MuSyC is a consensus framework that unifies multi-drug synergy metrics for combinatorial drug discovery

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Drug combination discovery depends on reliable synergy metrics but no consensus exists on the correct synergy criterion to characterize combined interactions. The fragmented state of the field confounds analysis, impedes reproducibility, and delays clinical translation of potential combination treatments. Here we present a mass-action based formalism to quantify synergy. With this formalism, we clarify the relationship between the dominant drug synergy principles, and present a mapping of commonly used frameworks onto a unified synergy landscape. From this, we show how biases emerge due to intrinsic assumptions which hinder their broad applicability and impact the interpretation of synergy in discovery efforts. Specifically, we describe how traditional metrics mask consequential synergistic interactions, and contain biases dependent on the Hill-slope and maximal effect of single-drugs. We show how these biases systematically impact synergy classification in large combination screens, potentially misleading discovery efforts. Thus the proposed formalism can provide a consistent, unbiased interpretation of drug synergy, and accelerate the translatable ability of synergy studies.

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Throughout the preceding century, two principles have been used to quantify synergy of drug combinations: the Dose Equivalence Principle (DEP), introduced by Loewe1,2, and the Multiplicative Survival Principle (MSP), introduced by Bliss3. In 1992, a committee was convened in Saariselkä, Finland seeking to find a consensus between these principles and unify the field4,5. Unable to reconcile their differences, the committee’s conclusion (The Saariselkä Agreement) did not reach a consensus and simply recommended that drug combination studies explicitly state how synergy was calculated6,7. Multiple synergy models have since emerged, often derived as extensions of either the DEP or MSP, further splintering the field8-12. In the absence of a consensus framework for drug synergy, discovery efforts for combinations often calculate all available synergy metrics13-15, as first recommended by Greco and colleagues following Saariselkä16. However, there remains no basis for choosing one metric over another, which becomes particularly problematic when synergy metrics conflict. This “calculate everything” paradigm thus hampers reproducibility between studies, delays progress in the discovery of synergistic drug combinations, and negatively impacts the translatability of combination discovery efforts.

Despite the lack of consensus on how to quantify synergy, drug combination screens remain essential to both pharmaceutical and academic discovery efforts, as shown by recent studies by AstraZeneca and the NCI-DREAM consortia17,18, as well as combinatorial CRISPR screens19. Yet, the paucity of successful clinical combinations explicable by true pharmacological interaction, rather than patient-to-patient variability20, is symptomatic of the challenges facing the field. Therefore, the need identified at Saariselkä still exists: a consensus framework to interpret drug combination pharmacology.

We recently introduced a framework to quantify synergy based on the Law of Mass Action, named Multi-dimensional Synergy of Combinations (MuSyC)21, that distinguishes between different synergy types (e.g., potency, efficacy, efficacy). In the present work, we build upon our previous findings to show how MuSyC generalizes the DEP and MSP, thereby unifying the field of drug synergy, as sought at Saariselkä. Further, we map the landscape of current synergy metrics, including: Bliss Independence3, Loewe Additivity1, Combination Index (CI)22, Highest Single Agent (HSA)23, Effective Dose model6, ZIP7, a partial differential equation (PDE) Hill model by Schindler3, BRAID8, and the General Pharmacodynamic Interaction (GPI) model9. In mapping relationships between these various frameworks, we identified systematic differences impacting the interpretation of synergy in drug combination experiments. Specifically, we found: (1) the conflation of synergistic potency and efficacy masks synergistic interactions; (2) MSP-based frameworks are biased toward antagonism for drugs with intermediate efficacy; and (3) DEP-based frameworks contain a Hill-slope dependent bias. The Hill-slope bias results from satisfying the famous “sham” combination thought experiment, thus arguing against the merit of sham-compliance as a measure of validity for synergy frameworks. Using five large combination datasets25-29, MuSyC identifies real-world examples where the conflicting assumptions of previous drug synergy frameworks misleads or impedes drug discovery efforts through these pervasive and predictable biases. Additionally, we show that MuSyC uncovers two consequential errors in the highly cited CF22,30 which has been proposed as the standard Mass Action-based, synergy framework31. We therefore propose MuSyC as a consensus framework to interpret combination pharmacology and signify its broad applicability to the study of drug mixtures.

Results

A state-transition model to measure multi-drug synergistic effects. The 4-parameter Hill equation is commonly used to fit dose-response data from in vitro and in vivo assays (see Box 1 Eq. (10) and Table 1 for parameter annotation). Here we derive this equation from the equilibrium of a two-state transition model of drug effect based on the Law of Mass Action (Fig. 1A, left). Traditionally, the parameters of the Hill equation are interpreted as a drug’s efficacy ((E₀ − Eₑ) potency (C), and cooperativity (h), also known as the Hill slope. These parameters correspond to three possible geometric transformations of a dose-response curve (Fig. 1A, right). To generalize this one-drug formalism to two concurrent drugs, we developed a four-state transition model of combination pharmacology (Fig. 1B, left)12. From this model, we derive a two-dimensional (2D) Hill equation for two drugs (Box 1, Eq. (15)) defining a dose-response surface (Fig. 1B, middle). The 2D Hill equation contains five additional parameters, not present in the single-drug Hill equation, which measure different types of drug interactions. These additional parameters measure changes in a drug’s efficacy (β), potency (α₁₂ and α₂₁), and cooperativity (γ₁₂ and γ₂₁) in a combination—corresponding to three distinct types of synergy (Fig. 1B, right, Table 1). See Supplemental Code 1 and Supplemental Section Interactive MuSyC Jupyter Notebook for an interactive demonstration of the 2D Hill equation parameters. As we show below, these parameters are conflated in traditional synergy metrics (e.g. Loewe, Bliss, and HSA), as well as in recently proposed ones obscuring the true origin and magnitude of drug synergy or antagonism.

Mapping the landscape of prominent synergy models within a consensus framework. Multiple alternative synergy models have been proposed, most broadly derived from one of two guiding principles: the Multiplicative Survival Principle (MSP) or the Drug Equivalence Principle (DEP) (Table 2). Prior work has shown contradictory results when comparing between MSP and DEP frameworks2,12,32, and a lack of consensus remains on the commonality between the two principles2,7,9,11. Here we show MuSyC satisfies both the MSP and DEP under specific parametric constraints (Fig. 2A, B), thereby unifying the foundational principles of drug synergy.

The MSP was first described by Bliss3 and is the foundation of the Bliss Independence framework. MSP assumes the probability of a cell surviving treatment by drug 1 (U₁) is independent of the probability of the same cell surviving treatment by drug 2 (U₂). Therefore, the probability of surviving both Drug 1 and Drug 2 is U = U₁ ⋅ U₂. Synergy or antagonism occur when U ≠ U₁ ⋅ U₂. A method to define an alternative Bliss null model has been reported for growth-rate data as the sum of growth-rate inhibition33, but this formulation is uncommon, and not classified as MSP. MuSyC satisfies the MSP under the following conditions: (1) the effect metric is expressed as a percent (E₀ = 1, and E₁ = E₂ = E₃), (2) there is no potency synergy (α₁₂ = α₂₁ = 1), and (3) there is and no cooperativity synergy (γ₁₂ = γ₂₁ = 1) (Fig. 2A, see Supplemental Section Multiplicative Survival Principle for details).

