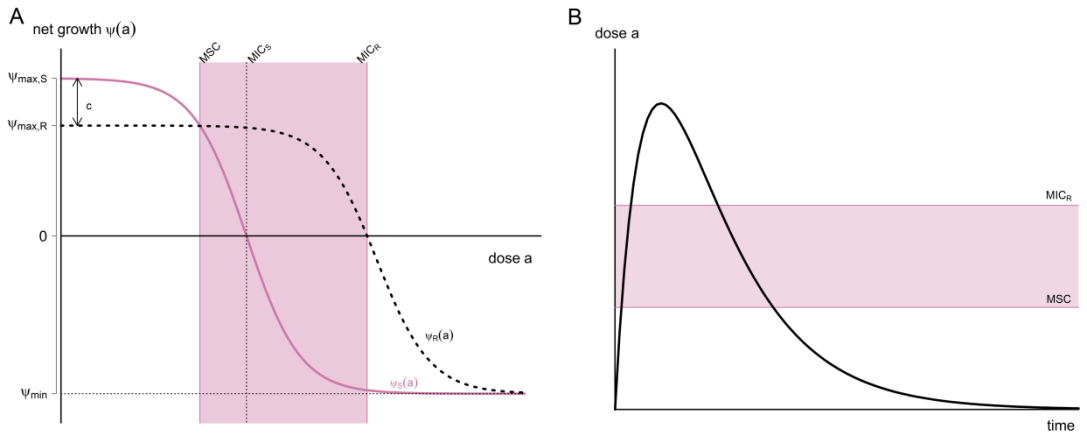

Fig 1. The revised mutant selection window and pharmacodynamic parameters. **(a)** The mutant selection window (MSW) is defined as the antimicrobial concentration range in which resistant mutants are selected (13). Following (14), we determine the MSW using net growth curves of a susceptible strain *S* and a resistant strain *R*. Mathematically, net growth is described with the pharmacodynamic function $\psi(a)$ ((20), see Materials and Methods and Fig S3 for details). In short, the function consists of the four pharamcodynamic parameters: net growth in absence of antibicrobials ψ_{max}, net growth in the presence of a dose of antimicrobials, which effects the growth maximal, ψ_{min}, the *MIC* and the parameter κ, which describes the steepness of the pharamcodynamic curve. Here, the two pharmacodynamics functions $\psi_S(a)$ (continuous pink line) and $\psi_R(a)$ (dotted black line) describe the net growth of the *S* and *R*, respectively, in relation to the drug concentration *a*. Cost of resistance c is included as a reduction of the maximum growth rate of the resistant strain $\psi_{max,R}$, with $c = 1 - \psi_{max,R}/\psi_{max,S}$. Note that with this definition, cost of resistance is expressed as reduction in net growth rate in absence of antimicrobials (a = 0). The lower bound of the MSW is the concentration for which the net growth rate of the resistant strain is equal to the net growth rate of the sensitive strain and is called the minimal selective concentration (MSC) (see Materials and Methods for analytic solution, see Fig S1 for how the MSC is influenced by pharamcodynamic parameters os the sensitive strain). The upper bound is given by the MIC of the resistant strain

MIC_R. We calculate the size of the MSW as: $size(MSW) = \frac{MIC_R}{MSC}$. **(b)** The boundaries of the MSW applied to the pharmacokinetics of the system.

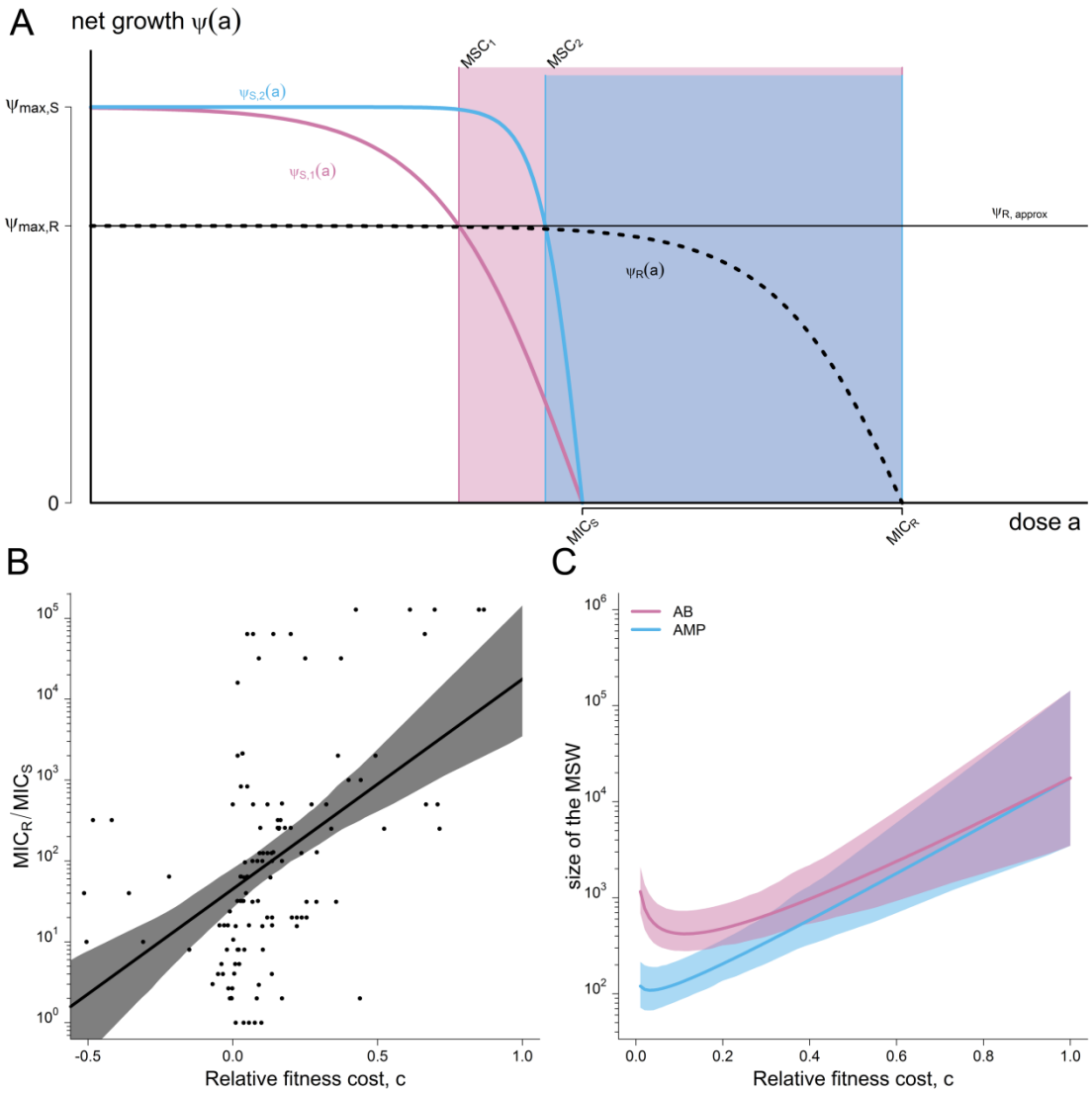

Fig 2. The mutant selection window for arbitrary mutant strains. The two boundaries of the MSW, MSC and MIC_R, are influenced differently by the pharmacodynamic parameters of the sensitive strain S and the resistant strain R. **(a)** The lower boundary of the MSW (MSC) depends primarily on the pharmacodynamic parameters of the sensitive strain, assuming that the net growth rate of the resistant strain below the MSC is approximately at the same level as without antimicrobials: $\psi_R(a) \approx \psi_{max,S}(1-c) = \psi_{R,approx}$, for $0 < a < MSC$ (ψ_R: dotted black line; $\psi_{R,approx}$:continuous black line) (see Materials and Methods for details). The effect of each of the four pharamcodynamic parameters and of the cost of resistance on the MSC is depicted in Fig S1. We plotted the pharmacodynamic function $\psi_S(a)$ of two sensitive strains with varying κ values: $\psi_{S,1}(a)$ representative for ABs with a small κ ($\kappa = 1.5$, pink) and $\psi_{S,2}(a)$ representative for AMPs with a large κ ($\kappa = 5$,

blue). Increasing the κ value results in increasing the MSC (MSC_1 (pink) <MSC_2(blue)). **(b)** The upper boundary of the MSW is per definition the MIC_R, which is linked to its fitness cost, i.e. the upper boundary MIC_R increases with costs c (data from(44)). Here, the log-linear regression and the 95% confidence interval are plotted. See materials and methods for details of the statistics. **(c)** The relationship between cost of resistance, other pharmacodynamic parameters, and the size of the MSW is complex. We show that because both boundaries of the MSW – the MSC and the MIC_R – are influenced by costs of resistance c, the lowest MSW window size is achieved at intermediate cost of resistance *c*. We plotted the size of the MSW (line) and the 95% confidence intervals for both AMP-like and AB-like pharmacodynamics, with $\psi_{max,S} = 1$, $MIC_S = 1$, $\psi_{min,S,AB} = -5$, $\psi_{min,S,AMP} = -50$, $\kappa_{S,AB} = 1.5$ and $\kappa_{S,AMP} = 5$. $\psi_{max,R}$ was calculated using the relationship $\log_{10}(MIC_R/MIC_S) = 2{,}59 * c + 1{,}65$.

