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

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Antimicrobial peptides (AMPs) and antibiotics reduce the net growth rate of bacterial populations they target. It is relevant to understand if effects of multiple antimicrobials are synergistic or antagonistic, in particular for AMP responses, because naturally occurring responses involve multiple AMPs. There are several competing proposals describing how multiple types of antimicrobials add up when applied in combination, such as Loewe additivity or Bliss independence. These additivity terms are defined ad hoc from abstract principles explaining the supposed interaction between the antimicrobials. Here, we link these ad hoc combination terms to a mathematical model that represents the dynamics of antimicrobial molecules hitting targets on bacterial cells. In this multi-hit model, bacteria are killed when a certain number of targets are hit by antimicrobials. Using this bottom-up approach reveals 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. While our approach idealizes the dynamics of antimicrobials, it provides a conceptual underpinning of the additivity terms. The choice of the additivity term is essential to determine synergy or antagonism of antimicrobials.

This article is part of the themed issue 'Evolutionary ecology of arthropod antimicrobial peptides'.

1. Introduction

Antimicrobial peptides (AMPs) are produced by virtually all multicellular organisms, for example by plants [1], insects [2] and humans [3]. Moreover, multiple AMPs are expressed in concert [4–8]. The co-expression is hypothesized to be due to synergy between AMPs [9,10].

Multiple agents are said to synergize if they are more effective in combination than one would expect by adding their individual effects. Thus, to experimentally determine if AMPs synergize or antagonize one needs to compare their observed combined effect with a reference model that predicts how individual effects would add up independently. To define such a reference model is surprisingly challenging [11]. In most instances, there is no unique reference model and its choice sensitively impacts whether agents are found to synergize or not.

In the case of pathogens, we are interested in how their growth can be inhibited. In this paper, we specifically define the effect of the inhibitor as the reduction of the pathogen’s growth rate. While we focus on the inhibiting effect of AMPs, the basic principles also apply to other important inhibitors, such as antibiotics and antibodies.
It has been shown that pathogen growth declines as a sigmoid function with the concentration of the inhibitor [9,12,13]. If the individual effects of inhibitors on pathogen growth were linear, instead of sigmoid, then they can simply be added to predict how the effects should combine without synergy (figure 1a). For nonlinear effect curves, however, the combined effect of two or more independent inhibitors is not necessarily obtained by a simple addition of the single effects.

There are two widely used proposals that describe how independent effects combine and that serve as reference models to determine synergy or antagonism: Bliss independence and Loewe additivity. Bliss independence is based on probabilistic arguments: the combined effect of two inhibitors on the survival is defined as the probability of being affected by at least one of the inhibitors [14]. Applied to the specific case of pathogen growth rates, Bliss independence is equivalent to adding all single effects of the various inhibitors.

Loewe additivity, in contrast, is built on the premise that an inhibitor cannot synergize or antagonize with itself [11,15]. Strictly speaking, inhibitor effects in combination are additive according to Loewe if two doses of the same inhibitor are added together. In combination, the dose of the first inhibitor is increased by the amount of the second inhibitor. Especially for nonlinear individual effect curves, this premise requires a sophisticated mathematical derivation. The complication arises from the fact that addition of inhibitors increases the effect by different amounts, depending on the amount of inhibitors already present. This is due to the slope of the nonlinear curve that is small for low and high inhibitor concentrations and large for inhibitor concentrations in between (figure 1b). As an example, addition of inhibitors at concentrations that by themselves already induce almost the maximum effect, i.e. at concentrations where the slope is small, do not increase the overall effect any more. While such behaviour could be interpreted as antagonism, according to Loewe it is defined as independent.

Loewe additivity is often used as a reference point for comparison with biological data. Loewe independence is equivalent to Bliss independence. For dependence, the combined effect is always higher according to Bliss independence than according to Loewe additivity. Of note, applied to linear effect curves, Loewe additivity and Bliss independence predict the same combined effects (figure 1a).

To resolve these discrepancies between the ad hoc combination terms, we developed a mechanistic mathematical model that describes the interactions between inhibitors at the level of molecules. In our models, the two inhibitors either target two distinct mechanistic components and thus interact in an independent fashion (molecular independence), or they target a single component in which case they interfere with each other at the level of individual molecules (molecular dependence).

Our approach is based on the multi-hit model framework originally introduced by Hedges [16] to examine the kinetics of colicin, an AMP produced by Escherichia coli. The multi-hit model idealizes the mechanistic pathways behind the inhibitor’s mode of action. According to this framework, a pathogen dies as soon as a certain threshold number of inhibitor molecules bind to or ‘hit’ pathogen receptors. We implemented independence by providing two distinct receptor types to which inhibitors can bind. In the alternative scenario of dependence, there is only one type of receptor for binding in our model.