The DEP was first established by Loewe1,2. DEP-based methods are characterized by linear isoboles (contours of equal effect) (Fig. S2A). A combination of doses d₁ and d₂ achieving effect E, satisfies the DEP when 𝑓 𝑑 (𝑑phinx) 1 1 2 1 2 + 𝑓 𝑑 (𝑑phinx) 1 1 2 1 2 = 1, where 𝑓 𝑑 (𝑑phinx) represents the monotherapy response of drug i. MuSyC satisfies the DEP under the following conditions: (1) the drugs’ actions are mutually exclusive (α₁₂ = α₂₁ = 0) and (2) h₁ = h₂ = 1 (Figs. 2B and S2B, see Supplemental Section Dose Equivalence Principle for details).

From the literature, we identified several prominent synergy models beyond Bliss and Loewie including: CF34, HSA23, Effective Dose model6, ZIP7, Hill PDE9, and GPIPD24. Table 2 compares
**Box 1 | Derivation of MuSyC**

Consider a reversible transition between an unaffected population (U) and an affected population (A) governed by

\[ U \xrightarrow{r_1 \cdot d} A \]

(7)

where \( d \) is the concentration of the drug, \( h \) is the Hill slope, often called cooperativity, and \( r_1 \) and \( r_{-1} \) are constants corresponding to the reaction rate (Fig. 1A). Applying the Law of Mass Action, steady state ratios of \( U \) and \( A \) are

\[
\frac{dU}{dt} = A \cdot r_{-1} - U \cdot r_1 \cdot d^h \equiv 0 \]

\[
\frac{dA}{dt} = \frac{r_1 \cdot d^h}{r_{-1}} \equiv (\frac{d}{C})^h
\]

(8)

When \( d = \left( \frac{C}{A} \right)^{\frac{1}{h}} \), then \( (A = U) \). This dose is commonly called the EC50 (herein denoted as \( C \)). Equation (8) is called the “median effect equation”, and has been shown to describe multiple distinct drug mechanisms of action.\(^{52} \) Because 100% of the population is either unaffected or affected, we also have the condition \( U + A = 1 \). This leads to the 2-parameter 1D Hill equation

\[ U = \frac{C^h}{C^h + d^h} = \frac{1}{1 + (\frac{d}{C})^h} \]

(9)

If the \( U \) and \( A \) differ by an observed effect (such as proliferation rate), the measured effect \( E \) at dose \( d \) will be a weighted average

\[ E = U \cdot E_U + A \cdot E_A \]

where \( E_U \) and \( E_A \) are the the effects characteristic of the \( U \) and \( A \), respectively. From this we find the final form of a 4-parameter Hill equation:

\[ E = E_U + E_1 \]

(10)

### 2D extension of the Hill equation for two-drug systems

Consider a system with 4 possible states, \( U_A, A_2, \) and \( A_{12} \) corresponding to populations that are unaffected, affected by drug \( 1 \) alone, affected by drug \( 2 \) alone, or affected by both drugs, respectively. The corresponding transitions between these states are:

\[ [U,N] = \begin{bmatrix} U_1 \ 
A_1 \ 
A_2 \ 
A_{12} \end{bmatrix} \]

(11)

Here, the \( \alpha \) parameters quantify the modulation of one drug’s EC50 (potency) due to the other drug. Similarly, the \( \gamma \) parameters measure the change of a drug’s Hill slope (cooperativity) due to the other drug.

As in the 1D case, finding the steady state of the system leads to the following system of equations

\[
\frac{dU_1}{dt} = -U_1 \cdot (r_1 \cdot d_1^h + r_2 \cdot d_2^h) + A_1 \cdot r_{-1} - A_2 \cdot r_{-2}
\]

\[
\frac{dA_1}{dt} = -A_1 \cdot (r_{-1} + r_2 \cdot (\alpha_2 \cdot d_2^h)) + U_1 \cdot r_1 \cdot d_1^h + A_2 \cdot r_{-2}
\]

\[
\frac{dA_2}{dt} = -A_2 \cdot (r_{-1} + r_2 \cdot (\alpha_2 \cdot d_2^h)) + U_1 \cdot r_2 \cdot d_2^h + A_1 \cdot r_{-1}
\]

\[
\frac{dA_{12}}{dt} = -A_{12} \cdot (r_{-1} + r_2 \cdot (\alpha_2 \cdot d_2^h)) + A_1 \cdot r_2 \cdot (\alpha_2 \cdot d_2^h) + A_2 \cdot r_2 \cdot (\alpha_2 \cdot d_2^h)
\]

(12)

At equilibrium, the Eq. (12) must all be equal to zero; however, the system only defines a rank 3 matrix. Taking the first three equations from (12) with the constraint \( U + A_1 + A_2 + A_{12} = 1 \), we define

\[
M := \begin{bmatrix}
- (r_1 \cdot d_1^h + r_2 \cdot d_2^h) & r_{-1} & r_{-2} & 0 \\
- (r_{-1} + r_2 \cdot (\alpha_2 \cdot d_2^h)) & 0 & 0 & (r_{-2})^h \\
- (r_{-1} + r_2 \cdot (\alpha_2 \cdot d_2^h)) & 0 & 0 & (r_{-1})^h \\
1 & 1 & 1 & 1
\end{bmatrix}
\]

(13)

such that

\[ M \cdot \begin{bmatrix} U_1 \ A_1 \ A_2 \ A_{12} \end{bmatrix}^T = \begin{bmatrix} 0 \ 0 \ 0 \ 1 \end{bmatrix} \]

or, solving for the proportions of each state,

\[ \begin{bmatrix} U_1 \ A_1 \ A_2 \ A_{12} \end{bmatrix}^T = M^{-1} \cdot \begin{bmatrix} 0 \ 0 \ 0 \ 1 \end{bmatrix} \]

(14)

If we again consider distinct effects \( E_0, E_1 \), and \( E_3 \) distinguishing populations \( U, A_1, A_2, \) and \( A_{12} \), we find the equation for the dose-response surface to be

\[ E = \begin{bmatrix} E_0 \ E_1 \ E_2 \ E_3 \end{bmatrix} \cdot M^{-1} \cdot \begin{bmatrix} 0 \ 0 \ 0 \ 1 \end{bmatrix} \]

(15)

As \( d \rightarrow \infty \) the equation reduces to

\[ E = E_3 + \frac{E_1 - E_3}{1 + (\frac{d_{EC50}}{C})^h} \]

(16)

by which we can see the 2D equation reduces to a 1D Hill equation at the boundaries (See Supplemental Section Proof of boundary behavior of 2D Hill equation).
Table 1 Annotation of MuSyC parameters.

| Parameter | Description |
|-----------|-------------|
| A1, A2   | Percent of affected population. |
| A12      | Percent of affected by drug 1 and drug 2, respectively. |
| d1, d2   | Drug concentrations for drug pair. |
| E1, E2   | Measured effect at (d1, d2). |
| C0, C1   | The concentration of drug required to achieve 50% of the maximal effect (i.e., EC50). |
| h0, h1   | Hill coefficients for dose-response curves of drug 1 and 2 in isolation. |
| E0       | The basal effect E0 (d1 = d2 = 0). |
| E1, E2   | Maximal efficacy of drugs 1 and 2 in isolation. |
| E3       | Maximal efficacy of the combination of drugs 1 and 2. |
| α, α2    | Percent increase (or decrease) in max effect with both drugs over the most efficacious single drug (β = E1/E0). |
| α01, α21 | Fold change in the potency (C0) of [d1] induced by drug 1, and drug 2, respectively. |
| γ, γ2    | Fold change in the cooperativity (h0) of [d1] induced by drug 1, and drug 2, respectively. |

