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Charting the fragmented landscape of drug synergy

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Abstract

Even as the clinical impact of drug combinations continues to accelerate, no consensus on how to quantify drug synergy has emerged. Rather, surveying the landscape of drug synergy reveals the persistence of historical fissures regarding the appropriate domains of conflicting synergy models – fissures impacting all aspects of combination therapy discovery and deployment. Herein we chronicle the impact of these divisions on: 1) the design, interpretation, and reproducibility of high-throughput combination screens; 2) the performance of algorithms to predict synergistic mixtures; and 3) the search for higher-order synergistic interactions. Further progress in each of these subfields hinges on reaching a consensus regarding the long-standing rifts in the field.

Keywords

Drug synergy; Loewe Additivity; Bliss Independence

The Fragmented Foundations of Drug Synergy

While experimenting with medicinal combinations stretches back to antiquity, one of the first quantitative models to measure synergy was advanced by Loewe in 1926 [1]. Loewe proposed what is now called the Dose Equivalence Principle (DEP) which asserts if the effect of reducing one drug's concentration can be compensated for by adding a second drug at a constant ratio, there is no synergy (also called additivity). This definition leads to the classic linear **isoboles** (see Glossary) of Loewe Additivity. Observed deviations from this expected ratio signify synergy or antagonism. The second principle, the Multiplicative

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Disclosure Statement

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Data and Code Availability

All code and data required for figure generation are included in the GitHub repository https://github.com/QuLab-VU/TIPS_Review_2020.git. The code for generating figures was Python 2.7. The authors would like to thank the DeepSynergy paper for making their software available as well as for excellent readability making it quickly deployable. All DeepSynergy training was run using a GeForce RTX: 2070 series GPU processor and Python 3.7.

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Survival Principle (MSP), was first proposed by Bliss in 1939 [2] then later independently by Webb [3]. In contrast to the DEP, the MSP takes a probabilistic approach to synergy asserting the probability of being unaffected by each drug individually (*U1,U2*) is independent. Therefore, the probability of being unaffected by the combination (*U1,2*) is equal to the product of the single drug probabilities (*U1,2=U1*U2*). This equation has been termed Bliss Independence. If the percent of the population unaffected by the combination is less or more than this product, the combination is synergistic or antagonistic, respectively. A third major synergy principle was proposed by Gaddum in 1940, which defined synergy as the difference between a combination's effect and the most efficacious single agent [4]. This model is commonly called Highest Single Agent (HSA). Together, these three principles, (DEP, MSP, and HSA) comprise the foundations of almost all subsequent synergy frameworks (Figure 1A, Foundational Principles) [5]. Several excellent reviews on the mathematical basis of these foundational principles are [6–8].

In the 1980s, two equations – one satisfying the MSP and the other the DEP – were derived from a mass action model of drug effect by Chou and Talalay (Figure 1A, Mass Action Interpretation) [9,10]. Because the mass-action based equations for MSP and DEP are not equivalent, Chou recommended using the DEP equation which assumes the inhibitors are **mutually exclusive**. This equation is called the Combination Index (CI) [10]. However, the existence of multiple equations for calculating drug synergy became increasingly problematic in the late 1980s and early 1990s as systematic comparisons between methods emerged. These comparisons highlighted the frequent contradictory results between the MSP and DEP frameworks [11,12]—inconsistencies that have been subsequently documented as recently as 2019 [13]. Seeking consensus in the field, a conference was convened in Saariselkä, Finland in 1993. The resolutions of the attendees were documented in what was termed the Saariselkä Agreement [14,15] (Figure 1A, Seeking Consensus) which concluded,

> "It is clear that the adherents of Loewe additivity and Bliss independence have heard all the most compelling arguments for and against each model, and cannot be persuaded to switch allegiances. Thus…we propose that both models be tentatively accepted…This recommendation is made even though predictions of combined-effects based on each of the rival reference models may be quite different"

Symptomatic of this internal division in the field, the preponderance of papers using the term "drug synergy" since 1993 (~84%) do not reference either the DEP or MSP (Figure 1B). Further, the adherents of the MSP- or DEP-based frameworks remain firmly entrenched in their respective camps – DEP-based studies being roughly twice as common as MSP-based – with less than6% of studies explicitly mentioning both (Figure 1B).

Nevertheless, this lack of consensus has not slowed the development of combination screening technology which has outpaced the analytical tools necessary to translate its potential for therapeutic discovery. Seeking to address this gap, an assortment of synergy frameworks have been postulated in recent years [16–23] (Figure 1A, Modern Expansion). In the next section, we review these recent advances and highlight how the persistence of divisions between the MSP and DEP debated at Saariselkä continue to impact all aspects of synergy studies in the modern era.