We found that our multi-hit model simulating independence of the molecule action predicts combined effects equivalent to Bliss independence. For dependence, the combined effect is consistent with Loewe additivity. Our results

Figure 1. The shape of the pharmacodynamic function determines the difference between predicted combination effects. Here, we show predicted effects for both Bliss independence and Loewe additivity (§2) dependent on the number of inhibitor molecules. We plotted the combined effect for a 1:1 mixture of the two inhibitors, scaled by a factor 0.5 to adjust for the amount of inhibitors in combination. With this scaling, we are able to plot both the single effect curves and the combined effect curves using the same x-axis. (a) In the hypothetical case of linear pharmacodynamic functions describing single inhibitor effects of inhibitor 1 and 2, respectively, combined effects according to Bliss and Loewe are congruent (independence). Here, the effect in combination is equal to the sum of the two individual effects. The addition of the individual effects of inhibitor 1 and inhibitor 2 with $n$ molecules each is equal to the effect of the combination of the inhibitors with $2n$ molecules. (b) For realistically sigmoid-shaped pharmacodynamic functions of single inhibitor effects of inhibitor 1 and inhibitor 2, Bliss independence and Loewe additivity models predict different effects of inhibitors in combination. The grey area marks all effects that are antagonistic according to Bliss independence and synergistic according to Loewe additivity.
suggest using Bliss independence as a reference model when the inhibitors target distinct components. Loewe additivity is the reference model of choice if the inhibitors target the same component. In the specific case of AMPs, these results strongly suggest that Bliss independence should be used for AMPs that kill cells by forming pores. Loewe additivity, on the other hand, is the appropriate reference model for AMPs that destabilize the bacterial cell membrane by introducing positive charges (see §4).

2. Methods

(a) Mathematical description of single effects: the pharmacodynamic function

We describe the effect of a single inhibitor on the net growth rate of the bacterial population by a pharmacodynamic function (also known as $E_{\text{max}}$ or Zhi model) [12,17,18]. Formally, the effect $E(A)$ of the inhibitor present at the concentration $A$ is given by

$$ E(A) = \frac{E_{\text{max}} \cdot A^r}{EC_{50} + A^r}. $$

Here, $E_{\text{max}}$ denotes the maximum effect of inhibitor, $EC_{50}$ is the concentration of the inhibitor resulting in 50% effect of $E_{\text{max}}$ and $r$ is the Hill coefficient, which describes the steepness of the curve (see electronic supplementary material, figure S1).

Assuming that the bacterial population grows at a rate of $r_0$ without inhibitors, the net growth rate in presence of an inhibitor, $r(A)$, is

$$ r(A) = r_0 - E(A). $$

(b) Mathematical definition of Bliss independence and Loewe additivity

To define the net growth rate in the presence of two different inhibitors, we need to consider two different pharmacodynamic functions,

$$ r_1(A_1) = r_0 - E_1(A_1) $$

and

$$ r_2(A_2) = r_0 - E_2(A_2). $$

Here, $A_1$ and $A_2$ denote the concentrations of each inhibitor, and $E_1$ and $E_2$ their effects.

We used the pharmacodynamic functions $r_1(A_1)$ and $r_2(A_2)$ as input for the two ad hoc reference models: Bliss independence and Loewe additivity. With both reference models, it is possible to determine independent effects of inhibitors in combination based on the individual effects of the inhibitors. Applying the reference models, we obtained the net growth rates predicted by each reference model, $r_{\text{Bliss}}(A_1, A_2)$ and $r_{\text{Loewe}}(A_1, A_2) = r_0 - E_{\text{Loewe}}(A_1, A_2)$ as a function of the concentrations of both inhibitors, $A_1$ and $A_2$.

We define Bliss independence [14] simply as the sum of the single effects. The net growth rate under Bliss independence, $r_{\text{Bliss}}$, is then

$$ r_{\text{Bliss}}(A_1, A_2) = r_0 - (E_1(A_1) + E_2(A_2)). $$

Bliss independence is defined as the product of effects [14], with the effect specified as normalized survival [11,14] or normalized growth rate [19]. In our opinion, an additive definition is more appropriate if effects on the net growth rate are considered. The net growth rate is the derivative of the population size of the pathogen. Using this relationship, multiplicative population size effect become additive when the population growth rate is considered (for detailed derivation, see the electronic supplementary material, SI).