Fig. 1 MuSyC is a mass-action, state-transition model of drug combination synergy. A Two-state transition model for a single drug system. The "unaffected" cells are indicated in red with the letter "U", while affected cells are indicated in cyan with "A1". The traditional equation for fitting dose-response relationships (middle) is the 4-parameter Hill equation. We derive this equation using the Law of Mass Action from a two-state transition model of drug effect (left). Edge notation is equal to the ratio of states’ percent occupancy at equilibrium (C1/C0) at dose (d). The Hill equation contains parameters measuring a drug’s efficacy (E0 – E1), potency (EC50), and cooperativity (h). Each parameter corresponds to distinct geometric transformations of the dose-response curve (right). B Two-drug model: MuSyC is derived from a four-state state-transition model of combination pharmacology (left) based on the Law of Mass Action and results in a 2D Hill-like equation describing a dose-response surface (middle). Cells affected by both drugs are indicated in the magenta circle and labeled "A12". Red to blue color gradient on the dose-response surface ranges from no effect (red) to maximum achieved effect (blue). The edge notations (left) refer to the ratio of the connected corners for the boundary condition. For example, edge #3 annotation means (C2/C0) → (C1/∞h) when d2 → ∞. Beyond the parameters of the single Hill equation, the 2D Hill equation has additional parameters (β, α, γ) corresponding to distinct transformations of the dose-response surface (right) (Video S1). These transformations describe changes in a single drug’s efficacy, potency, and cooperativity due to the combination, and, therefore, are interpreted as synergistic efficacy (β), synergistic potency (α), and synergistic cooperativity (γ). There are two values for α and γ because each drug can independently modulate the potency and cooperativity of the other6,7 (edge 3 vs. edge 4 of the state transition model). In contrast, the single β parameter describes the percent increase in maximal effect due to both drugs (effect E3 at A12). See Fig. S1 for MuSyC extension to three drugs.

generated synthetic dose-response surfaces using MuSyC (Eq. (15)) across a range of α and β values and calculated the synergy according to Loewe, Bliss, and Highest Single Agent (HSA) at the EC50 of both drugs (Fig. 3A, D, G and Video S2). In each case, many distinct sets of (α12, α21, β) are indistinguishable (e.g., the black contour line on the spheres).

Figure 3A shows that near the boundary between synergism and antagonism, Loewe is insensitive to changes in synergistic potency, tracking instead with synergistic efficacy. Consequently, in the anti-cancer dataset from O’Neil et al. 26, Loewe misses potency antagonism in combinations with synergistic efficacy (Fig. 3B middle distribution, see Fig. S5 for an example surface). This reflects Loewe’s assertion of infinite potency antagonism (α12 = α21 = 0, Fig. 2A) in its null model. Therefore, combinations that are antagonistically potent (α < 1) are all synergistic by Loewe in the absence of sufficient antagonistic efficacy (values above black contour in Fig. 3A). Indeed, Loewe is frequently synergistic even in cases of antagonistic potency and efficacy

Conflating synergistic potency and efficacy masks synergistic interactions. To determine how conflation of distinct synergy types impacts the interpretation of drug-response data, we...
mutually non-exclusive case. The mutually exclusive case has been widely adopted and is the model compared here. The concentration-dependent synergy of Bliss and Loewe is explored in Supplemental Section S2, Figs. S12–S16, and Supplemental Code 1, an interactive Jupyter Notebook. This notebook shows how different MuSyC synergy parameters may be reflected at specific concentrations of Bliss or Loewe synergy.

| Combination Principle | Multiple | MuSyC | HSA | GPDI (HSA) | Other |
|-----------------------|---------|-------|-----|------------|-------|
| Dose Equivalence Principle | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ |
| Effective Dose | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ |
| ZIP | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ |
| Hill PDE | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ |
| BRAID | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ |
| Leavens | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ | – – – – – | ✓✓✓✓✓ |

MSP is biased against combinations of drugs with intermediate efficacy. MSP frameworks, such as Bliss, explicitly expect drug effects to measure the “percentage of cells affected”, which is by definition bounded within the closed interval $E \in [0, 1]$. Nevertheless, dose-response data is usually not a measure of percent effect, but rather of relative percent effect. As an example, Bliss calculates the halofantrine (inhibits polymerization of heme molecules) reduces the potency of melfoxquine (targets phospholipids) against the multi-drug resistant malaria strain HB3 (Fig. 3F).

HSA is commonly thought to quantify synergistic efficacy. However, for antagonistically potent combinations, HSA cannot distinguish synergistic and antagonistic efficacy because it does not account for the topology of the dose-response surface (compare (log($\alpha_{12}$), log($\alpha_{21}$), $\beta$) = (-, -, +) and (-, -, -) quadrants of Fig. 3G and Video S2). In the anti-cancer combination dataset, Bliss will strictly classify a combination as either synergistic or antagonistic (Fig. 3E bottom distribution) despite the asymmetric interactions. As an example, Bliss calculates that halofantrine (inhibits polymerization of heme molecules) reduces the potency of melfoxquine (targets phospholipids) against the multi-drug resistant malaria strain HB3 (Fig. 3F).

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example, the synergistic efficacy of paclitaxel (targets microtubule stability) and mk-2206 (AKT inhibitor) in KPL1 cells is masked by Bliss’s high expectation for moderately efficacious drugs (Fig. 4E, gray plane). In several datasets, the magnitude of this MuSyC-predicted bias was sufficient to obfuscate many of the strongest synergies and antagonisms according to Bliss (Fig. S6A, B). Other MSP-based methods, such the Effective Dose model also assume data measures percent affect and fit a simplified 2-parameter Hill equation enforcing $E_0 = 1$ and $E_1 = 0$. This assumption can lead to poor fits of percent effect data for moderately efficacious drugs, and thus invalid synergy scores (Fig. S7). Therefore, the distinction between percent affect and percent effect is a critical component of MuSyC.

Re-examining the sham experiment: Sham compliance introduces Hill-dependent bias in DEP models. A new synergy model’s consistency is traditionally tested with the “sham” combination thought experiment. In a sham experiment, a single drug is considered as though it were a combination, with the expectation that the drug should be neither synergistic nor antagonistic with itself. DEP frameworks, characterized by linear isoboles, are known to satisfy the sham experiment, while MSP frameworks famously do not. In Box 2, we show MuSyC only satisfies the sham experiment when $h = 1$, which makes sense as MuSyC produces linear isoboles only in this condition (Fig. S2B and Eq. 25). Further, our analysis in Box 2 revealed that sham combinations exhibit unique biochemistry, only equivalent to true combinations in the case $h = 1$. When $h \neq 1$, combinations contain intermediate states representing mixed-inhibition (black circles, Fig. 5A, B). In sham combinations, these mixed-inhibition states are equivalent to single drug complete-inhibition states (Fig. 5A, cyan circles), while for real combinations, these are not equivalent (Fig. 5B). When $h = 1$, these intermediate mixed-inhibition states do not exist, explaining the concordance between sham and true
We expect when $h \neq 1$, enforcing sham compliance leads to predictable systematic biases. We expect when $h < 1$, DEP frameworks will overestimate synergy (Fig. S2C), and when $h > 1$, DEP frameworks will overestimate antagonism (Fig. S2D).