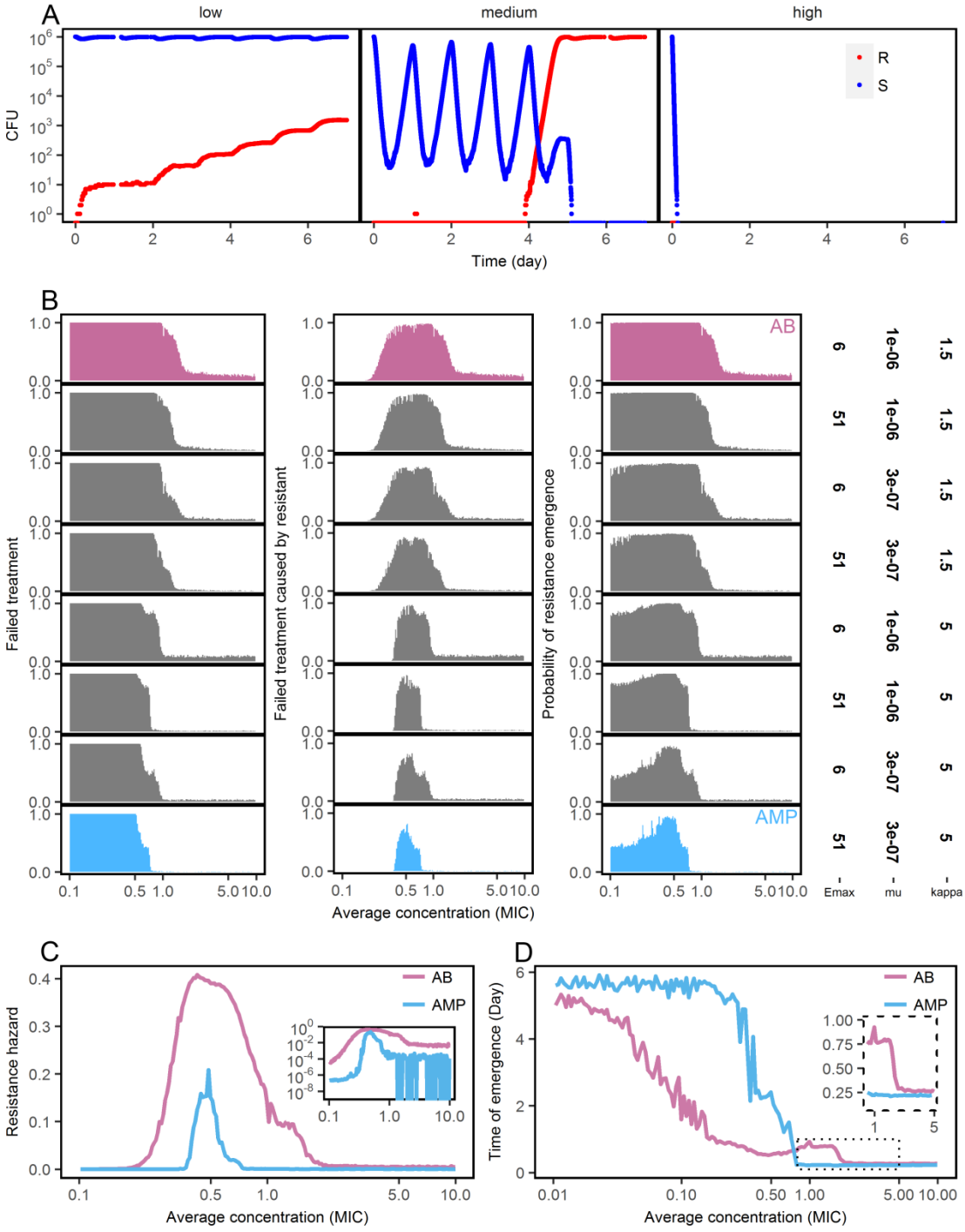

Fig 3. Evolution of drug resistance determined by pharmacodynamics.

(a) At high dose antimicrobials achieve maximal effects and rapidly kill most of the population, preventing resistance evolution (left). At medium dose, the sensitive strain will not be eliminated immediately, and resistant mutants emerge (central). At low dose, the sensitive strain will not be removed, the mutants emerge as well, but will not quickly reach equilibrium due to substantial fitness costs (right, resistant: pink,

susceptible: blue), **(b)** Simulations comparing the range from 'pure' antimicrobials peptides (AMP) to 'pure' antibiotics (AB) by altering μ, ψ_{min} and κ. We find that the probabilities of treatment failure (left), of failure caused by resistant strains (middle) and of resistance emergence are always higher under the AB-scenario than the AMP-scenario. A successful treatment requires less AMP than AB. **(c)** Following (29) we calculate the resistance hazard as the time-averaged proportion of mutants in a patient under a particular treatment dose. We find that AMPs are much less likely to select for resistance across concentrations than antibiotics (inset graph: a log-scale view). **(d)** Time to resistance is much longer under AMP than AB treatment when the average concentration is below MIC, but shorter around MIC and equal in higher concentrations (inset graph). The parameters are: $\psi_{max,S} = 1$, $\psi_{max,R} = 0.9$, $\kappa_{AB} = 1.5$, $\kappa_{AMP} = 5$, $\psi_{min,AB} = -5$, $\psi_{min,AMP} = -50$, $MIC_S = 10$, $MIC_R = MIC_s * 10^{[2.59*(\psi_{max,S} - \psi_{max,R}) + 1.65]}$. $\mu_{AB} = 10^{-6}$, $\mu_{AMP} = 3 * 10^{-7}$, $k_a = 0.5$, $k_e = 0.2$, $d_n = 0.01$, $\tau = 1/24$.

Appendix

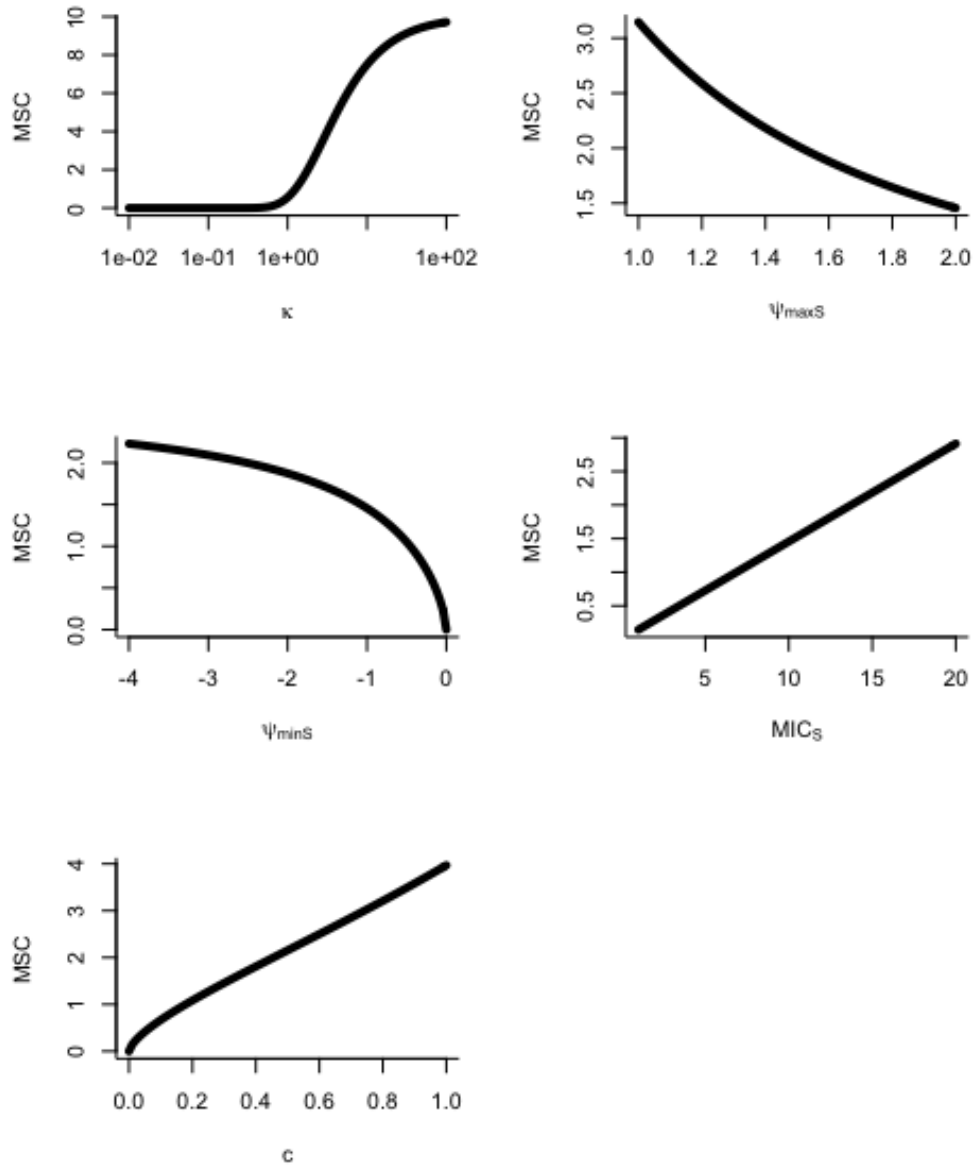

Figure S1: The MSC **is dependent on the pharmacodynamic variables of the susceptible strain *S* and the cost of resistance *c***. For parameter values, see fig 1a in main text.

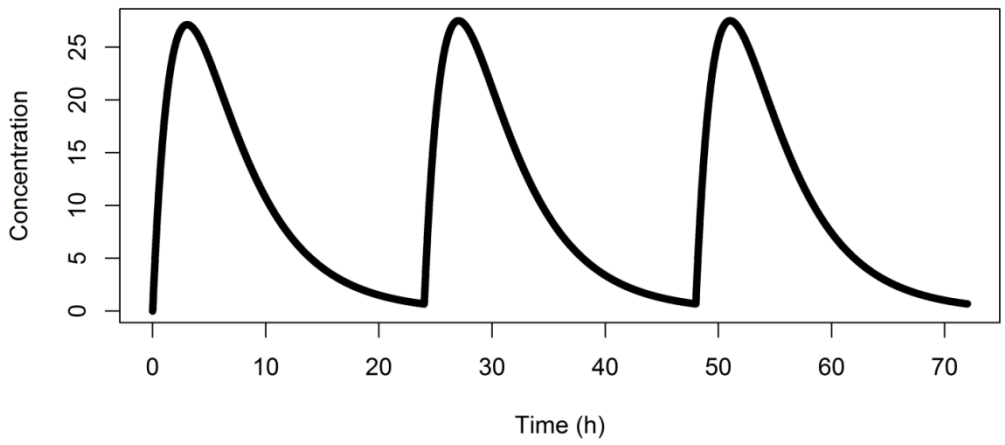

Fig. S2. **Pharmacokinetics of a given antimicrobial.** The curve can be captured by equation (6). This pharmacokinetics depicts that drug concentration reaches maximum shortly after dosing, then declines gradually before next dosing. The parameters are: $k_a = 0.5$, $k_e = 0.2$, $\tau = 1/24$, $D = 50$.