Loewe & Muishkevich stated that an inhibitor does not synergize with itself [15], which gives rise to the isobole equation [11] (electronic supplementary material, figure S2)

$$ 1 = \frac{A_1}{A_1^{\text{isoeffective}}} + \frac{A_2}{A_2^{\text{isoeffective}}}. $$

Hereby, $A_1^{\text{isoeffective}}$ and $A_2^{\text{isoeffective}}$ are the concentrations of inhibitor 1 and 2, respectively, that are as effective as a combination of both inhibitors with the concentration $A_1 + A_2$.

$$ A_1^{\text{isoeffective}} = EC_{50} \left( \frac{E_{\text{Loewe}}(A_1, A_2)}{E_{\text{max}}(A_1, A_2)} \right)^{1/K} $$

and

$$ A_2^{\text{isoeffective}} = EC_{50} \left( \frac{E_{\text{Loewe}}(A_1, A_2)}{E_{\text{max}}(A_1, A_2)} \right)^{1/K}. $$

The combined effect under Loewe additivity $r_{\text{Loewe}}$ is then

$$ r_{\text{Loewe}}(A_1, A_2) = r_0 - E_{\text{Loewe}}(A_1, A_2) = r_0 - E_1(A_1^{\text{isoeffective}}) - E_2(A_2^{\text{isoeffective}}). $$

This definition is problematic for mixtures of inhibitors where the combined effect exceeds the maximum effect of one of the inhibitors [20]. In this range, Loewe additivity predicts suppression, i.e. more of an inhibitor is required in combination than alone to achieve the same effect (electronic supplementary material, figure S3).

(c) Multi-hit model

(i) Model equations and parameter definitions

In the multi-hit model, the total bacterial population $N_{\text{tot}}$ is structured according to the number of inhibitor molecules attached to bacterial receptors [16]. The ‘pathogen receptors’ conceived by Hedges are not receptors in the biochemical sense of signal transmission, but represent any target site that the inhibitors in question can hit. A ‘hit’ comprises the mechanisms of action of the inhibitor, i.e. the travel to the target site, the attachment to the receptor and the subsequent biochemical reactions. Hereafter, we will use the terminology of Hedges who reduced a potential complex hit to the simple attachment of the inhibitor molecule to a receptor.

We built a multi-hit model with two inhibitor molecules (figure 2a). The number of molecules of each inhibitor type attached to cell receptors is denoted with $i$ and $j$, respectively. How we limit the range of $i$ and $j$ is described below.

For each number of attached inhibitors of type 1, $i$, and inhibitor of type 2, $j$, the temporal development of the subpopulation $N_{ij}$ is described by the following ordinary differential equation:

$$ \frac{dN_{ij}}{dt} = \phi(N_{ij}) - d_{ij}N_{ij} + \alpha_{1,ij}N_{i-1,j} + \alpha_{2,ij}N_{i-1,j-1} + \mu_{i+1,j}(i+1)N_{i+1,j} + \mu_{2,i+1,j}(j+1)N_{i+1,j+1} - \alpha_{i-1,j}N_{ij-1} - \alpha_{i,j-1}N_{i-1,j} - \mu_{1,ij}N_{ij} - \mu_{2,ij}N_{ij}. $$

Hereby, $\phi(N_{ij})$ is a term describing the bacterial replication. Based on the carpet model of AMP action [21,22], we assume that inhibitor molecules are distributed randomly on the pathogen cell surface and that the inhibitors stay attached when the cell is replicating. During replication, the inhibitors are redistributed to both daughter cells and each possible event of redistribution of $i$ molecules of inhibitor 1 and $j$ molecules of inhibitor 2 has a fixed probability $1/(i+1) \times 1/(j+1)$ (for detailed explanation, see
Cells enter into a zombi class when the respective killing threshold \( \tau_1 \) or \( \tau_2 \) is reached (figure 3).

(d) Pharmacodynamic parameter estimation

We solved the described differential equation (2.12) numerically to determine the net growth rate \( r(A_1, A_2) \) for any given combination of the two inhibitors. After a short phase during which the attachment classes converge towards a quasi-equilibrium, the growth dynamics is approximately exponential (with a possible negative growth rate). We calculate the net growth rate, \( r(A_1, A_2) \), from the rate of change of the total number of bacteria \( N_{tot} \) at the times \( t_1 = 80 \) min and \( t_2 = 120 \) min

\[
r(A_1, A_2) = \frac{\log(N_{tot}(t_2)) - \log(N_{tot}(t_1))}{t_2 - t_1}.
\]  

From these estimates of the net growth rate for various combinations of inhibitor concentrations, we derived the pharmacodynamic parameters \( r_0, E_{\text{max}}, EC_{50} \) and \( \kappa \). \( r_0 \) is determined by evaluating the multi-hit model without inhibitors present (\( A_1 = A_2 = 0 \)). \( E_{\text{max}} \) is calculated as the difference between \( r_0 \) and the net growth for high inhibitor concentrations (\( A_1 \to \infty \) and/or \( A_2 \to \infty \)). \( EC_{50} \) is estimated as the concentration...
that describes the following relationship best:

$$r_0 - \frac{E_{\text{max}}}{2} = r(\text{EC}_{50}).$$  \hfill (2.15)

To obtain $\kappa$ for one of the inhibitors, the derivative of the pharmacodynamic function with respect to the concentration of this inhibitor at the EC_{50}, $\frac{\text{d}r(\text{EC}_{50})}{\text{d}A}$, was solved for $\kappa$

$$\kappa = -\frac{4(\text{d}r(\text{EC}_{50})/\text{d}A)\text{EC}_{50}}{E_{\text{max}}}.$$  \hfill (2.16)

Using this relationship, $\kappa$ can be estimated from the rate of change of the net growth rate at the EC_{50} in our multi-hit simulations.