In combinations from the anti-cancer dataset, the average trend of Loewe synergy closely follows the Hill slope bias predicted by MuSyC (Fig. 5C). Further, subtracting the MuSyC-predicted bias from Loewe values for each combination results in a distribution independent of Hill slope (bottom panel). The bias toward synergy is particularly large for drugs with low Hill slopes. As an example, both doxorubicin (DNA damaging agent) and mk-4827 (PARP inhibitor) have small Hill slopes when applied to MBA-MB-436 cells, and their combination is synergistic by Loewe. However, using MuSyC, we see this combination is both antagonistically efficacious and antagonistically potent (Fig. 5D). In one dataset (Cokol et al.), this MuSyC-predicted bias revealed a screen-wide underestimation of synergy by Loewe (Fig. S6A,C).

**Fig. 3 Conflating potency and efficacy synergy masks synergistic interactions in large drug combination datasets.** A The colors on the sphere (radius on $\beta$ axis bottom left) represent the value of Loewe (colorbar to right) for a drug combination with a MuSyC synergy profile ($\alpha_{12}$, $\alpha_{21}$, and $\beta$) (axes bottom left). For all combinations: $E_2 = 1$, $E_1 = E_2 = 0$, $h_1 = h_2 = 1$, $d_1 = d_2 = C_0 = C_2$, $\gamma_{21} = \gamma_{12} = 1$. The solid line marks the boundary between Loewe synergy and antagonism. Along this contour, which includes many different sets of ($\alpha_{12}$, $\alpha_{21}$, and $\beta$), Loewe is the same ($-\log(\text{Loewe}) = 0$). Gray planes correspond to $\beta = 0$, $\log(\alpha_{21}) = 0$, and $\log(\alpha_{12}) = 0$. The hole in the upper-right quadrant represents sets for which Loewe is undefined. B Distribution of Loewe for anti-cancer drug combinations grouped by their synergy profiles according to MuSyC. Loewe was calculated as detailed in Methods section, including the Hill slope correction. The background color distinguishes antagonism (purple, “Ant”) from synergism (yellow, “Syn”). C The anti-cancer combination methotrexate and L-778123 is antagonistically potent and efficacious against HT29 cells, by MuSyC; however, it is designated by Loewe to be synergistic. Left panel shows the MuSyC-fitted dose-response surface, right panel shows the edges of the MuSyC surface. Color on the dose-response surface indicates effect (% Viable), with colorbar given next to the surface. On the right, the open circles mark the EC50 for each drug in isolation, closed circle is the shifted EC50 due to antagonistic potency. Brackets are 95% confidence intervals for each parameter based on Monte Carlo sampling (see Methods section). D Sphere for Bliss as in A. E Distribution of Bliss for anti-malarial drug combinations. Combinations for which each drug alone achieves $E_{\text{max}} < 0.1$ were selected, ensuring $E_1 \cdot E_2 \approx E_3 \approx 0$. Under this condition, the differences between MuSyC and Bliss are due only to asymmetric potency synergy (all combinations near the $\beta = 0$ plane in D). F Mefloquine increases the potency of halofantrine (red curves) but halofantrine decreases the potency of mefloquine (blue curves) in the HB3 strain of *P. falciparum*. G Sphere for HSA as in A. H Distribution of HSA for anti-cancer combinations grouped by MuSyC synergy profile. In antagonistically potent combinations, HSA can miss synergistic efficacy. I Combination of dexamethasone and mk-8669 in DLD1 cells is antagonistically potent, but synergistically efficacious.
Fig. 4 Bliss is biased against combinations of moderately efficacious drugs. A The null dose-response surface according to Bliss such that Bliss is zero at all doses for different single agent efficacy. $\Delta$ is defined as the expected increase in percent effect of the combination over the stronger single agent at saturating doses. The left and right panels have the same expected increase according to Bliss, $\Delta = 0.09$, while the combination of moderately efficacious drugs (middle panel) has a expected increase of $\Delta = 0.25$. The color of the surface indicates the drug combination effect, from no effect (green) to maximum (purple). The solid color lines on the left and back sides show the single-drug responses. B Calculation of $\Delta$ (colorbar bottom) for surfaces with different pairings of $(E_1, E_2)$. Color indicates the difference, with range given on the colorbar below the image. C Median Bliss for anti-cancer combinations$^{26}$ grouped by the maximal efficacy of their single agents. Ranges for each surface: cyan square: [0.35, 0.65], blue square: [0.1, 0.9], and magenta square: [0.0, 1.0]. Bliss is calculated at the maximum tested concentrations of both drugs. D Heatmap of the median Bliss score (colorbar left) for each combination across the cancer cell-line panel$^{26}$. Rows and columns are ordered by the average efficacy of each drug alone over all cell-lines ($E_{max}$) (bar graph top and right). Colored boxes correspond to groupings denoted in the legend (bottom). Boxplots show Bliss trends toward antagonism for combinations of moderately efficacious drugs (assuming approximate normality, one-sided $t$-test, blue < yellow < green < blue < green < yellow). The bottom and top of the boxes are the first and third quartiles, respectively. The red line shows the median. The whiskers extend to 1.5 times the interquartile range below and above the first and third quartiles. The boxplots represent $n=967$ biologically independent combinations (yellow), $n=6047$ combinations (cyan), and $n=7773$ combinations (green). E Dose-response surface of paclitaxel and mk-2206 in KPL1 cells$^{26}$. Gray plane is the expected effect of the combination over the stronger single agent at saturating doses. The left and right panels have the same expected increase according to Bliss, $\Delta = 0.25$. The color of the surface indicates the drug combination effect, from no effect (green) to maximum (purple). The solid color lines on the left and back sides show the single-drug responses. F Calculation of $\Delta$ (colorbar bottom) for surfaces with different pairings of $(E_1, E_2)$. Color indicates the difference, with range given on the colorbar below the image. G Boxplots show Bliss trends toward antagonism for combinations of moderately efficacious drugs (assuming approximate normality, one-sided $t$-test, blue < yellow < green < blue < green < yellow). The bottom and top of the boxes are the first and third quartiles, respectively. The red line shows the median. The whiskers extend to 1.5 times the interquartile range below and above the first and third quartiles. The boxplots represent $n=967$ biologically independent combinations (yellow), $n=6047$ combinations (cyan), and $n=7773$ combinations (green).

Therefore, satisfying sham compliance biases models toward synergy for drugs with low Hill slopes, regardless of with what these drugs are combined. This bias—which stems from enforcing a biochemical reaction scheme only appropriate for sham combinations—should be sufficient grounds for dismissing the sham experiment as a measure of a new synergy framework’s validity.