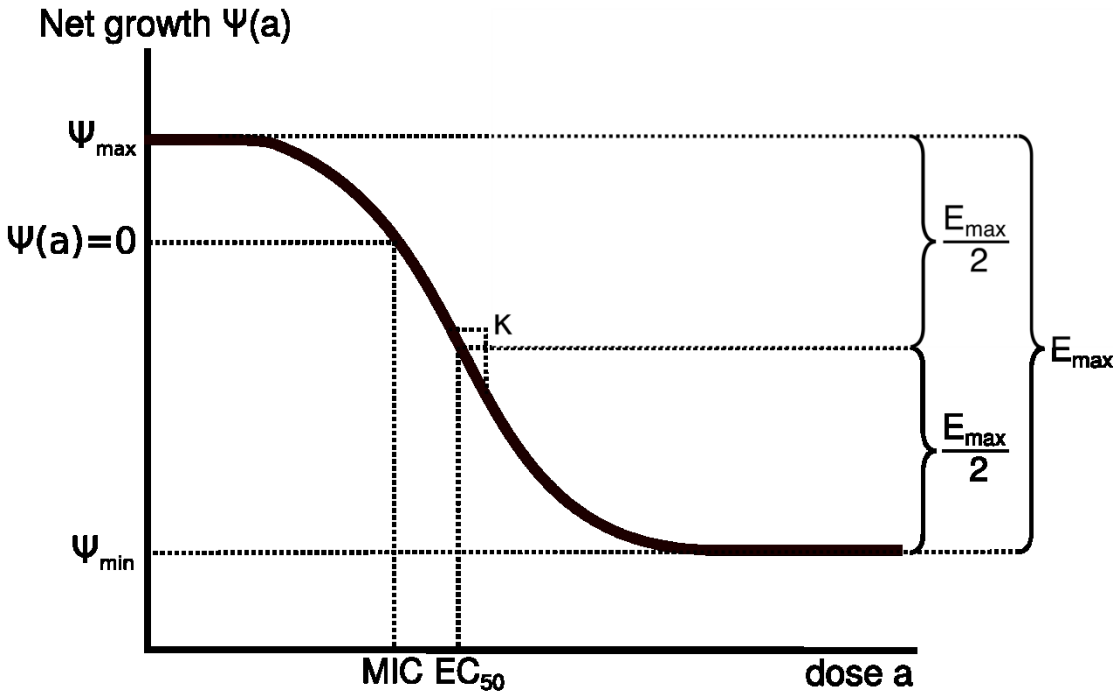

Fig S3. **Concept figure of the pharmacodynamic function and the pharmacological parameters.** The pharmacodynamic function $\psi(a)$ describes the net growth rate of a pathogen population in the presence of an antimicrobial with the dose a:

$$\psi(a) = \psi_{max} - d(a)$$

Here, ψ_{max} is the maximal net growth rate, i.e. $\psi_{max} = \psi(a = 0)$, and $d(a)$ represents the impact of the antimicrobial on the growth of the pathogen. In Regoes et al. (2004), two options are given to mathematically describe the term $d(a)$ with pharmacodynamic parameters:

$$d(a) = \frac{(\psi_{max} - \psi_{min})\left(\frac{a}{MIC}\right)^{\kappa}}{\left(\frac{a}{MIC}\right)^{\kappa} - \frac{\psi_{min}}{\psi_{max}}}$$

$$= \frac{E_{max}\left(\frac{a}{EC_{50}}\right)^{\kappa}}{1 + \left(\frac{a}{EC_{50}}\right)^{\kappa}}$$

ψ_{min} is the minimal net growth rate with $\psi_{min} = \psi(a \to \infty)$, E_{max} is the maximum effect of the antimicrobial, with $E_{max} = \psi_{max} - \psi_{min}$, MIC is the dose at

which the net growth equals 0 ($\psi(a = MIC) = 0$), κ is the slope parameter that describes the steepness of the curve, and EC_{50} is the dose of the antimicrobial at which half of the maximum effect is achieved ($\psi(a = EC_{50}) = \psi_{max} - \frac{E_{max}}{2}$). Note that $EC_{50} = MIC\left(-\frac{\psi_{min}}{\psi_{max}}\right)^{\frac{1}{\kappa}}$. All pharmacodynamic parameters are indicated in the figure.

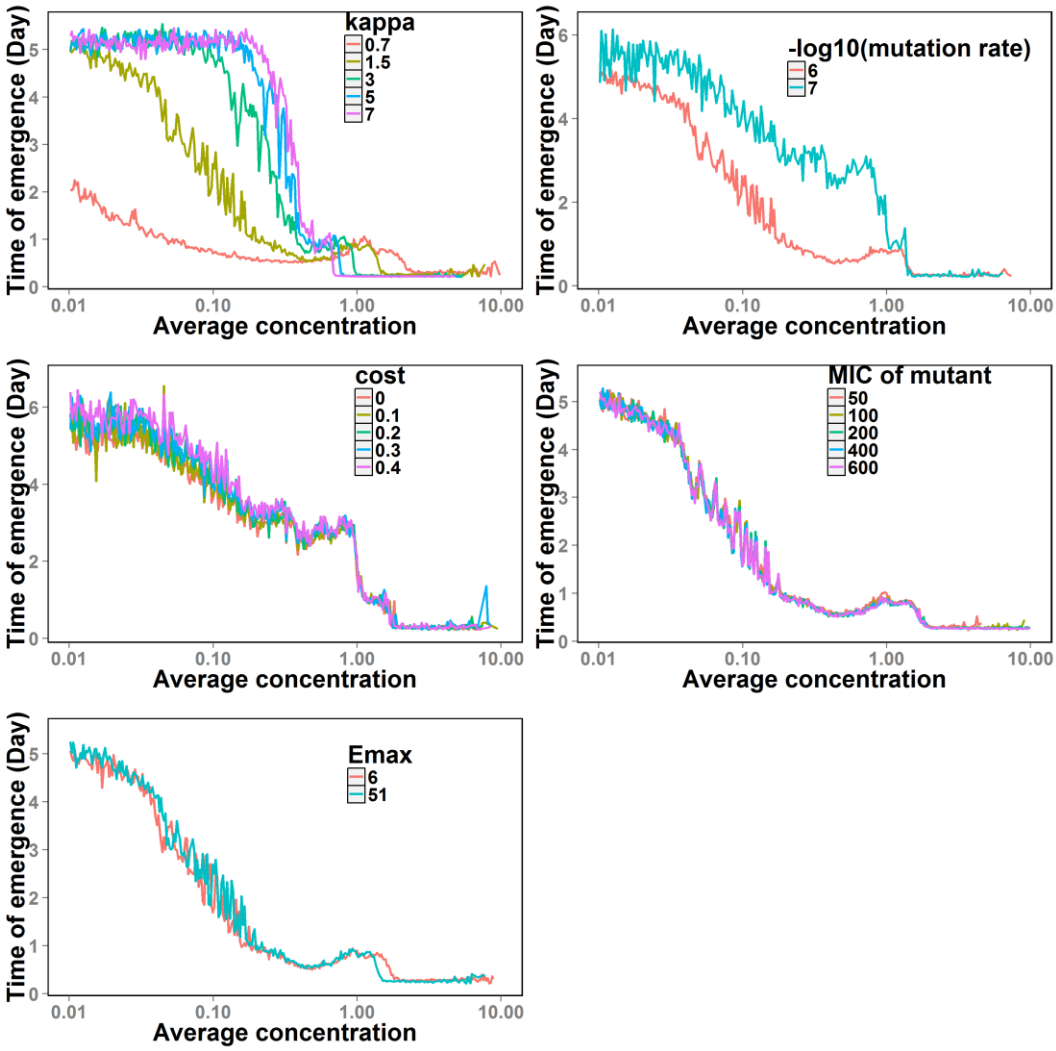

Fig. S4. **Average time of emergence of mutants.** Kappa and mutation rate determine the time of emergence of mutants. Higher kappa and lower mutation rate will result latter emergence of mutants. However, MIC of mutants, cost of mutants and maximal effect of antimicrobials do not significantly effect the time of emergence of mutants.

Parameters	Value	Unit	Description
a	0~1000	×MIC	The concentration of drugs
ψ	-50~1	h^{-1}	The growth rate of bacterial population
MIC	1~10	-	Minimal inhibitory concentration
κ	1.5, 5	-	Shape parameter of pharmacodynamic curve
d	-	h^{-1}	Death rate of bacterial population
c	0~1	-	Cost of resistance
S	0~10^6	CFU	Population size of sensitive strain
R	0~10^6	CFU	Population size of resistant strain
K	10^6	CFU	Capacity of system
μ	10^{-6}, 10^{-7}	-	Mutation rate
k_a	0.5	h^{-1}	Rate of drug absorption
k_e	0.2	h^{-1}	Rate of drug decay
D	-	×MIC	Dosage of a given drug
τ	1/24	h^{-1}	The dose frequency
$p_{S\to R}$	0~1	-	Probability of a treatment developing resistance

Table S1. Parameters and their values used in this study.

Psimax	Psimin	Kappa	MIC	Antibiotics
0.368	-5.959	0.740	0.426	Ampicilin
0.052	-5.927	1.242	0.848	Ciprofloxacin
0.045	-5.866	1.853	0.067	Gentamicin
0.205	-5.918	1.621	0.527	Kanamycin
0.159	-5.876	1.808	0.480	Neomycin
0.218	-4.171	0.445	1.371	Rifabutin
0.280	-0.783	0.904	1.627	Spectinomycin
0.008	-6.407	1.866	2.993	Tetracycline

Table S2. The measured pharmacodynamic parameters of different antibiotics for reference in this study.