(e) Eigenvalue analysis
For only one type of inhibitor present, at a concentration $A$, the system of linear differential equations describing the multi-hit model (equation (2.12)) can be rewritten in the matrix form

$$\begin{bmatrix}
\frac{\text{d}N_0(t)}{\text{d}t} \\
\vdots \\
\frac{\text{d}N_i(t)}{\text{d}t}
\end{bmatrix} = M(A)\begin{bmatrix}
N_0(t) \\
\vdots \\
N_i(t)
\end{bmatrix},$$  \hfill (2.17)

where $N(t) = (N_0(t), N_1(t), \ldots, N_i(t))$ is the $(\tau + 1)$-dimensional vector representing the number of cells in each class at time $t$ and $M(A)$ is the $(\tau + 1) \times (\tau + 1)$ transition matrix representing the fluxes between the cell subpopulations $N_i$. The term $M(A)N(t)$ in equation (2.17) is therefore the time increment of the cells in the classes $N(t)$. $M(A)$ is given by

$$M(A) = \begin{bmatrix}
b - d - \alpha A & \frac{b}{2} + \mu & \frac{b}{3} & \ldots & \frac{b}{\tau - 1} & 0 \\
bA & b - d - \alpha A - \mu & \frac{b}{2} + \mu & \ldots & \frac{b}{\tau - 1} & 0 \\
0 & bA & b - d - \alpha A - \mu & \frac{b}{2} + \mu & \ldots & \frac{b}{\tau - 1} \\
0 & 0 & bA & b - d - \alpha A - \mu & \frac{b}{2} + \mu & \ldots & \frac{b}{\tau - 1} \\
\vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots \\
0 & 0 & 0 & 0 & \alpha A & bZ - dZ
\end{bmatrix}.$$  \hfill (2.18)

The exponential evolution of the number of cells as given by numerical solutions of the model allows solving the system of differential equations using a dominant eigenvalue approach. The maximal eigenvalue can be approximated by $r(A)$ and is the solution to the eigenvalue equation

$$M(A)y = r(A)y,$$  \hfill (2.19)

where $y$ is the eigenvector associated with $r(A)$. Equation (2.19) can be solved explicitly only in the case of no inhibitor or when an infinite number of inhibitor molecules are present. In the case of no inhibitor molecules, we obtain the number of pathogen cells in each class

$$N_0(A = 0, t) = N_0(A = 0, t = 0)e^{(b - d)t}$$

$$N_i(A = 0, t) = 0 \quad \text{for } 1 \leq i \leq \tau,$$  \hfill (2.20)

and when an infinite number of inhibitors of one type is present

$$N_i(A \to \infty, t) = N_i(A \to \infty, t = 0)e^{(bZ - dZ)t},$$  \hfill (2.21)

The net growth rate in the absence of inhibitors $r_0$ is therefore only dependent on the birth and death rates of class $i = 0$, which contains the cells without bound inhibitors: $r_0 = b_0 - d_0$. In the presence of an infinite number of inhibitor molecules, the class where the threshold-hit value $\tau$ is reached is dominating the system. For high inhibitor concentrations, the total number of bacteria $N_\text{tot}$ is dependent on the parameters of the zombi class only: $r_{A \to \infty} = \alpha A$. The maximum effect of the inhibitor $E_{\text{max}}$ is the difference between $r_0$ and $r_{A \to \infty}$: $E_{\text{max}} = (b_0 - d_0) - (bZ - dZ)$. No general formula for $r(A)$ can be obtained analytically for intermediate concentrations. We could, however, obtain numerical solutions.

(f) Implementation
The model equations are written in R (v. 3.1.3) [23] using RStudio (v. 0.98.1103) [24]. We solved equation (2.12) numerically with the package deSolve [25]. The code is available upon request.

3. Results
(a) Introducing additivity principles into the multi-hit framework
To relate the pharmacodynamic functions to the level of individual molecules involved in the interaction between bacteria and inhibitors, we used the multi-hit model framework proposed by Hedges [16]. In this model, we described the binding of inhibitor molecules to bacterial receptors. Here, receptors represent any target sites that inhibitors hit and the term 'hit' describes the processes of an inhibitor travelling to the target site, binding to a receptor and the mechanisms of action following the binding. We structured the bacterial population by the number of inhibitor molecules that are attached to receptors on the bacterial cells. The binding is governed by the attachment and detachment rate constants $\alpha$ and $\mu$. Bacteria divide at a rate $b_0$ and die at a rate $d_0$.