MuSyC reveals errors in the derivation and application of the Combination Index. Recent reports have identified potential flaws with the use of CI$^{11,36}$, yet it remains the most highly cited synergy metric$^{30,34,37}$. CI has recently been proposed to the Food and Drug Administration (FDA) and National Institutes of Health (NIH) as the de facto definition of drug synergy$^{31}$. Due of its prominence in the field, here we specifically examine its behavior with respect to the biases discussed above. We find CI and MuSyC have the same null model when $h_1 = h_2 = 1$, $\alpha_{12} = \alpha_{21} = 0$, $E_0 = 1$, $E_1 = E_2 = 0$ (Supplemental Section Relationship between different synergy frameworks). The presence of constraints on $h$ and $E$ indicates CI could be impacted by both a Hill-slope and efficacy range bias, like those we reported above for DEP and MSP-based frameworks, respectively. Like MuSyC, CI is based on the Law of Mass-Action, facilitating a direct comparison of their formulations. In doing so, we found two errors in CI with significant consequences: (1) a fundamental math error in its derivation involving combinations with $h \neq 1$ (Box 3, Fig. 6A–C), and (2) a fitting error that arises when applied to drugs with partial efficacy ($E_1$ or $E_2 > 0$) (Fig. 6D–F).

Details of the error in the derivation of CI are in Box 3. Because of this error, the CI equation is only valid for combinations of drugs with Hill slopes equal to one ($h_1 = h_2 = 1$). In this regime, MuSyC also results in linear isoboles (Fig. S2B). When $h \neq 1$ (Figs. 6A and S8A), the CI equation incorrectly factors the exponent outside the sum, introducing the exact same cross terms that we show (Box 2) are only valid for sham combinations, and not valid for all combinations when $h_1 \neq h_2 \neq 1$. Therefore, when $h \neq 1$, CI suffers from the same Hill-slope dependent bias we show in Fig. 5. We show the consequence of this bias in illustrative two-drug examples using synthetic (Figs. 6B and S8B) and experimental combinations (Figs. 6C and S8C).
Even when \( h = 1 \), CI requires that the maximal and minimal effects \( (E_0, E_1, \text{Fig. 1}) \) be fixed at 1.0 and 0. Subsequently, CI fits the single-drug dose-response curves using the two-parameter median-effect equation \(^{30} \) (Eq. (8)), see also Supplemental Section Percent Affect vs Percent Effect—Combination Index. In contrast, MuSyC is based on a four-parameter Hill equation \(^{31} \) (Box 1, Eq. (10)), and thus can describe metrics of drug effects with arbitrary ranges. It is common to observe in many dose-response assays, such as percent viability, drug effects that do not reach 0% \((i.e. \ E_1 > 0)^{38} \). For such assays, the two-parameter median-effect equation fits poorly (Fig. 6D). These poor fits lead to an effect-dependent error in CI quantification of synergy as observed in synthetic (Fig. 6E) and experimental combinations (Fig. 6F). We note, this effect-dependent error is not the same as the effect-range bias we report for MSP-based frameworks in Section “MSP is biased against combinations of drugs with intermediate efficacy”.

### Table 3 Summary of the datasets used for comparisons and validating theoretical predictions by MuSyC.

| Model                          | # of Combinations | Metric of drug effect | Effect range     | Refs. |
|-------------------------------|-------------------|-----------------------|------------------|-------|
| \( P. \) falciparum (Strains:3D7,HB3,Dd2) | 773               | Percent response      | [0,100]          | 25    |
| 37 cancer cell lines          | 22,738            | Percent viable        | [0,1]            | 26    |
| 60 cancer cell lines          | 330,064           | Percent growth        | \([-100, 100]\)   | 27    |
| HIV                           | 116               | Infectivity           | [0,1]            | 28    |
| S. cerevisiae                 | 175               | Area under growth curve | [0,484]         | 29    |

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**Fig. 5** Hill-slope dependent bias results from enforcing sham compliance DEP-based frameworks. A An illustration of the unique biochemistry of the sham experiment. The red circle represents an undrugged molecule with three binding sites. In a sham experiment, a drug is treated as though it were two separate drugs (green and blue polygons). Mixed states in which the binding sites are bound by both green and blue drugs (black circles) are equivalent to fully drugged states (cyan circles). We highlight three paths (green, blue, magenta arrows) that can be followed to reach a mixed-drugged state. These three paths correspond to the coefficient of \( c_{3} \) in Eq. (19) in Box 2. B In a combination of mutually exclusive drugs (triangle and polygon), targeting the same molecule, and with the same number of binding sites, the mixed states (black circles) are not equivalent to fully drugged (cyan circles) accounting for the discrepancy between MuSyC and the sham experiment (Box 2). C Loewe synergy is biased by Hill slope in the anti-cancer drug screen \(^{26} \). The orange shaded regions show moving window percentiles (window width is 0.1) of Loewe (10th through 90th percentiles, in steps of 10). The top panel shows how many data points are present in the window. The blue curve in the middle plot shows the median MuSyC-predicted bias as a function of the geometric mean of the Hill slopes (see Methods section). Subtracting the MuSyC-estimated bias (calculated for each data point) from Loewe yields the bottom plot. D The antagonistically efficacious and potent combination of mk-4827 and doxorubicin \(^{26} \) is misidentified as synergistic by Loewe, because both drugs in isolation have Hill slopes \( h < 1 \).
which are well-established quantities used to describe sigmoidal geometric transformations of effect. Further, because drugs 1 and 2 are the same, their synergy parameters describe directly to molecular inhibition, its synergy parameters describe based on the data measured and shape of dose-response curves.

In contrast, MuSyC frameworks facilitate rigorous investigation on a common landscape, MuSyC facilitates rigorous investigation of connections between these principles has remained unknown. Most synergy frameworks over the last century; however, the connection between these principles has remained unknown. Here, approaching combination pharmacology using the Law of Mass Action applied to a state-transition model results in a single framework unifying both principles. By mapping all frameworks on a common landscape, MuSyC facilitates rigorous investigation of oft-cited, contradictory conclusions between existing frameworks—contradictions that preclude reproducibility between synergy studies. Specifically, as is seen in Fig. 2C, there is no combination which can simultaneously satisfy the conditions required by both DEP and MSP synergy frameworks. Previous works advocate prioritizing combinations that are synergistic by all methods, choosing a synergy model carefully when the two drugs do not target the same molecule or are mutually exclusive or have the same number of binding sites, by far the preponderance of real combinations, the diagonal states are ill defined yet remain embedded in the sham equation.

One key advance of MuSyC, facilitating this unification, was the decoupling of $\alpha$, $\beta$, and $\gamma$. These synergy parameters correspond directly to classic, pharmacological measures of a drug’s potency, efficacy, and cooperativity. By calculating synergy in this way, interpretation of synergy does not depend on arbitrary expectations or thresholds. Rather, an alpha of 10 corresponds to a 10-fold increase in a compound’s potency, as a result of the other drug, regardless of whether we define $\alpha = 1$ or $\alpha = 10$ as the “threshold” for synergy. As practical advice for accurately fitting all synergy parameters, we recommend sampling $(d_1, d_2)$ around the four corners $(0, 0), (d_{1,max}, 0), (0, d_{2,max}), (d_{1,max}, d_{2,max})$ to best constrain synergistic efficacy, and around the four edges $(C_{1,0}, 0), (C_{1,d_{2,max}}, 0), (d_{1,max}, C_{2}), (d_{1,max}, C_{2})$ to best constrain synergistic potencies and cooperativities, where $d_{max}$ is asymptotically high dose of drug $i$. We refer interested readers to Supplemental Section “Interactive MuSyC Jupiter Notebook”, Figs. S12–S16, and Supplemental Code 1, which provide an interactive demonstration that shows how each synergy parameter results in different outputs across multiple concentrations. We envision distinguishing synergies of potency, efficacy, and cooperativity will be of differential consequence in alternate contexts. For example, in cancer synergistic efficacy may be most important, while for neurological disorders, synergistic cooperativity—i.e. sharp on-off drug response—may be preferred. In an analysis of clinical trials of combination therapies, we find highly cited CI including a mathematical, derivational error which impacts its reliability for synergy quantification.