Chapter 6

The evolution of antimicrobial resistance in a model combining a multiple-step mutations and pharmacodynamics

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Abstract

The evolution of antimicrobial resistance is a major health threat. Various factors contribute to the evolution of antimicrobial resistance. Here, we develop a mathematical model with multi-step mutations to predict resistance evolution from a perspective of antimicrobials. We describe each mutant with a set of pharmacodynamics parameters and monitored the time of its emergence. The results show that the mutants emerge sequentially with medium increment of MIC will emerge earlier than those with higher or lower increment of MIC. Mutation with fitness cost slow down resistance evolution in terms of late emergence. The fitness cost in resistant mutants is likely to be compensated as lately as possible, otherwise will hinder the emergence of later fitter mutant and thus slows down the resistance evolution. For a given mutant, the shape of dose-response and maximal killing rate that can be achieved by antimicrobials nearly have no influence on the time of their emergence. We further find that high dosing frequency and low drug clearing rate facilitate resistance emergence and render treatment in low concentration into failure. However, the rage of concentration that allows resistance emerging and fixation is not affected by the dosing frequency and drug clearing rate. Taken together, our results suggested the resistance evolution from a perspective of antimicrobials, which suggests that the pharmacodynamics of antimicrobials plays an important role in the evolution of antimicrobial resistance.

Introduction

Infectious pathogens, such as bacteria, continuously evolve resistance to newly developed drugs in clinical treatment as well as the environment. The evolution of antimicrobial resistance could be very well captured by the mutation and selection dynamics. Bacteria evolve high level of resistance to antimicrobials through horizontal gene transfer, gene recombination, standing genetic mutation and phenotypic adaptation [1-3]. Rare mutants which are beneficial under unfavorable conditions, such as antimicrobial concentrations above MIC (Minimum Inhibitory Concentration), can be selected quickly through so-called purifying selection [3, 4]. The rate of evolution is determined by the specific genetic mutation, the fitness cost related to mutation and the selecting pressure imposed by antimicrobials.

Predictive outcomes from a given treatment are highly desirable, especially if the probability of resistance emergence could be predicted [4, 5]. Many of the clinical treatment failed due to the emergence and/or presence of resistant strains in the course of treatment. However, predicting evolution of antimicrobial resistance is notoriously difficult as it involves many variables from both, antimicrobials and bacteria [5]. On the antimicrobial side, treatment strategies were widely explored regarding dosing quantity, dosing frequency and drug combination [6, 7]. They have significant and predictable impact on the emergence and evolution of resistance. For example, it is highly recommended to apply aggressive treatment to minimize the probability of resistance emerging. When there is still active immune response and no resistant strain before treatment, this strategy applies as high concentration of drug could remove bacteria rapidly which in turn reduce the probability of resistance emerging [6, 8]. In the presence of resistant strains, aggressive treatment strategies may fail [9], as high concentrations of antimicrobials result in competitive release of resistant strains [9, 10]. Thus, a more carefully designed strategy based on the principle of maximal tolerance in the host is developed to adjust the dosage of treatment [11]. In addition, patients' low adherence and ways of administration will also largely facilitate the

evolution of drug resistance [12-14].

On the bacterial side, the prediction of resistance evolution relies on mutation, mutation related fitness, interaction between resistant and sensitive strain and specific selection pressure imposed by antimicrobials [5]. Although genetic mutations which cause resistance to various antimicrobials have largely been identified in different bacterial species [15], it is difficult to predict and identify these them in real time. Second, the fitness related to a given mutation is not available either. However, beneficial mutants under strong antimicrobial selection are usually very rare [16]. For example, only five mutants are able to resist high level of β-lactamase across the whole genome; these mutants form only a few accessible mutational pathways on the fitness landscapes [16]. Chevereau *et al.* measured the fitness of ~4000 *E. coli* mutants under different antibiotic treatments. They found that the distribution of fitness of these mutants is related to the dose-response property of antibiotic [17]. In particular, wider distribution of fitness is observed in antibiotics with a steeper dose-response curve, which will select faster resistance if this distribution is constantly maintained by adjusting drug concentration during treatment [17]. The degree of resistance could also be predicted by specific mutations and its profile of gene expression. Suzuki *et al*. evolved the bacteria under different antimicrobial treatment and showed that the observed phenotypic resistance in a mutant was linearly correlated with the level of gene expression [18].

An overlooked perspective in the evolution of antimicrobial resistance lies in the interaction between bacteria and antibiotics, especially the dose-response relation. Classic theory of mutation selection window (MSW) predicts resistance evolution relying on the assumption in which a particular mutant is present [19-22]. It predicts that the concentration below the mutants prevention concentration (MPC) and minimal inhibitory concentration (MIC) will select the resistance [23]. However, a necessary step before mutant selection is mutant emergence. The emergence of certain mutants is not captured by the predictive frame work of the classic MSW theory. We

think this could be determined by the dynamics of antimicrobial killing, which can be very well described by the Michaelis–Menten kinetics, which is an important base of pharmacodynamic Emax models [24, 25]. A modified version of this model with more detailed parameter setting can be easily tested in the lab [25]. This allows us directly compare the pharmacodynamic properties of different antimicrobials [25, 26]. Recently, we combined this model with simple population model to investigated the resistance evolution of bacteria under *in-silico* treatment of different antimicrobials with distinct pharmacodynamics properties. Our results showed that the resistance evolution is largely determined by the hill coefficients which controls the shape of dose-response curve. In particular, larger hill coefficients (κ in our model) together with higher maximal effects and lower mutation rate determine narrower range of mutation-selecting concentration in terms of *de novo* resistance emergence. Previous work with antivirus drug showed the dose-response slope is correlated with drug resistance [27, 28].

Here, we extend our previous model which is based on the single mutation to a multiple mutation model. Mutations with high resistance usually result from accumulative multiple-step mutations, each of which increase certain level of resistance [16]. The process is well captured by the theory of fitness landscape [29-32]. Under strong antimicrobial selection, few beneficial mutants would emerge and form few mutational trajectories. By tracking the fittest mutation in each mutation step, we follow a chain of mutations which represent the peaks of fitness landscapes. We define mutations based on the pharmacodynamic parameters. A unique set of pharmacodynamic parameters could capture each of these mutants. Mutations not only alter the MIC, but also change other pharmacodynamics parameters, such as κ in our previous model. In doing so, we investigate the *de novo* emergence of each step of mutation under certain treatment. We then explore a wide range of parameters which will influence the emergence of resistance with a focus on the difference between AMPs and antibiotics [26]. We further investigated how the treatment strategy affects the emergence and evolution of resistance.

Materials and Methods

The model

We consider a homogeneous environment (for example an organ in human) that has a maximum carrying capacity. Bacterial populations grow with a rate *r,* and suffer death with rate *d*. In the presence of antimicrobials in the environment, the rate of bacterial death is mainly controlled by the concentration of bacteria. Beneficial mutants (*R*) that are resistant to antimicrobials emerge from the wild-type (*WT*) or strains with lower resistance with rate μ. The discrete dynamic process can be described as follows,

$$\begin{aligned} &WT \xrightarrow{r_{WT}} WT + WT, \quad WT \xrightarrow{d_{WT}} \varnothing, \\ &WT \xrightarrow{\mu_i} R_i, \\ &R_i \xrightarrow{r_R} R_i + R_i, \quad WT \xrightarrow{d_R} \varnothing, \\ &R_i \xrightarrow{\mu_j} R_j, \quad i \neq j. \end{aligned} \tag{1}$$

High-level resistance to antimicrobials is achieved through accumulative multiple mutational steps. Under low selective pressure, a number of mutations with similar fitness will emerge and fluctuate in the population, evolution in such conditions is generally considered as neutral. These mutations likely contribute to a fitter "quasi-species" through more steps of mutations. The system is balanced by the mutation and selection with fitness peaks fluctuating with selection pressure. The system can be described by the reaction-diffusion model in the limit of an infinite population [33].

Antimicrobials exert strong selection that allows beneficial mutations to emerge and survive. Thus accessible mutation pathways could be built upon these mutations (see Fig. 1). The mean-field approximation of above discrete dynamics of mutation and selection process could be written as

$$\frac{dN_i}{dt} = \sum_j r_j N_j \mu_{j\to i} + r_i N_i \left(1 - \frac{N_{tot}}{K}\right)\left(1 - \sum_i \mu_{i\to j}\right) - d_i N_i, i \neq j, i, j = 1, 2, 3 \ldots \tag{2}$$

In the limit of strong selection, few mutational pathways are accessible. One fitter

mutants will emerge from previous one, take over the whole population and further contribute to the next fitter mutation. Continuing such iteration forms the dynamics of resistance mutation. This dynamics can be simplified into

$$\frac{dN_i}{dt} = r_{i-1}N_{i-1}\mu + r_i N_i\left(1-\frac{N_{tot}}{K}\right)(1-\mu) - d_i N_i,\ i = 1, 2, 3\ldots \tag{3}$$

In equation 2 and 3, N_i represents the population size of different bacterial strains, which include both wild-type and resistant strains. We have $N_{tot} = \sum N_i$. The new mutants comes from the older ones with a fixed mutation rate μ. The doubling rate rates r of all the individuals in the population are constrained by the carrying capacity K. The death rate d_i is mainly determined by the pharmacodynamic properties of the drug. The death rate is mutation- and antimicrobial-dependent. It follows the form,

$$d_i(a) = \frac{(r_i - \psi_{i,min})(a/MIC_i)^{\kappa_i}}{(a/MIC_i)^{\kappa_i} - \psi_{i,min}/r_i},\ i = 1, 2, 3, \ldots \tag{4}$$

Where ψ_{min} is the minimal growth rate, which should be negative with the presence of antimicrobials. a is the concentration of antimicrobials. MIC is the minimal inhibitory concentration of a particular strain. The Hill coefficient κ, is a shape parameter of pharmacodynamics.