The multi-hit model owes its name to how the effect of the inhibitor is conceptualized: bacteria are assumed to die as soon as a certain number of inhibitors bind to or 'hit' them. This threshold number is denoted as $\tau$. We extended this framework by an additional zombi class. The zombi class is reached after $\tau$ inhibitor molecules are attached to a bacterial cell and describes a state in which the cell is doomed to die. We assumed that zombi cells cannot divide any more ($b_Z = 0$) and that zombi cells die at an increased
rate of \( d_Z \). This extension is necessary to obtain a finite maximum effect at very high inhibitor levels: without the zombi class, multi-hit models predict that bacterial growth rate declines for increasing inhibitor concentrations without boundary for increasing inhibitor concentrations.

We expanded the multi-hit approach to describe the effect of two distinct inhibitors. To this end, we structured the bacterial population with respect to the numbers of inhibitor molecules of both types that are attached to receptors on the bacterial cells. As a result, the variables describing these bacterial subpopulations feature two indices (see §2), instead of just one as for a single inhibitor.

To conceptualize killing in the two-inhibitor multi-hit model, we considered two scenarios: molecular independence and molecular dependence. In the first scenario, molecular independence, the inhibitors hit two different targets (figure 4d). Therefore, the effects of inhibitor 1 and inhibitor 2 are independent and consequently, two threshold numbers, \( \tau_1 \) and \( \tau_2 \), are required to characterize the effect of the inhibitors. We also defined two zombi classes with birth and death rates \( b_{Z1}, b_{Z2}, b_{Z12} \) and \( d_{Z1}, d_{Z2}, d_{Z12} \) (figure 4b). This scenario is equivalent to assuming that the two inhibitors attach to distinct receptor types on the surface of the bacterial cell. On a more abstract level, molecular independence is a simplistic description of inhibitors that affect different bacterial components.

In the scenario of molecular dependence, we assumed that it does not matter which inhibitor molecule ‘hits’ the bacterial cell target. This scenario is characterized by a single threshold parameter \( \tau \) and a single zombi class (figure 4c) with birth and death rates \( b_Z \) and \( d_Z \), and is equivalent to assuming that the two inhibitors attach to the same receptor type (figure 4d).

To quantify the effects predicted by the two-inhibitor multi-hit model, we determined the total population size for two time points \( t_1 \) and \( t_2 \) under different inhibitor concentrations by numerical simulations of the model (see §2). All parameter values used are listed in table 1. The total population size is the sum of all living bacterial subpopulations, including zombi classes. The increase or decrease of the total population size between the two time points was used to estimate the net bacterial growth rate.

We compared the net growth rates from the multi-hit model with the prediction of the ad hoc combination terms. To calculate the combination terms, the pharmacodynamic parameters characterizing each of the two inhibitors are required. To estimate these, we fitted the pharmacodynamic function to net growth rates obtained under single inhibitors in our multi-hit model. Figure 4c,f shows these fits. The functional form of the effect derived from the multi-hit model agrees surprisingly well with the predictions of the pharmacodynamic function, although there are subtle deviations for concentrations above the EC50 when the effect starts to saturate.

(b) Linking the multi-hit model to pharmacodynamic functions

Using a dominant eigenvalue approach (see §2e), we derived that, in the absence of inhibitors, the bacterial population grows at a net rate of \( b_0 - d_0 \) in the multi-hit model. This parameter combination corresponds to the pharmacodynamic parameter \( r_0 \). Furthermore, we showed that the maximum effect in the multi-hit model is given by the death rate in the zombi class \( (b_0 - d_0) - (b_Z - d_Z) \), which parallels the pharmacodynamic parameter \( E_{\text{max}} \). The number of inhibitor molecules \( \tau \) needed for bacterial cell killing in the multi-hit model is related to the pharmacodynamic parameters \( \kappa \)—describing the steepness of the pharmacodynamic relationship—and EC50—the concentration which affects the net growth rate by 50% of \( E_{\text{max}} \). The relationship between \( \tau \) and EC50 can be extracted only numerically, and is depicted in figure 5. The numerical solutions of equation (2.19) show that EC50 increases linearly with \( \tau \) and that the Hill coefficient \( \kappa \) raises to an asymptotic value for large \( \tau \). When \( \tau \) increases, the net growth rate curves thus keep a similar shape but are shifted to the right: the larger \( \tau \), the higher the concentration of inhibitors has to be to obtain the same effect.

(c) Multi-hit approach provides mechanistic underpinning of additivity reference models

We compared the multi-hit model with the two ad hoc additivity reference models Bliss independence and Loewe additivity. The independent-hit model gives rise to a complex pattern of isoboles (lines of equal effect), convex for low while concave for high concentrations (figure 6a—note the linear scale for the inhibitor concentrations). Despite these complexities, the pattern agrees in great detail with the predictions of Bliss independence (figure 6b). There is a small deviation between the predictions from the two approaches around (and not just above) the EC50 (electronic supplementary material, figure S4e).