The DEP and MSP have formed the foundational principles of most synergy frameworks over the last century; however, the connection between these principles has remained unknown. Here, approaching combination pharmacology using the Law of Mass Action applied to a state-transition model results in a single framework unifying both principles. By mapping all frameworks on a common landscape, MuSyC facilitates rigorous investigation of oft-cited, contradictory conclusions between existing frameworks—contradictions that preclude reproducibility between synergy studies. Specifically, as is seen in Fig. 2C, there is no combination which can simultaneously satisfy the conditions required by both DEP and MSP synergy frameworks. Previous works advocate prioritizing combinations that are synergistic by all methods, choosing a synergy model carefully when the two drugs do not target the same molecule or are mutually exclusive or have the same number of binding sites, by far the preponderance of real combinations, the diagonal states are ill defined yet remain embedded in the sham equation.

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synergistic efficacy is statistically higher in clinically efficacious combinations than clinically non-ef ficacious combinations (see Supplemental Section MuSyC statistically distinguishes efficacious and non-ef ficacious drug combinations in clinical trials based on combination screens, Figs. S9 and S10).

The relationship between MuSyC and the MSP and DEP frameworks (Fig. 2) is constrained by monotherapy parameters (E1, E2 for MSP, h for DEP). These constraints suggested systematic biases in MSP and DEP frameworks contingent on a single drug’s ef ficacy (MSP, Fig. 4) and Hill slope (DEP, Fig. 5). These systematic biases merit consideration when using these frameworks for drug discovery in large screens, or when accounting for batch-effects across different datasets (Fig. S6). Such systematic biases can confound machine learning models to predict synergy, decreasing their utility. Additionally, the constraint on h highlighted a discrepancy between the biochemistry of true sham experiments and real combinations. The centrality of the sham experiment in the drug synergy literature cannot be overstated; however, we argue enforcing sham compliance comes at the cost of improperly modeling real combinations, leading to a unpredictable Hill-dependent bias.

CI has previously been criticized for its procedure to fit the median-effect equation. Specifically, CI depends on a log-transformation in order to linearize the two-parameter median effect equation “in similar logic to the defunct Scatchard analysis in pharmacology, which has been replaced by non-linear modeling”36. That is, this log-transformation alters the noise profile such that small deviations for ef fects at low concentrations result in large deviations in the synergy calculations. Additionally, this transformation results in information loss, since undefined values for ef fects outside the range of (0,1) are forcibly removed, and as a consequence, CI synergy estimates become “statistically unstable” for noisy experimental data11. Beyond these valid points regarding CI’s practical application, here we uncover a mathematical error in the derivation of CI (Box 3) causing a systematic bias depending on the Hill slope. Because these flaws compound in non-linear ways, the expected error when applying CI is unique to each particular combination and assay design.

The prospects of higher-order synergies (i.e., interactions beyond pairwise) and scaling laws for drug mixtures, while provocative, have remained contentious6,12,39,40. MuSyC’s cubic geometry allows it to be easily extended to three or more drugs (Fig. S1), and we expect MuSyC will enable a more refined search for higher-order interactions. For instance, combinations that mix different synergy prof les (e.g., drugs 1 and 2 are synergistic, drugs 2 and 3 are synergistically ef ficacious) may exhibit different higher-order interactions than combinations all sharing a single synergy type. However, the number of synergy
parameters in MuSyC scales as \(2^N(N + 1) - 3N - 1\) (including \(\gamma\)) where \(N\) is the number of drugs (Fig. S1), and the commensurate data necessary to fully constrain MuSyC hyper-surfaces invokes a parameter identifiability problem ("the curse of dimensionality"). Nonetheless, MuSyC's geometry could be leveraged to guide sampling schemes to constrain the boundaries, allowing the solution to be built up step-wise from the boundaries (see Supplemental Section Proof of boundary behavior of the 2D Hill equation).

MuSyC expects single-drug dose-response curves to be sigmoidal well fit by the 1D Hill equation (Eq. (10)), and dose-response surfaces to be well fit by the 2D Hill equation (Eq. (15)). In our experience, these expectations are met by real data, as most single drugs have monotonic, sigmoidal responses, and even complex drug interactions can be modeled using various mixtures of \(\alpha, \beta, \text{and } \gamma\) (96% and 88% of combinations in anti-cancer and anti-malarial datasets had \(R^2 > 0.7\), respectively). However, it is possible for drugs to have multiphasic responses due to polypharmacology which are not well fit by a Hill curve. It may be possible to extend MuSyC to encompass such drugs—for instance by including a multiphasic Hill model or modeling effects of "partially affected" states (Fig. 5A, B and Box 3). In extreme cases, it may only be possible to apply non-parametric frameworks such as Bliss, Loewe, or HSA. Nevertheless we note that without fitting dose-response curves to a parametric model, these metrics are sensitive to noise in individual data-points. Replicate measurements may be able to reduce this sensitivity. Additionally, MuSyC assumes all drugs are administered concurrently, whereas patient treatments are often staggered. New theory and experimental methods are needed to address the synergy of combinations which are staggered temporally, bridging the synergy of pharmacodynamics with the synergy of pharmacokinetics. Finally, in the datasets we analyzed here, we did not find a role for synergistic cooperativity (\(\gamma\)). Future studies in other systems are needed to better understand situations when synergistic cooperativity is expected.

By viewing the landscape of drug synergy through the lens of mass-action, we have demonstrated the underlying assumptions, limitations, and biases of commonly applied synergy methods. We have shown how MuSyC unifies the DEP and MSP thus providing a consensus framework for the study of combination pharmacology. These findings provide much needed clarity to the
field and should promote the reproducibility of drug synergy studies across drug combination discovery efforts. Such a rigorous approach to the discovery and application of drug combinations will open the door to the discovery of new and previously discarded avenues for therapeutic mixtures.

Methods
We note the synergy calculations conducted for the different published datasets were not necessarily the same as those used in the original paper. Indeed the limitations of the current frameworks forced customized analysis for each publication highlighting the need for a consensus framework. However, in order to compare between datasets, we have calculated Bliss, Loewe, HSA, and other synergy frameworks, as described below, from the raw data.

Software
Implementation and website. A web application to calculate MuSyC synergy parameters for users’ data is available at https://musyc.labolx.xyz/. Experimental data are uploaded in comma-separated value (CSV) format; data format details and usage instructions are in the supplemental materials. The application fits dose-response surfaces using MuSyC and offers the results both as a CSV download of fit parameters, and interactive plots of the dose-response surface.