For drug dosing, it follows a multiple dosing regime in which the drug is given every certain period of time. Assume the dosing going through two stages: absorbing and clearing, the concentration-time relation of a given drug with multiple dosing can be described by the solved one compartment model:

$$a(t) = \sum_n \frac{Dk_a}{k_a - k_e}\left(e^{-k_e[t-(n-1)\tau]} - e^{-k_a[t-(n-1)\tau]}\right),\ n = 1, 2, 3\ldots \tag{5}$$

where k_a is the absorption rate, and k_e is the decay rate. D is the dose given each time, n is the number of doses, τ is the dose frequency. We use the average concentration in the course of treatment to represent the dose level of treatments. Then we can calculate the average concentration over the course of treatment,

$$\langle a(t)\rangle_{\Delta t} = \frac{1}{\Delta t}\int_{\Delta t} a(t)\,dt \tag{6}$$

The definition of mutations

The sequence of mutations is defined according to the pharmacodynamic properties. We assume that every mutation has a unique set of pharmacodynamic parameters which is captured by equation (4), and emerge sequentially by changing the phramcodynamic parameters in each step [25]. We also assume that the highest fitness of mutations could be reached by three accumulative mutational steps by change of one or more of these parameters in each step. Using this model, several cases can be investigated.

1) How much MIC likely increase in each mutational step? Previously work assumes beneficial mutations increase MIC but suffer fitness cost. In this study, we assume sequential beneficial mutants to display increased MICs. In order to determine how much increase in MIC in the sequence of mutations could be most likely selected and evolved, we set different increment of MICs between two adjacent mutations in sequential mutants. At the same time, we keep all the other pharmacodynamic parameters constant (Fig. 2A).

2) When are fitness costs most likely to evolve and to be compensated? In our model, the fitness and fitness cost is defined as the growth and growth reduction in the drug free environment, respectively. The fitness of wild-type is set to 1, which is represented by r in equation (2). In this scenario, we fix the increase of MIC in sequential mutations and other parameters as the same in all the mutations, then change r values in the sequential mutations.

3) How likely the mutation will change the dose-response sensitivity of bacteria or κ in the pharmacodynamics. Like in point 2), we would also like to see what the potential trend could be, for example, decreasing or increasing in dose-response curve, which is the parameter κ in equation (3). Keeping other parameters as constants, we

set different κ values in the sequence of mutations.

4) Whether the mutation could change the ψ_{min} of the drug. In this case, the MIC of a given mutants might not change but the tolerance of bacteria to antimicrobials increases.

The treatment strategy for different drugs with different pharmacokinetics

The treatment strategies, which include categories of antimicrobials, quantity of dosing and frequency of dosing, play important roles in resistance development. Antimicrobials with different pharmacokinetic and pharmacodynamic properties select resistance differently. For example, drugs absorbed quickly and excluded slowly could quickly reach effective concentrations in body. This will result a quicker elimination of bacteria and slower resistance development. Drugs which possess a steeper dose-response relation also slow resistance development as it suppress the *de novo* resistance emergence. In this study, we mainly choose two *in-silico* drugs with resemblance to antibiotics and AMPs, as these two classes of drugs have proven distinct pharmacodynamics. In addition, rational dosing, as another important treatment strategy, is also very critical for resistance evolution. Rational choice of dose could also minimize the development of resistance either with presence or absence of resistant [9, 10]. With our model (equation (1-5)), we examine resistance development in a multiple mutation situation with combined input of above mentioned strategies to investigate the time of emergence and resistance evolution.

Simulations

All pharmacodynamic parameters are taken from experimental measurements (Table (1)). Bacterial growth in drug-free environment is set to 1. Costs are defined as growth reduction in drug-free environments. Bacteria in infection sites are constantly removed with a fixed rate of 0.01. Additionally, antimicrobials play an important role in removing bacteria, the rate is largely depended on their pharmacodynamics and pharmacokinetics. We determined the *in-vitro* killing rate of different antimicrobials,

such as antibiotics and antimicrobial peptides. Their pharmacodynamic properties could be very well captured by above equation (4). With the stress on these two antimicrobials, we set κ of AMPs and antibiotics are 5 and 1.5, respectively. ψ_{min} for AMPs is fixed as -50 $hour^{-1}$; and for antibiotics is fixed as -5 $hour^{-1}$. We assume mutation rate in AMPs is 3 times lower than in antibiotics and is set to 10^{-5} and $3x10^{-6}$, respectively [34].

In order to test the resistance evolution during a treatment process, we model a disease progression that the infection develops observable symptoms when bacterial population reaches a maximal capacity. Then the drug is immediately administered to treat the infection. We assume that the symptom could emerge when the bacterial load reaches 10^6, which is set as the initial population size as well as the capacity of infection site. During the simulation, we do not consider the spatial distribution of drug concentration or spatial sensitivity of bacteria on the infection site. Bacteria grow with constraint of the capacity while being removed and killed by antimicrobials. The mutation emerges with a fixed probability which is independent of the antimicrobials. We particularly collected the time of emergence of each steps if beneficial mutations to exam how likely a mutant could emerge in particular situations. We implemented the model (Equation 1-5) in R. For deterministic numerical analysis we used the package *deSolve*; for stochastic simulation we used the package *adaptivetau* implemented with Gillespie algorithm [35].

Results

The change of parmacodynamic parameters in mutants

We investigated how the parameters that determine pharmacodynamics influence the emergency of sequential mutants. We first investigated the increment of MIC. During the treatment, mutations will result in strains with different degrees of resistance. Our results show that mutants with small increment (less than 10 times of MIC of wild-type) and significant increment (100 times of MIC in each increment) in each mutational step will take much longer time to be fixed in the population. Strains with an intermediate increase in MIC (around 10-100 times of MIC to those of wild-type strains) will be fixed in much shorter time (Fig. 2B). When mutants develop high degree of resistance, it is likely to suffer fitness cost, which we refer as a decreased growth in drug free environment. Thus resistant strain with fitness cost will be outcompeted by the sensitive strain in drug-free environment. Compensatory mutations will emerge to finally restore the normal growth. It is interesting that when the compensatory mutation will most likely emerge in the sequential mutations. Our results show that the pattern of compensatory evolution is rather complicated. If the mutants suffer a fitness cost in early mutational steps, it would hinder its emergence. But fitness in latter mutational steps will not influence the time of emergence (Fig. 2C). Fig. 2C shows that fitness compensation will rise as lately as possible, while earlier compensation will hinder the subsequent beneficial mutations. Gradual compensating the fitness cost is also less likely as it will take much longer to arise. We simulated the changes of κ accompanied by increased MIC. The results should that either decreasing κ or increasing κ will not affect the time of resistance emergence (Fig. 2D). Additionally, we also found that changes of ψ_{min}, either increasing or decreasing, in the mutations will not affect their rising in population (Fig. 2E).

The dose-frequency and pharmacokinetics

We then studied how dosing frequency and pharmacokinetics affects resistance

evolution and treatment success in the background of multiple mutations. We evaluated the treatment success and development of MIC of whole populations factored by the dose, dosing frequency and pharmacokinetics. We found that higher dosing frequency of antimicrobials will facilitate treatment success (in terms of higher treatment success) in a lower treatment dose (Fig. 3). But once the treatment is not successful, the resistance will emerge quickly and develop faster than treatment in terms MIC increasing in lower treatment dose frequency (Fig. 3). The rate of drug clearance influence on above process in a similar way (Fig. 4). Lower rate of clearance will result successful treatment in lower dose, while also accelerate resistance evolution.

More importantly, our results showed that the mutant selection window (the range of concentration in which treatment failure is caused by the resistant strain) is independent of the treatment strategy and pharmacokinetics of drugs (Fig. 3&4). This implies that the capacity of resistance selection of antimicrobials is likely determined by the intrinsic factors of the drugs.

The AB *vs* AMP

With a particular focus on difference in pharmacodynamics (dose-response relations), we compare the resistance emergence in the treatment of AMPs and antibiotics in the multiple-step mutation model. The result showed that it takes much longer for mutants emerging from AMP treatment, in which the time of emergence is nearly two orders of magnitudes higher that in antibiotics (Fig 2F). Taken together, this indicates the time of resistance emergence is highly correlated to the pharmacodynamic properties of antimicrobials, especially the shape of dose-response curve (κ). This also result a narrow range of concentration in antibiotics with higher κ, which will select resistant as a main cause of treatment failure (Fig. 3&4).

Discussion

In this study, We extended our previous model to a stepwise mutational model, which allows us to define the resistant mutants with different set of pharmacodynamic parameters rather than only changes in MIC [10, 19]. Dynamics of evolution under strong selection demonstrated that the degree of resistance in a population usually increases in a stepwise fashion [16, 36]. This indicates beneficial mutants emerging through mutation which takes time to emerge and establish. This can be confirmed by the genotype-phenotype analysis [37]. For example, bacterial resistance to beta-lactam is only provided by a few mutations, out of a 120 accessible pathways [16]. We not only define the mutants by changes in fitness under a particular environment or concentration, but also map the mutants into changes of the pharmacodynamics that covers the range of concentrations.

In our simulation, we assumed relative low concentrations (around MIC of sensitive strain) to "treat" the infection and monitor the emergence of subsequent resistant strains. The growth rate of bacteria in the continuum of concentration is controlled by the pharmacodynamics. When the concentration is below the MIC, bacterial populations show positive growth; when the concentration is above MIC the population suffers declining as the growth rate is negative. We project this framework into the evolution framework of mutation-selection in which resistance evolution in concentrations below MIC fits into framework of mutation-selection regime. Assuming a resistant strain would gain more fitness, the selection coefficient for beneficial mutants lies between the maximal growth rate and the growth rate controlled by the pharmacodynamics. However, when the concentration is above MIC, the situation of evolutionary rescue applies. As the population will decline, mutations with absolute increase in MIC will be immediately selected and take over the population. The probability of fixation of beneficial mutants is a function of total population and the time [38]. In the range of concentration the population is controlled by the killing rate of antimicrobial peptides, the other pharmacodynamics

properties except MIC in resistant strains become not important. Instead, the κ value and ψ_{min} in our model are critical for the fixation of beneficial mutants. Increasing both parameters will leading the decreasing of the probability of fixation.