According to Bliss independence, the maximum effect is the sum of the two individual maximum effects: \( E_{\text{max} \, 1,2} = E_{\text{max} \, 1} + E_{\text{max} \, 2} \). This can be accomplished in the multi-hit model with molecular independence by choosing the death rates of the three zombi classes appropriately. An agreement with the prediction of Bliss independence is obtained by setting the death rate of the double zombi class to the sum of the death rates in the single zombi classes minus the death rate in the other classes: \( d_{Z \, 12} = d_0 + d_{Z \, 1} + d_{Z \, 2} \) (for derivation, see electronic supplementary material, S3). Thus, to achieve a quantitative agreement with Bliss independence, the death rate parameter in the double zombi class \( Z_{12} \) must be higher than in the single zombi classes. This implies that even cells doomed to die from one of the inhibitors can be driven to death at an accelerated rate by the additional effect of the remaining inhibitor. Importantly, Bliss independence is consistent with only the very particular form of molecular independence in which the zombi classes are characterized by the equation above.

We found that molecular dependence predicts linear isoboles when the net growth rate is plotted against both the inhibitor concentrations (figure 6c). Combining the individual effects of the two inhibitors according to Loewe, we also obtained linear isoboles (figure 6d). As expected for Loewe additivity, the maximum effect of two inhibitors combined is the same as that of a single inhibitor. On a more quantitative level, the predictions of the dependent-hit model agree well with Loewe additivity, except for a small range of concentrations above the EC50 (electronic supplementary material, figure S4f)—similarly to the deviation between the multi-hit model and pharmacodynamic function for a single inhibitor.

4. Discussion

Bliss independence [14] and Loewe additivity [15], as well as the median effect equation for multiple drugs [26] are commonly used as reference models for the combined action of
two independent inhibitors [11,27]. In this paper, we concentrated on Bliss independence and Loewe additivity because, unlike the median effect equation, these two reference models can be applied to inhibitors with different Hill coefficients—a situation often encountered when the effect on the net growth rate of pathogens is considered.

Bliss independence and Loewe additivity are defined ad hoc from abstract principles. Using the multi-hit framework,
we linked these two common reference models to the mechanistic level on which the inhibitor molecules interact with molecular targets on the bacterial cell.

The results presented in this paper provide guidance for choosing the reference model that is most appropriate for a specific pair of inhibitors: Bliss independence should be the model of choice if distinct components are targeted, whereas Loewe additivity is more appropriate if the inhibitors target the same bacterial component.

The choice of the reference model is essential to determine if two inhibitors synergize or antagonize. Two or more inhibitors synergize if their combined effect is greater than the effect predicted by the reference model. If the combined effect is smaller than the predicted effect, then the combination...
Table 1. Parameter values used for the independent- and dependent-hit model. Unless otherwise specified, the parameter values listed in this table were used to obtain the results.