The web application uses the Django web application framework (djangoProject.com) and Python 3.7. Fitting tasks are processed asynchronously using a message queue (RabbitMQ; rabbitmq.com) and task-worker framework (Celery; celeryProject.org). Data are organized in a Postgres relational database (postgresql.org). The following packages were used for fitting, data analysis, or visualization: Scipy v1.1.0, NumPy v1.14.3, Pandas v0.23.0, Matplotlib v2.2.3, uncertainties v3.0.4.

Fitting 2D Hill equation. Here we describe fitting protocol for drug metrics where the metric of drug effect decreases as dose increases (E0 > E3) (e.g., anti-proliferative drugs); however, the framework is equally valid if increasing the drug corresponds to increases the effect (E0 < E3) (e.g., percent effect).

Previously, we found it necessary to use a Metropolis Hastings Monte Carlo (MCMC) seeded with a particle swarm optimization (PSO) to fit the 2D Hill equation15. This was prompted by the inconsistent performance of standard nonlinear least squares (NLLS) regression. In particular, we observed instances of calculated uncertainties in NLLS which were several orders of magnitude greater than the parameter value. This, we have discovered, is because the multi-collinearity between the Hill slope and the EC50 (\(\log C\)) depends on the slope of the line (\(h\)). The correlation is problematic when trying to estimate the parameter uncertainty from NLLS regression because \(h\) is estimated as the square root of the inverse Hessian, approximated to be \(J^TJ\) (where \(J\) is the Jacobian at the solution). (1)

\[
\log E_0 - \log(C_0/C_1) = h \log(d) - h \log(C)
\]

This correlation is problematic when trying to estimate the parameter uncertainty \(\sigma\) from NLLS regression because \(\sigma\) is estimated as the square root of the inverse Hessian, approximated to be \(J^TJ\) (where \(J\) is the Jacobian at the solution). (2)

\[
\frac{\partial I}{\partial E_0} - \frac{\partial I}{\partial C} = 0
\]

When the Hill slope is large (e.g., \(h > 5\)), the second two terms of the \(J\) cause the numerical approximation of the inverse of \(J\) to be undefined. This problem is compounded in the 2D Hill equation where the addition to \(h\) and \(C\) of the parameters \(a\) and \(y\) are co-linear. However, this does not affect the accuracy of the fitted parameter values from the NLLS regression—only the parameter uncertainty47. For the fitting the 2D Hill equation in this study, we adopted a Monte Carlo sampling approach to calculate the fit parameter uncertainty. This is significantly faster than our previous method (PSO + MCMC)15 while maintaining reasonable calculations of the parameter uncertainties accounting for the multi-collinearities described above. The Monte Carlo algorithm for fitting the 2D Hill equation is as follows. First, the 4-parameter 1D Hill equation (Eq. (10)) is fit to the dose-response of each drug in isolation. The fit uses the Trust Region Reflective (TRF) algorithm implemented in the curve_fit() module of the scipy optimization package. \(h\) and \(C\) were unconstrained while \(E_0\) and \(E_3\) are constrained for each dataset as annotated in the Methods section, data acquisition, preparation, and analysis. In general, adjusting the parameter bounds to closely match what is feasible for the given dataset will lead to better parameter estimates, helping the curve fitting algorithm to avoid becoming stuck in a suboptimal local minima. The initial 1D Hill fits provide estimates for \((E_0, E_3, E_C, C, H, I_2)\), because the 2D Hill equation becomes equivalent to the 1D Hill equation in the limit as \(d \to 0\). In practice, best fits of these parameters in the 2D Hill equation which have counterparts from the 1D Hill equation tend to be similar to their monotherapy fits published as used as initial guesses. However we note it is possible for these values to differ significantly from their monotherapy best guesses when the monotherapy data are noisy, and thus can have wide uncertainties. Next the 2D Hill equation (Eq. (15)) is fit using the TRF algorithm with initial values based on the 1D Hill equation fits and with bounds based on the parameter uncertainty calculated for the 1D Hill fits. The final values for parameters unique to the 2D Hill equation, \(E_{C12}, E_{C13}, E_{C12}^*, E_{C13}^*\), are \((\min(E_i, E_j), 1, 1.1)\). For all combinations \(r_1 = r_2 = 100\). The bounds for \(\log(E_0), \log(C_0)\) are set to \([-4, 4]\). From this initial fit, 100 Monte Carlo samples are used to calculate the parameter uncertainty as described by Motulsky and Christopoulos97 (Chapter 17: Generating confidence intervals) for the 2D Hill equation, \(E_{C12}, E_{C13}, E_{C12}^*, E_{C13}^*\) (Fig. 10A). Specifically, noise, with a distribution \(N(0, \sigma)\), where \(\sigma\) is equal to the root mean square (RMS) of the best fit, is added to best-fit values of the 2D Hill equation for all drug doses. The data plus noise is then fit again initializing the optimization from the best-fit parameters of the original data. This is done 100 times. From this ensemble of fits, the 95% confidence interval of each parameter can be calculated. This Monte Carlo approach results in asymmetric confidence intervals which better captures the non-Gaussian distribution of uncertainty for many fits (e.g. the distribution of \(h\) is log-normal) as well as being robust to the co-linear parameters in the 2D Hill equation. The asymmetric confidence interval is particularly salient when the dose-range is insufficient to observe the lower plateau of the dose-response. Only combinations for which \(R^2 > 0.7\) and the fitted EC50s of both drugs was less than maximum tested dose for each \((C_0 < \max(d1_1), C_0 < \max(d2))\) were included for subsequent analysis.

Comparing fitting algorithm robustness between different synergy frameworks. We additionally examined the performance of the MuSyC fitting when the raw data is subject to different types of noise (Fig. S3A). We synthetically generated 10 random samples of 5400 noise and synergy profiles and compared between all parameterized models of synergy the percent convergence (Fig. S3B), fit quality assessed by \(R^2\) (Fig. S3C), and variation in synergy parameters assessed by Z-score (Fig. S3D). Fitting algorithms for the different models is described in Section Calculation of other synergy metrics. Overall, we find MuSyC performs as well or better than comparable parameterized models of synergy on the tested synergy profiles. We note this analysis is hampered by the lack of a “true” standard for synergy—dose-response surfaces to be generated based on a defined model—in this case the MuSyC model. Bliss, Loewe, and HSA in general do not require fitting an equation to the data, and were thus excluded from this analysis. Nevertheless we note such synergy metrics may be more sensitive to noise in the data, as noise in individual datapoints is not smoothed out via a curve fit.

Data acquisition, preparation, and analysis
Onei et al. anti-cancer screen. The anti-cancer drug combination data were downloaded from the supplemental materials of Onei et al.26. Single agent and combination datasets were merged. Drug effect was the mean normalized percent viability (\(X_{viability}\) column) calculated as detailed in Onei et al.26. The minimum and maximum bounds for \((E_0, E_3, E_C)\) during 2D Hill equation fits were \([0.99,0.0,0.0,0.0,0.0,0.0]\) and \([1.01,1.5,2.5,2.5,2.5,2.5]\), respectively.