Using our frame work we investigated the increase of MIC in sequential mutants. We found that intermediate increase of MIC in the mutants will most likely to be selected as it takes much shorter time to emerge in our results. Experimental evolution on the spatial gradient of antibiotics also revealed fastest rate of resistance evolution in medium-increase concentrations [39]. In our stepwise mutation model, the concentration around MIC used in our simulation will also kill the mutants with small increase in MIC. Although mutant with large increase in MIC will maintain, if without fitness cost, the maximum growth, it leaves little marginal fitness increase for the emergence of future mutations unless the fitness excessing those of their ancestors. Thus, the mutants with medium increase in MIC will be most likely selected.

Increasing in MIC is usually accompanied by reduction of fitness of mutants [40-42]. We tested how the change of fitness in mutants would influence the emergence of future resistant. We simulated evolution with increasing in resistance with variation in fitness. Our results showed that fitness cost would increase the time of emergence for future strains, as reduced in growth rate leading to slow growing of population and longer waiting time to accumulate beneficial mutants. Thus, we propose that the fitness cost would most likely to appear lately. In reference [18] and [43], their results collectively showed that in most cases the fitness cost will appear lately in the mutants with relative higher resistance. Similar experimental evolution in malaria parasites demonstrated that accumulative increase in resistance by stepwise mutation will result fitness cost in later time [36].

Compensatory mutations of the fitness costs in resistant mutations are of great importance to the evolution of resistance [44, 45]. An unresolved question is when fitness costs will be compensated. Our model assumes that the fitness cost can be

maintained in further mutation and be compensated without loss of resistance. For example, MICs-increase associated fitness costs can be maintained in first two mutations and then compensated in third mutation. In addition, as the evolution of compensatory mutation is influenced by environment and genetic structure [46, 47], we particularly investigated its evolution under constant drug pressure. In that condition, our results showed that the fitness cost is likely to be compensated by latest mutations. Because the early compensation will hinder the emergence of future mutations, the gradual compensation is also less likely to happen.

Our results also showed that the κ value which controls the shape of dose-response curve and the ψ_{min} which depicts the maximum killing rate have no significant influence on the emergence of resistance strains. This is due to the concentration used in the simulation is around the MIC of sensitive strain. In this range of concentrations, resistant strains almost maintain the growth rate round ψ_{max}.

We also found that the treatment strategy and pharmacokinetic properties are important for the resistance emergence. Higher treatment frequency and lower clearing rate will result earlier resistance emergency. But the range of concentration that fails the treatment due to resistance emergence is nearly the same and independent of treatment strategy and pharmacokinetic properties. As we previously found, the mutant selection window might be only dependent on the pharmacodynamics, which describes how the antimicrobials interact with bacteria. Also, the MIC increase during selection also shares the same patterns. With a stress on the difference between antibiotics and antimicrobial peptides, we found the same trend as before, which implies AMPs are less likely to select resistance. When the concentration is high enough, AMP will rapidly kill bacteria and leave no resistance emergence.

Our model also allows us to investigate the evolution of antimicrobial resistance in a variable range of antimicrobial concentration with multiple steps of mutations. The

patterns of mutation and selection differ significantly in different concentrations. As what proposed before [10], we connected the fitness of any mutants across the range of concentration by the pharmacodynamics [25, 26].

The treatment strategy and pharmacokinetics play critical role in emergence and evolution of resistance. It is not surprising that high treatment frequency and lower drug-clearing rate will facilitate the evolution of resistance as they maintain drug concentration with a relative high level. However, the range of concentration that will select the resistance differs in antibiotics and AMPs. As noted widely, bacteria rapidly develop resistance to antibiotic than AMPs. This might be due to the narrower mutation selection window in AMPs.

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Figures

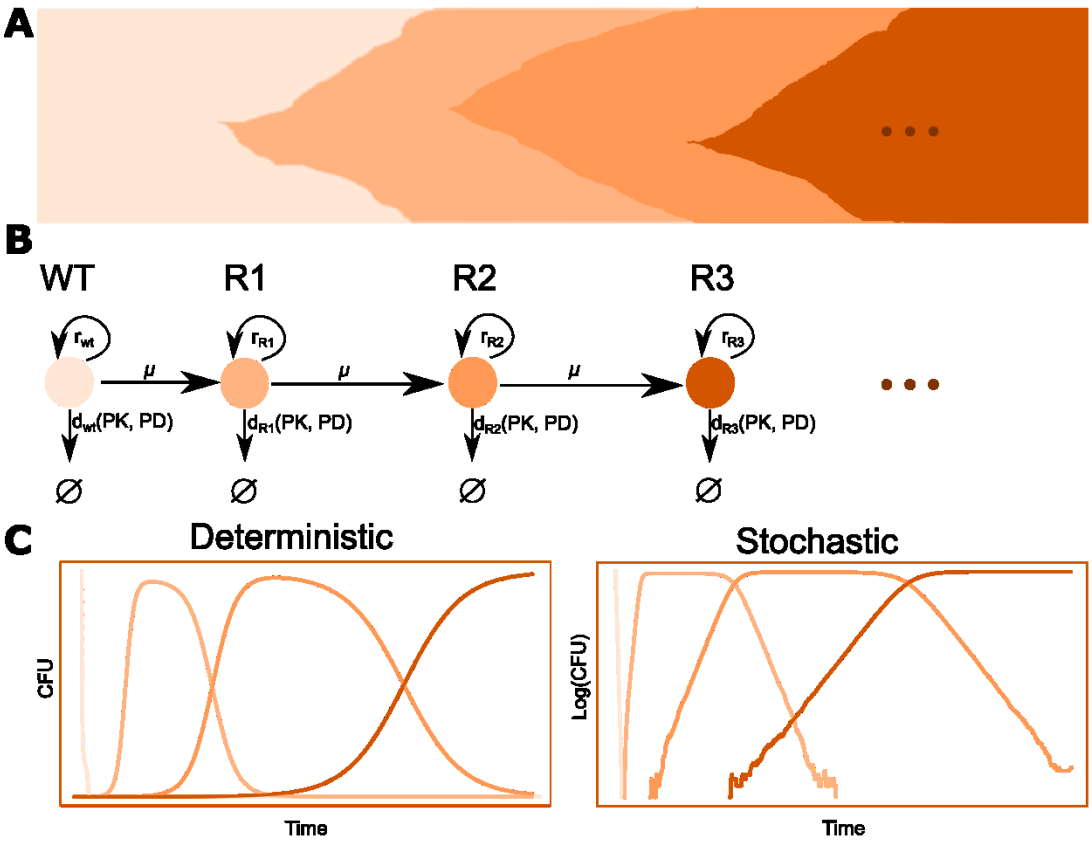

Figure 1. (A) The schematic view of evolutionary model with multiple step of mutation. Here, we only consider the most beneficial mutation which will take the whole population, thus we assume that higher resistance are evolved through steps of mutations. Each mutation increases certain level of resistance. (B) We assume the population dynamics of each mutation is controlled by a unique pharmacodynamics (equation 1) which describes the rate of self-replication, rate of death killed by antimicrobials and constant mutation rate that generates more resistant strain. (C) A deterministically and stochastically numerical realization of above model showed the dynamics of strains of mutations. Stochastic realization allows us to estimate the time of resistance emergence of each mutation.