<table>
<thead>
<tr>
<th>parameter</th>
<th>value</th>
<th>unit</th>
<th>description</th>
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<td></td>
<td></td>
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<tr>
<td>$t_1$</td>
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<td>min</td>
<td>first time point at which the multi-hit model is evaluated</td>
</tr>
<tr>
<td>$t_2$</td>
<td>120</td>
<td>min</td>
<td>second time point at which the multi-hit model is evaluated</td>
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<td>$N_{o1}(t = 0)$</td>
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<td>number of cells in class with $i = 0$ and $j = 0$ at the time point $t = 0$ h</td>
</tr>
<tr>
<td>$N_{o2}(t = 0)$</td>
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<td>1</td>
<td>number of cells in all classes with $i &gt; 0$ and $j &gt; 0$ at the time point $t = 0$ h</td>
</tr>
<tr>
<td>$b$</td>
<td>0.71</td>
<td>h$^{-1}$</td>
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</tr>
<tr>
<td>$d$</td>
<td>0.01</td>
<td>h$^{-1}$</td>
<td>death rate of bacterial cells in all classes $N_{ij}$ with $i &lt; \tau_1$ and $j &lt; \tau_2$</td>
</tr>
<tr>
<td>$\alpha_1$</td>
<td>$10^{-10}$</td>
<td>h$^{-1}$</td>
<td>attachment rate of inhibitor 1 molecules in all classes $N_{ij}$ with $0 &lt; i \leq \tau_1$ and $0 &lt; j \leq \tau_2$. For $i = 0$, $\alpha_1 = 0$ h$^{-1}$</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>$10^{-10}$</td>
<td>h$^{-1}$</td>
<td>attachment rate of inhibitor 2 molecules in all classes $N_{ij}$ with $0 \leq i &lt; \tau_1$ and $0 &lt; j \leq \tau_2$. For $j = 0$, $\alpha_2 = 0$ h$^{-1}$</td>
</tr>
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<td>$\mu_1$</td>
<td>1.00</td>
<td>h$^{-1}$</td>
<td>detachment rate of inhibitor 1 molecules from all classes $N_{ij}$ with $0 &lt; i \leq \tau_1$ and $0 &lt; j \leq \tau_2$. For $i = 0$, $\mu_1 = 0$ h$^{-1}$</td>
</tr>
<tr>
<td>$\mu_2$</td>
<td>1.00</td>
<td>h$^{-1}$</td>
<td>detachment rate of inhibitor 2 molecules from all classes $N_{ij}$ with $0 \leq i &lt; \tau_1$ and $0 &lt; j \leq \tau_2$. For $j = 0$, $\mu_2 = 0$ h$^{-1}$</td>
</tr>
<tr>
<td>independent-hit model parameter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>5</td>
<td>1</td>
<td>number of inhibitor 1 molecules attached to the bacterial cell necessary to kill the cell</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>3</td>
<td>1</td>
<td>number of inhibitor 2 molecules attached to the bacterial cell necessary to kill the cell</td>
</tr>
<tr>
<td>$b_{1,1}$</td>
<td>0.00</td>
<td>h$^{-1}$</td>
<td>birth rate of bacterial cells in zombi classes $N_{ij}$ with $i = \tau_1$ and $j &lt; \tau_2$</td>
</tr>
<tr>
<td>$b_{1,2}$</td>
<td>0.00</td>
<td>h$^{-1}$</td>
<td>birth rate of bacterial cells in zombi classes $N_{ij}$ with $i &lt; \tau_1$ and $j = \tau_2$</td>
</tr>
<tr>
<td>$b_{2,1}$</td>
<td>0.00</td>
<td>h$^{-1}$</td>
<td>birth rate of bacterial cells in zombi class $N_{ij}$ with $i = \tau_1$ and $j = \tau_2$</td>
</tr>
<tr>
<td>$d_{1,1}$</td>
<td>4.00</td>
<td>h$^{-1}$</td>
<td>death rate of bacterial cells in zombi classes $N_{ij}$ with $i = \tau_1$ and $j &lt; \tau_2$</td>
</tr>
<tr>
<td>$d_{1,2}$</td>
<td>3.00</td>
<td>h$^{-1}$</td>
<td>death rate of bacterial cells in zombi classes $N_{ij}$ with $i &lt; \tau_1$ and $j = \tau_2$</td>
</tr>
<tr>
<td>$d_{2,1}$</td>
<td>7.70</td>
<td>h$^{-1}$</td>
<td>death rate of bacterial cells in zombi class $N_{ij}$ with $i = \tau_1$ and $j = \tau_2$</td>
</tr>
<tr>
<td>$\alpha_{1,1}$</td>
<td>$10^{-10}$</td>
<td>h$^{-1}$</td>
<td>attachment rate of inhibitor 1 molecules in zombi classes $N_{ij}$ with $0 &lt; i$. For $i = 0$, $\alpha_{1,1} = 0$ h$^{-1}$</td>
</tr>
<tr>
<td>$\alpha_{1,2}$</td>
<td>$10^{-10}$</td>
<td>h$^{-1}$</td>
<td>attachment rate of inhibitor 1 molecules in zombi classes $N_{ij}$ with $0 &lt; j$. For $j = 0$, $\alpha_{1,2} = 0$ h$^{-1}$</td>
</tr>
<tr>
<td>$\mu_{1,1}$</td>
<td>1.00</td>
<td>h$^{-1}$</td>
<td>detachment rate of inhibitor 1 molecule in zombi classes $N_{ij}$ with $1 \leq i \leq \tau_1$. For $i = 0$, $\mu_{1,1} = 0$ h$^{-1}$</td>
</tr>
<tr>
<td>$\mu_{1,2}$</td>
<td>1.00</td>
<td>h$^{-1}$</td>
<td>detachment rate of inhibitor 1 molecule in zombi classes $N_{ij}$ with $1 \leq j \leq \tau_2$. For $j = 0$, $\mu_{1,2} = 0$ h$^{-1}$</td>
</tr>
<tr>
<td>dependent-hit model parameter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1, \tau_2$</td>
<td>5</td>
<td>1</td>
<td>number of inhibitor 1 and inhibitor 2 molecules necessary to kill a bacterial cell</td>
</tr>
<tr>
<td>$b_2$</td>
<td>0.00</td>
<td>h$^{-1}$</td>
<td>birth rate of bacterial cells in zombi classes $N_{ij}$ with $i = \tau_1$ and/or $j = \tau_2$</td>
</tr>
<tr>
<td>$d_2$</td>
<td>5.00</td>
<td>h$^{-1}$</td>
<td>death rate of bacterial cells in zombi classes $N_{ij}$ with $i = \tau_1$ and/or $j = \tau_2$</td>
</tr>
<tr>
<td>$\alpha_{2,1}, \alpha_{2,2}$</td>
<td>0.00</td>
<td>h$^{-1}$</td>
<td>attachment rate of inhibitor 1 and inhibitor 2 molecules in all classes $N_{ij}$ with $i = \tau_2$ and/or $j = \tau_2$</td>
</tr>
<tr>
<td>$\mu_2$</td>
<td>0.00</td>
<td>h$^{-1}$</td>
<td>detachment rate of inhibitor molecules from all classes $N_{ij}$ with $i = \tau_1$ and/or $j = \tau_2$</td>
</tr>
</tbody>
</table>

is called antagonistic. The deviation of the combined effect from the reference model provides a quantitative measure of the degree of synergy or antagonism based on either Bliss independence, such as in [28], or Loewe additivity, such as in [29–31].