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Recent Advances Building on Fractured Foundations

Over the last decade, there has been an expansion of synergy frameworks, almost all of which continue to derive from either the MSP or DEP [5] (Figure 1A, Modern Expansion). However, the implicit assumptions and limitations of each new framework often go unnoted as there are no standardized criteria for comparison. Table 1 catalogs the most critical features of all frameworks. These features can broadly be grouped into implicit assumptions (Table 1, Equation Assumptions) and restrictions on the types of data that can be analyzed (Table 1, Data Assumptions).

Commensurate with the expansion of computational resources, most modern frameworks have transitioned to fitting **parametric equations** to calculate synergy (Table 1 Parametric). ZIP and Effective Dose Model (EDM) [17,19] are parameterized models for the MSP while BRAID, Hill PDE, and BIGL [18,20,22] are the same for the DEP. The GPDI model [21] fits two different equations, one for the DEP and one for the MSP, in an approach similar to the CI. MuSyC fits a 2D Hill equation which reduces to either the DEP or MSP in particular instances subsuming both into a more general model [5,16]. All of these methods assume a Hill-like **dose-response curve** for each single-drug, an assumption first used by Chou [9], which is not an original assumption of Bliss, Loewe, or HSA (Table 1 Hill Approx). Finally, the recently rediscovered Hand model [23] is also a parameterized version of the DEP which only requires the dose-response function to be differentiable and invertible, and, therefore, could be applied with non-Hill dose-response curves, though it was not considered in these instances.

The form of the Hill equation (Figure S1A) used by these methods constrains the range and type of drug effects for which they are applicable (Figure S1B). The EDM, Hill PDE, CI, and BIGL are derived with a 2-parameter Hill equation. This form of the Hill equation has parameters only for **cooperativity** (also known as the Hill slope) and the **potency**, measured as the concentration of drug required to achieve the half-maximal effect (EC50) and assumes a minimum and maximum effect of 100% and 0% (Figure S1B). When the maximal effect does not reach 0%, these methods can result in poor fits[5]. In contrast, ZIP, BRAID, and MuSyC all use the 4-parameter Hill equation as their base making them generally applicable to non-percent data with arbitrary effect ranges (Table 1 Non-% Data). Because the 2-parameter Hill equation is a special case of the 4-parameter equation (Figure S1B), these methods are also applicable to percent data. The distinction between the 2- and 4-parameter equations stems from the distinction between the **percent affect** and the **percent effect** of a drug. Percent affect measures the discrete change in the number of *affected* individuals in a population (*i.e.* an individual is either affected or not). Therefore, the percent of affected individuals can never exceed 100%. In contrast, the percent effect measures the continuous change in the behavior of individuals or populations relative to a control. Because percent effects are relative, they can be greater than 100% (*e.g.* see Figure 2A (0,0) condition). MSP frameworks assume the measurement is the percent affect; however, most phenotypic screens measure the percent effect. As a result, it is common to observe maximal *effects* which do not reach 0% (Figure S2A,B left two panels). This can happen when all cells are affected by the drug, but growth inhibition is partial, resulting in maximal percent effects >0%. A recent study [5] has shown this distinction between percent effect and percent affect

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results in a systematic bias toward antagonism when applying Bliss to drugs with maximal effects between 35% and 65%). Comparing an anti-cancer dataset [24] and an anti-malarial dataset [25] shows the frequency of drugs with intermediate maximal efficacy is assay/model dependent (Figure S2C,D). Therefore, careful investigation of the single-drug dose-response curves should precede using methods derived with a 2-parameter Hill equation (Table 1 Unbounded Drug Effect). Both Excess Over Bliss (EOB) [26] and BIGL [22] propose rescaling arbitrary drug effects to range between 0% and 100% in order to apply Bliss and Loewe respectively; however, it is unclear how rescaling impacts synergy calculations when comparing combinations with different maximal efficacy.

In summary, the modern expansion of drug synergy frameworks has increased confusion, rather than clarified, the appropriate domain of each synergy model. This has stemmed, in part, from the unstated limitations and assumptions of each model (Table 1). Careful consideration of the assay read-out, the single-drug dose-response curves, and the assumptions of a particular synergy framework is critical to avoid systematic biases and improve reproducibility.

A Field Divided

Due to the lack of a consensus regarding the best synergy framework, several software packages, including SynergyFinder [27] and Combenefit [28], have been developed which calculate multiple synergy metrics, commonly including Bliss, Loewe, and HSA. Other software has been developed to directly couple multiple synergy calculations with image analysis in high throughput studies [29,30]. However, we find that even on the same dataset, SynergyFinder and Combenefit can give opposite results for the same synergy metric (Figure 2A,B). This likely stems from different data transformations, normalizations, and manipulations which are not standardized in the field. We find divergent predictions to be the case for up to 36% of combinations comparing between SynergyFinder and Combenefit for Bliss in an anti-malaria dataset [25] (Figure 2C, green bars).