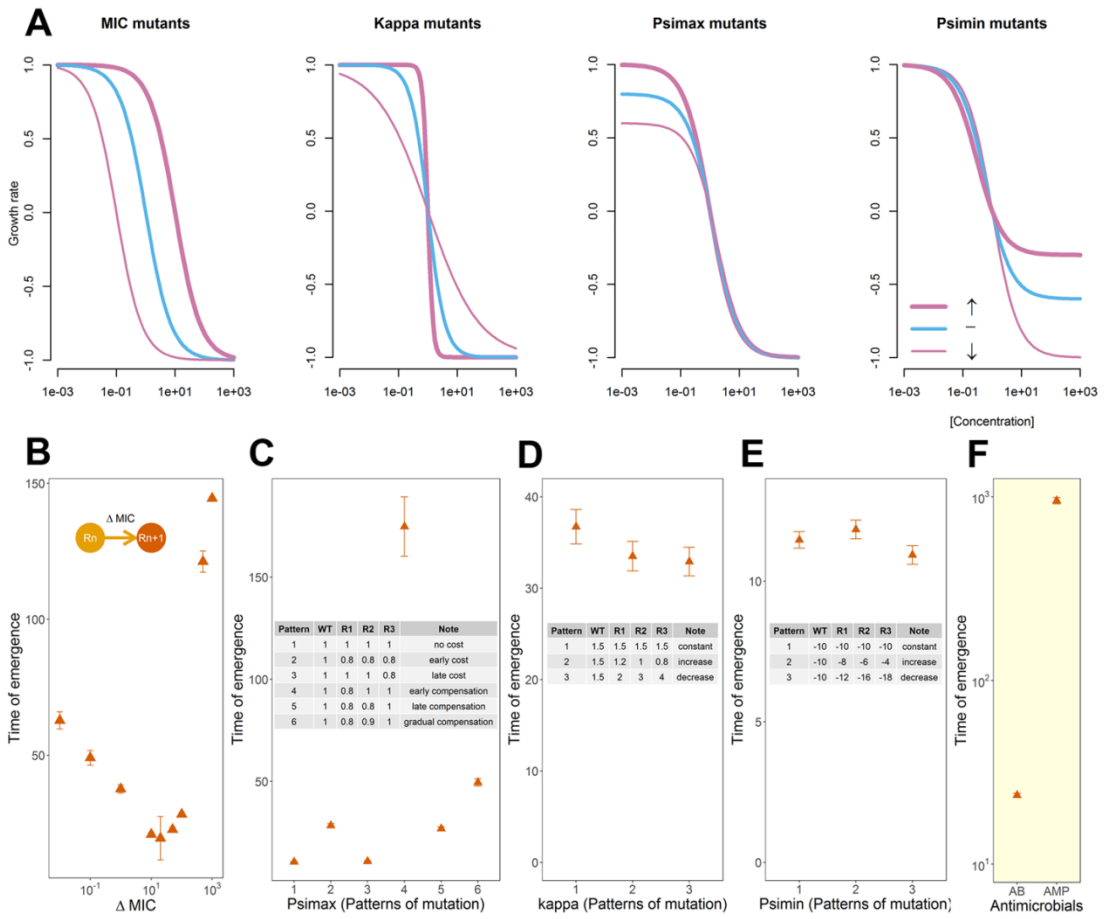

Figure 2. The time of resistance emergence in different parameters setting. (A) The definition of mutants is characterized by the changing of different pharmacodynamics parameters. (B) Medium increase in MIC between two consecutive mutations would take the shortest time to emerge. Mutants with both small increase in MIC and large increase in MIC will emerge lately. (C) Early fitness cost slightly delays the future resistance emergence. Fitness cost is likely to be compensated in late mutants as early compensation significantly delay the resistance emergence.(D, E) Mutants with alternated K and ψ_{min} do not affect the emergence of future mutations. (F) When incorporating the changes in mutants, we showed that resistance emerging in AMPs treatment is more than 10 times lower than antimicrobial treatment.

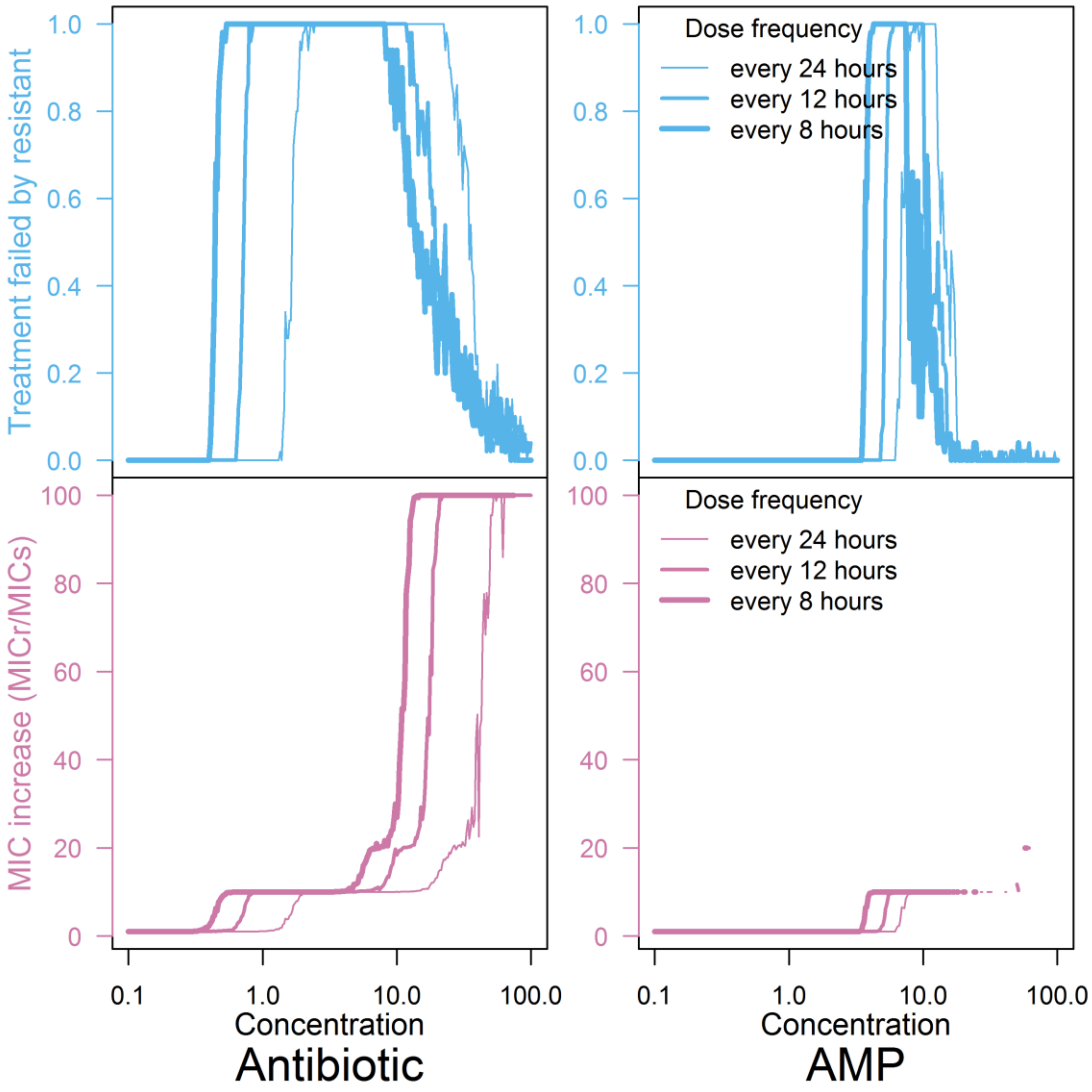

Figure 3. Dosing-frequency has a significant influence on treatment success. When compared with low-frequency treatments, high frequency of treatment will result resistance-induced treatment failure in lower concentrations and reach treatment success in a lower concentration. But the range of concentration that results resistance-caused treatments is independent of the treatment frequency. Instead, it is depended on the pharmacodynamics, where AMPs has narrower range of concentration. The increase of resistance shares the same patterns as treatment success with stress that bacteria are not likely to evolve to higher resistance.

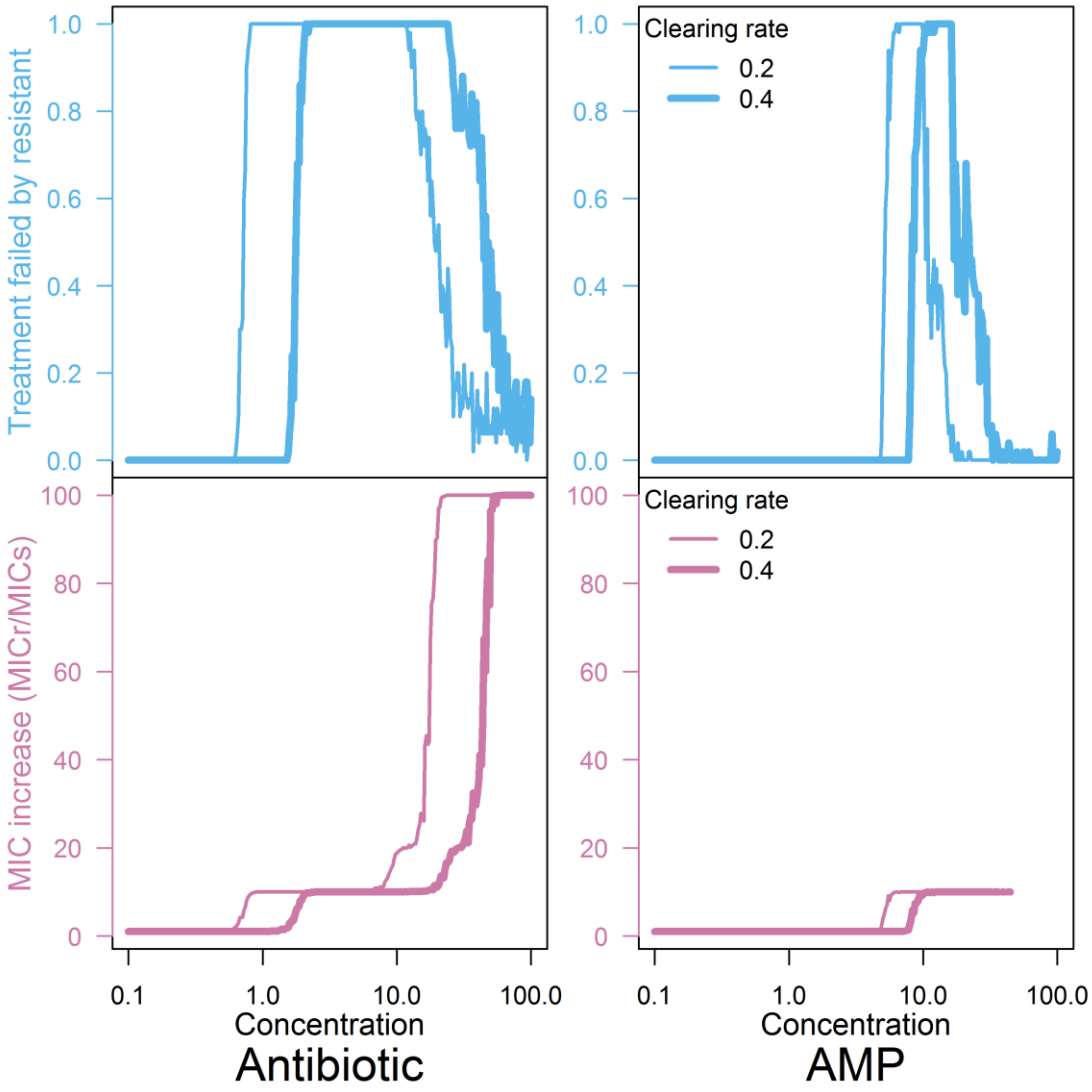

Figure 4. The influence of pharmacokinetics on the success of treatment and resistance evolution.

Appendix

Table S1. Parameters and their description in this study.