Mott et al. anti-malarial screen. The anti-malarial drug combination data were downloaded from https://tripod.nih.gov/matrix-client/ from the Malaria Matrix project. Blocks downloaded for analysis were \([1601,1602,1603,1701,1702,1703,1761,1764]\). Only blocks with a mqConfidence of \(>0.9\) were included. The drug effect was calculated as described in Mott et al.25. Effects \(<-20\%\) and \(>120\%\) were removed. The minimum and maximum bounds for \((E_0, E_3, E_C)\) during 2D Hill equation fits were \([90.0,0.0,90.0,0.0,0.0,0.0,0.0]\) and \([110.200,200,200]\), respectively.

Tan et al. anti-HIV screen. The anti-HIV drug combination data were downloaded from the supplemental table four of Tan et al.24. Drug effect was one minus the normalized infection rates as detailed in Tan et al.24. The minimum and maximum bounds for \((E_0, E_3, E_C)\) during 2D Hill equation fits were \([0.99,0.0,0.0,0.0,0.0,0.0,0.0,0.0]\) and \([1.01,1.5,2.5,2.5,2.5,2.5]\), respectively.

Cokol et al. anti-fungal screen. The anti-fungal drug combination data were downloaded from supplemental dataset one in Cokol et al.28. The raw cell growth measurements for all 200 drug-drug interaction assays were stored as a 96 × 64 matrix of numbers. Rows were time points with 15 min intervals and columns are the indices of an \(8 \times 8\) drug matrix. Drug dilutions were linear between the maximum reported in Table 1 of Cokol et al.28 and 0. The drug effect was quantified using the area under the growth curve (AUGC), calculated using Simpson’s integration, after the first 10 time points (150 min). The background unique to each experiment was removed by subtracting the minimum observed growth for each pair in a drug pair. The minimum and maximum bounds for \((E_0, E_3, E_C)\) during 2D Hill equation fits were \([0, 0, 0, 0, 0, 0, 0]\) and \([\infty, \infty, \infty]\), respectively.

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The ALMANAC anti-cancer drug combination data were downloaded from https://wiki.nci.nih.gov/display/NCITDTPData/ALMANAC. The matching single dose-response data were downloaded from https://wiki.nci.nih.gov/display/NCITDTPData/NCI-ALMANAC+CombDrugGrowth+Nov2017.zip.

**Calculation of other synergy metrics**

Bliss, Loewe, and HSA: Bliss, Loewe, and HSA depend on the concentration of drugs so a combination can be synergistic at one dose, but antagonistic at another dose. Several methods have been proposed for extracting a single synergy metric per combination from these frameworks to enable comparisons between combinations. For our analysis, we calculate the synergy score at the combination of each drug’s EC50 (Figs. 3 and 5) as proposed by Malyutina et al. or at the maximum tested concentration of each drug (Fig. 4). The EC50 of each drug was calculated from the fits to the 2D Hill equation (15) which we have observed to be more robust to noise when estimating the single drug pharmacologic profile. Assuming the notation for the 1D Hill equation and inverse Hill equation—which calculate effect (E) given a dose (d) and a dose given an effect, respectively—are given by

\[
H(d) = E_x + \frac{(d - E_x)}{\left(k + E_x\right)}
\]

where \(E_x < E_0\), then equations for Bliss, Loewe, and HSA at the EC50 are:

1. Bliss = \(H(1)(C_1) + H(2)(C_2) - E(C_1, C_2)\)
2. Loewe = \(\frac{H_{\text{EC50}}(C_1) + C_2}{H_{\text{EC50}}(C_1) + C_2}\)
3. HSA = \(\min\{H(1)(C_1), H(2)(C_2)\} - E(C_1, C_2)\)

where \(H(C_1, C_2)\) is the measured effect of combining \(C_1\) of \(d_1\) and \(C_2\) of \(d_2\). And equations for Bliss at the max of each drug is:

\[
\text{Bliss} = H(1(\max(d_1))) + H(2(\max(d_2))) - E(\max(d_1), \max(d_2))
\]

Thus, Loewe synergy is calculated using an equation similar to CI, while Bliss and HSA are calculated using an “excess over” approach, which calculates the raw difference between the expected and observed responses. While the reference models are always the same, we note alternative equations may be used to quantify synergy of a combination, though the biases we report are intrinsic to the reference models, not the synergy quantification approach. These equations assume the metric of drug effect decreases as the dose increases. Because many single agents did not reach 0% maximum efficacy, the EC50s (\(C_0\)) were not necessarily 50% (Fig. S7). If \(E(C_1, C_2) < E_1, E_2\) then Loewe was undefined. We apply a -log10 transformation the scale Loewe to match the ranges Bliss and HSA are synergistic; \(\text{Bliss} < 0\) and \(\text{HSA} < 0\). Loewe was unde

The Hill PDE model has no parameters to estimate from experimental data, but does not propose a method to estimate synergy from experimental data, but postulates some implementation of perturbation theory could be used to fit experimental data. Therefore, to calculate the synergy of this model, we defined the sum of residuals between the null surface and the experimental data as the metric of synergy.

**Drug combination database analysis**

Initial possible matching drug names between the in vitro experiments and the Drug Combination Database (DCDB) were determined using fuzzy string matching in Python (https://github.com/scipy/fuzzywuzzy v0.17.0). Drugs which had a sorted token ratio of 85 were initially included. Of the 427 drugs, there were 172 single drug matches. Matches included structural analogs. See matching_drug_names—11-29-2019_final.csv for complete matching list. Of these matching drugs, there were 126 tested combinations in clinical trials according to DCDB. Outliers in the synergy calculations were considered values 1.5 times the interquartile range (Q1–Q3) above or below Q1 or Q3 respectively.

**Data availability**

The datasets analyzed in this study were obtained from publicly available sources with DOIs Mott (10.1038/srep13891, Figs. 3, 6, 54, 56, and 59), O’Neill (10.1158/0008-5472.CAN-17-0489, Figs. 7, 8, 50, and 59), Holbeck (10.1158/0008-5472.CAN-19-0489, Figs. 7, 8, 50, and 59), and Cobol (10.1158/1068-937X.CAN-2017-2177, Figs. 6 and 59). Clinical trial data was collected from the Drug Combination Database (DCDB) (10.1093/database/baxu124, http://public.ubiyte.com/dcdb/), Figs. S9–S10). The synergy datasets generated in this study are available in a repository at https://bitbucket.org/maryc11/muyc_theory/.

**Code availability**

All code required for recreating manuscript analyses from the MuSyC fits are available for review in the repository https://bitbucket.org/maryc11/muyc_theory/. A web application to calculate MuSyC parameters is available at https://muyc.jlab.org/. The code for the interactive Jupiter notebook demonstration of MuSyC is available at https://github.com/djwooten/naturecomms-muyc2021/.

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**Effective dose model.** To fit Zimmer et al.’s Effective Dose Model we used the scipy.optimization.curve_fit module in Python 2.7. Specifically, the Effective Dose Model, Eq. 2 in Zimmer et al. (Eq. 30 in Supplement), contains parameters \((C_1, C_2, C_0, h_1, h_2, b, h)\) where the \(a\) parameters are the synergy values. In the model, there are no parameters for efficacy because it is assumed the drug effect ranges between zero and one. When this is not true, the Effective Dose Model results in poor fits to the data (Fig. S7A, B).

**Schindler’s Hill PDE model.** The Hill PDE model has no parameters to fit as the dose-response surface is derived the single dose-response curves. In fact, Schindler does not propose a method to estimate synergy from experimental data, but postulates some implementation of perturbation theory could be used to fit
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