Except for a few clear-cut cases in which one of the inhibitors is effective only in combination with the other [10,32], or in the case of suppression [19,33], the classification of a combination as synergistic or antagonistic depends on the reference model. For high concentrations of inhibitors, Bliss independence is the more conservative choice of a reference model if one seeks to determine synergistic effect. Loewe additivity is more conservative when determining antagonism.

Synergy and antagonism are deviations from the reference models, and conform neither to the molecular dependence nor the molecular independence scenarios of our multi-hit models. As an example, if an inhibitor targeting the cell membrane facilitates the travel of a second inhibitor to its molecular target within the cell, the rate at which the second inhibitor hits, $\alpha$, depends on the number of hits the cell has received from the first AMP—a clear instance of synergy. This type of interaction has been observed in the case of combinations of AMPs [10,32] and antibiotics [19]. The reference models describe additive interactions, and our multi-hit models are only used to describe additivity of mixtures based on the two approaches introduced by Loewe and Bliss.
The classification of mixtures as synergistic, additive or antagonistic is essential for successful treatment of pathogen infection. While it is obvious that the efficacy of synergistic mixtures is higher than for additive and antagonistic mixtures, synergistic combinations have been found to also favour emergence of resistant strains [34,35].

We generically referred to ‘inhibitors’, because our multi-hit model approach is applicable to a wide variety of effectors, such as AMPs, antibiotics and antibodies. Hedges [16] developed the multi-hit model for AMPs but did not consider inhibitor combinations. However, combinations of AMPs are common in host–microbe interactions [4–8]. The vast majority of AMPs are cationic, hence mostly targeting bacterial cell membranes that are negatively charged. They also often possess hydrophobic residues that result in interactions with fatty acids in the bacterial cell membrane. Most AMPs hence target the cell membranes and only very few AMPs enter the cells and target intercellular compounds [36]. Individual AMPs such as magainin can also interact with different targets on the cell surface [8], or, in the case of α-defensin and cathelicidin, mediate competition between bacteria [37].

Our multi-hit models reduce the molecular modes of action to a sequence of generic hits. Nevertheless, they can be applied to derive the reference model for a concrete pair of inhibitors. To this end, we need to assemble all the relevant molecular processes involved in the action of each inhibitor into a ‘hit’ and also need to gather information on how many hits are required for action (our parameter τ). For protegrin peptides that target the cell membrane, for example, a ‘hit’ would comprise the travel to the cell membrane and attachment. This AMP forms pores consisting of eight AMP molecules [38], and 100 pores are required for lethal cell damage [38]. Therefore, the threshold number of hits needed to kill the cell, τ, would be 800 for protegrin peptides.

To derive the reference model of a combination of two AMPs, we need to assess if the threshold number of hits of one of the AMPs depends on the number of hits the bacterial cell has already received from the other AMP. If we combine an AMP that targets the cell membrane with an AMP that hit intracellular targets, the two thresholds are independent of the number of hits of the other AMP. We would therefore apply the scenario of molecular independence, which we found to be equivalent to Bliss independence. On the other hand, if we combine two AMPs that both hit the cell membrane, we would apply the model of molecular dependence and would use Loewe additivity as the reference model.

Nevertheless, for a specific inhibitor pair, it might not always be easy to determine if their effects are truly independent or dependent, because our multi-hit models only describe molecular processes on an abstract level. Moreover, inhibitors may act in multiple ways which limit a straightforward categorization of modes of action. AMPs may use several mechanisms to kill target cells [39]. In such cases, we suggest considering the more conservative ad hoc term, which is either Bliss independence or Loewe additivity, depending on the individual pharmacodynamic curves and the concentrations of both inhibitors.

The multi-hit model approach should be applied with care to antibiotics and antibody inhibition. The efficacy of antibiotics is dependent on pathogen density [40] and in combination antibiotics display complex interaction patterns [41], neither of which we addressed in this study. Moreover, we also considered that the cells die after a certain number of hits are reached. While this may be a valid assumption for AMPs [9], many antibiotics inhibit the replication of pathogens in addition to killing them. To use the approach to describe the action of antibiotics will thus require to extend the multi-hit models by an effect on the replication rates [42]. This applies even more to antibiotics that primarily inhibit pathogen replication and induce death only through very specific mechanisms, such as antibody-dependent cell-mediated cytotoxicity.

Authors’ contributions. R.R.R. initiated this project. D.Y.B., G.Y., J.R. and N.H. 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. All authors revised the manuscript and gave final approval for publication.

Competing interests. We have no competing interests.

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