Parameters	Unit	Description
A	×MIC	The concentration of drugs
Ψ	h^{-1}	The growth rate of bacterial population
R	h^{-1}	Growth rate of bacteria without drug stress
MIC	-	Minimal inhibitory concentration
K	-	Shape parameter of pharmacodynamic curve
D	h^{-1}	Death rate of bacterial population
C	-	Cost of resistance
N	CFU	Population size of a particular strain
N_{tot}	CFU	Population size of all strains
K	CFU	Capacity of system
M	-	Mutation rate
k_a	h^{-1}	Rate of drug absorption
k_e	h^{-1}	Rate of drug decay
D	-	Dosage of a given drug
τ	h^{-1}	The dose frequency

Chapter 7

Concluding remarks and outlook

General conclusion

This thesis mainly focuses on the interaction of antimicrobial peptides (AMPs) and the predictive factors that influence resistance evolution in antimicrobials.

The research reported in chapter 2 has investigated the interaction between different AMPs. Testing with six AMPs from different organisms in two-way and three-way combination, A broad and strong synergism was observed between these combinations, which are measured in the framework of Loewe Additivity combined with pharmacodynamics. The results demonstrated a general effect of combination in in natural immune systems. They imply that innate immune systems might evolve different anti-infective components, e.g. different AMPs, not only to attack different pathogens but also to reduce the total cost of immune response through synergism between these components [1]. The reduction of immune cost can be achieved by the synergism between different anti-infective components. This also suggests cutting down the expense and efficacy of treatment by using drug combinations with synergism. Recent results verified the synergism between AMPs in nature immune systems [2, 3]. In Bumblebee, Abaecin and Hymenoptaecin function synergistically leading to reduction in total cost for immune reaction [2]. Additionally, It is also very interesting to note that AMPs and antibiotics have distinct pharmacodynamics. In particular, AMPs or some peptides antibiotics have very steep dose-response curve which is captured by the Hill coefficient κ in the pharmacodynamics. This is partially due to their rapidly killing rate in high concentrations [4, 5].

Followed by a theoretical investigation in chapter 3, a multi-hit model was proposed to explain the synergism between AMPs. AMPs target on the cell membrane and change its integrity by forming pores [6]. The balance of attaching rate and detaching is critically important in determining the killing efficacy. The attaching rate is higher that detaching rate due to the absolute positive charge on AMPs. The model assume that bacteria membrane only bears limited numbers of AMP molecules before

decomposed by AMPs. Once the number of molecules reaches the limit (which we call “zombie stage”), bacteria will go into a lysis process and eventually die. The model is reasonably explained by the experimental data which shows that bacteria grow normally blow certain AMP concentration. Once the concentration is higher that the critical limit, bacteria will be killed in a fast rate [7]. This multi-hit model also can project to the mean-field model with Hill function. Combined with the framework of Loewe additivity and Bliss independence, the multi-hit model successfully predicted the combination effect of two different AMPs [8]. In addition, with details in molecular interaction, this model also illustrates the rational choices of frameworks when determining the combination effects of drugs [8]. For example, Bliss independence are more suitable for drugs with different targets and Loewe additivity are more suitable for drugs with the same targets.

The different pharmacodynamics between AMPs and antibiotics are found in chapter 2, such as the difference in maximal killing rate and shape of dose response curve. We propose that the difference in pharmacodynamics determining emergence and evolution of resistance. In chapter 4, the mutant selection window (MSW), including subMSW and traditionally defined MSW, was calculated based on the pharmacodynamics of antimicrobials. The traditional MSW was calculated based on the relation of fitness cost and degree of resistance. A theoretical model combining pharmacodynamics and population dynamics with mutation showed that the emergence of resistance is associated with κ, the dose-response relation, and ψ_{min} maximal killing rate of a given drug. In particular, steeper dose-response and higher maximal killing rate delay emergence of resistance. This is consistent with results of experimental evolution by parallel evolve bacteria in AMPs and antibiotics. And it indicates that the pharmacodynamics is important in determining the effect of antimicrobials and characterizing the resistance selection and evolution.

The definition traditional MSW theory is also based on the pharmacodynamics, but requires a clearly defined “mutant” [9]. Once the pharmacodynamic parameters of the

"mutant" and wild-type are measured, the MSW is then defined accordingly. However, the theory paid little attention to the difference in the pharmacodynamics of different antimicrobials. The model proposed in chapter 4 hopefully can fill this gap. As the resistant strain needs to emerge before taking over the population, the model thus only inspects the resistance emergence, which is considered the very first step of resistance development. From this point, it is important to compare how likely a given antimicrobial will allow resistance emerging across the range of concentration and to connect this relation to pharmacodynamics.

In chapter 4, only one mutational step is considered. High level of resistance to antimicrobials in bacteria is usually reached by multi-step mutation. Unlike the traditional definition, the mutants in the model are defined according to the changes of pharmacodynamic parameters. With the multi-step model, several evolutionary dynamics are discussed in chapter 5. An intermediate increment of MIC in each mutational step is favored in the resistance evolution as shown with shortest emerging time. This is consistent with experimental evolution with gradients of antibiotics [10]. In addition, fitness cost will delay emergence of resistance and likely be compensated lately. Mutants having alternated κ and ψ_{min} will not change the time of emergence in the condition of multi-step mutation. The κ and ψ_{min} only influence the emergence of future mutants, and not vice versa. Incorporated with treatment strategy pharmacokinetics, the results of multi-step model showed that dose frequency and rate of drug-clearing will change the pattern of resistance emergence at particular dose, but do not change the range of dosage that select resistance. The results suggest that selection and evolution of drug resistance are largely associated with pharmacodynamics of antimicrobials.

Outlook

The simple mathematical model combined easy accessible experimental data allows

us to obtain a general picture of antibiotic resistance evolution especially in the treatment of antimicrobials with different antimicrobials and treatment strategy. The beneficial mutants are rare when bacteria are under strong selection in antimicrobial treatment. It is important to characterize the fitness and the related pharmacodynamics parameters of those mutants, especially those with high degree of resistance. Furthermore, for some important antimicrobials, the most frequent mutational pathways that lead to highest resistance should be also characterized. This is important to develop a more predictable method which correlated degree of resistance to the time and certain treatment strategies.

Our models predict that resistance evolution under treatment is highly correlated to the important pharmacodynamic parameters, such as κ, the slope of dose-response curve. Previous experimental evolution using antimicrobials with distinct pharmacodynamics showed different patterns of evolution, for example, evolution in AMPs are particularly slow [11, 12]. More experiments need to be carried out to enhance the conclusion. The comparison should be done not only between AMPs and antibiotics but among antibiotics or AMPs with different pharmacodynamics. Thus, we would obtain a more general and predictive model that can be used in practice of drug development and treatment of infectious disease.

The results in this thesis probe some most important features of innate immune systems, in particular synergism among co-expressed antimicrobial peptides [1, 13]. Our in-vitro experiments suggest synergism can reduce the total cost of immune response, such as the absolute quantity of AMPs. However, this waits to be further tested *in vivo*. Moreover, it is also intriguing to investigate how synergism in immune system influence the other life-history traits of organism [14, 15] and host-pathogen coevolution.

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Author contributions

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G.Y., D.Y.B., R.R.R., and J.R. conceived and designed the experiments. G.Y. performed the experiments. G.Y. and D.Y.B. analyzed the data. G.Y. and J.R. wrote the manuscript.

Manuscript 2: Desiree Y. Baeder, Guozhi Yu, Nathanaël Hozé, Jens Rolff, and Roland R. Regoes. Antimicrobial combinations: Bliss independence and Loewe additivity derived from mechanistic multi-hit models. Phil. Trans. R. Soc. B (2016), 371: 20150294. DOI: 10.1098/rstb.2015.0294

R.R.R. initiated this project. D.Y.B., G.Y., J.R. and R.R.R. conceived the model. D.Y.B. wrote the R script. All authors took part in the analysis of the results. D.Y.B., R.R.R., J.R. and N.H. drafted the manuscript.

Manuscript 3: Guozhi Yu, Desiree Y. Baeder, Roland R. Regoes and Jens Rolff, Predicting drug resistance evolution: insights from antimicrobial peptides and antibiotics. Submitted.

All authors participated in the design and interpretation of the results. G.Y. was primarily responsible for the predictive modeling, D.Y.B. for the PD work. All authors contributed to the writing of the paper. J.R. wrote the first draft, R.R.R. led the mathematical work.

Manuscript 4: Guozhi Yu, Desiree Y. Baeder, Roland R. Regoes and Jens Rolff, The evolution of antimicrobial resistance in a model combining a multiple-step mutations and pharmacodynamics. In preparation

All authors participated in the design and interpretation of the results. G.Y.,D.Y.B., R.R.R and J.R. conceived the model. G.Y. wrote the R script and performed analysis of the results. G.Y. and J.R. drafted the manuscript. R.R.R. led the mathematical work.

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Desiree Y. Baeder, **Guozhi Yu**, Nathanaël Hozé, Jens Rolff, Roland R. Regoes. Antimicrobial combinations: Bliss independence and Loewe additivity derived from mechanistic multi-hit models. Phil. Trans. R. Soc. B 371, 20150294; doi: 10.1098/rstb.2015.0294.	2016
Guozhi Yu, Desiree Y. Baeder, Roland R. Regoes, Jens Rolff. Combination effects of antimicrobial peptides. Antimicrob. Agents Chemother. 60:1717–1724. doi:10.1128/AAC.02434-15.	2016

Conferences and presentations

Anti-effective PK/PD: integrating knowledge and innovating therapies, April 21, Vienna, Austria. (**oral presentation:** Predicting drug resistance evolution: antimicrobial peptides vs. antibiotics)	2017
PhD student Meeting: Conflict and cooperation–bridging evolution, ecology and immunology, March 16-18, Bautzen, Germany. (**oral presentation:** Predicting drug resistance evolution: antimicrobial peptides vs. antibiotics)	2017
AMP international symposium on antimicrobial peptides June 6-8, Montpellier, France. (**poster presentation:** Combination Effects of Antimicrobial Peptides)	2016