The difficulty in comparing software calculations is augmented by the lack of consensus on what summary statistics should be used for these dose-dependent models of synergy. Malyutina *et al.* argued synergy scores at the IC50 of both drugs are more therapeutically relevant [31]; however, numerous studies have depended on a summary statistic for comparing combinations. In the anti-malarial dataset, we find the most antagonistic combinations by the mean (top 5%) are all present in the top 32% of most synergistic combinations by the maximum observed synergy (Figure 3A, Figure S3A Spearman rank correlation=0.13). This is because dose-dependent synergy frameworks, such as Bliss and Loewe, were not designed to compare different drug combinations, but rather different doses of the same combination. We find anti-malarial combinations where 3 samples at different doses are all antagonistic by Bliss (Figure 3B,C) and Loewe (Figure S3B,C), while the combination is synergistic by the mean over the whole **dose-response surface**. The discrepancy points to a philosophic quagmire regarding whether synergy is a property of drug pairs or drug dose pairs (Outstanding Questions Box). If synergy is a property of drug pairs, then the optimal drug concentrations should be determined based solely on maximizing efficacy with therapeutically tolerable doses. However, if synergy is a property

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of particular drug concentrations, then optimal doses should be selected based on both synergy and efficacy of a particular dose pair. The implications of these divergent worldviews have not been previously discussed, yet they represent a critical fault-line in the field with far-reaching ramifications for the discovery and deployment of combination therapy.

In summary, not only do the often cited contradictions between synergy models persist, but even the same metric can be contradictory between different software packages. Reliance on summary statistics to compare drug combinations for dose-dependent synergy frameworks (Table 1) further compounds this problem. Such conflicts undermine the reproducibility and translatability of synergy studies and therefore should motivate concerted efforts toward establishing data handling standards in the field (Outstanding Questions Box).

Tackling the Combinatorial Complexity of Synergy Studies

One challenge, common to all synergy studies, is the looming **curse of dimensionality** [32–35]. Each additional drug exponentially increases the requisite number of conditions to measure making an exhaustive search of combinatorial space intractable. Three subfields have evolved to deal with the inflationary cost of combination screens. First minimal sampling designs have been proposed for both MSP and DEP frameworks. Second, computational algorithms are being developed to predict synergy. And finally, the existence and nature of synergistic interactions beyond pair-wise remains an active area of study. The lack of such interactions would mean the effects of >2-drug mixtures could be predicted based on pair-wise measurements substantially reducing the number of conditions required to measure for **higher-order combinations**.

Minimalistic sampling heuristics

The most direct way to decrease the cost of drug combination screens is to reduce the number of data points required to calculate synergy. Several minimalistic sampling schemes have been proposed (Figure S4) [31–33,36–39]; however, not all sampling methods are equally appropriate for different frameworks. Specifically, the particularly stringent sampling patterns annotated Minimal MSP and DEP in Figure S4, require only 3 data points per combination, but can only be analyzed by Bliss/HSA or Loewe, respectively. The CSS sampling design [31] was proposed as a compromise between accuracy and cost and can be used to calculate both Bliss and Loewe. However, its accuracy depends on the intrinsic noise in the assay. Synergy studies should therefore carefully consider a framework's limitations before depending on a particular sampling scheme (Table 1). Additionally, as discussed above, the selection of dose becomes critical for these minimalistic sampling schemes (Figure 3C,S3B).

An alternative approach to reduced sampling schemes is the use of scaling laws to infer effects for under-sampled combinations [40]. These scaling laws have been used to calculate drug response in resistant mutants based on responses of sensitive cells. However, this approach is also sensitive to the magnitude of an assay's experimental noise. Further, it implicitly assumes that drug interactions are unidirectional (*i.e.* drug A changes the potency

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of drug B, but drug B does not change drug A's potency). Bidirectional synergistic potency has subsequently been assumed by several groups [16–19,21].

In summary, because the robustness of synergy calculations to different sampling strategies has not been rigorously addressed and depends on intrinsic experimental noise, we recommend subsequent screens should sample the full matrix of combinations when possible for greater versatility in analysis and robustness.

Predicting Synergy

In silico algorithms to predict synergy have seen substantial recent activity including two DREAM challenges [41,42]. Broadly, the algorithms can be grouped into either machine learning (statistical) methods or mechanistic models.

Two excellent reviews on common types of machine learning algorithms employed in predicting drug synergy as well as available databases for training models are [43,44]. Machine learning algorithms can be broadly categorized as network-based, gene expression-based, drug-centric, or some combination thereof. However, because the algorithms depend on the structure of the training dataset, most algorithms cannot transfer between different studies. Therefore, the wider adoption of these algorithms depends on the interoperability and accessibility of synergy datasets. DrugComb [45] is a recent effort to make synergy datasets FAIR (Findable, Accessible, Interoperable, and Reusable). Subsequent efforts are needed to link drug combination screens to their cognate molecular and cellular feature sets. Combining data modalities, specifically, information about gene expression and drug-target interaction was shown to improve the predictivity of machine learning algorithms by the most recent DREAM challenge [41].

However, an understudied aspect of these approaches is how each algorithm's predictions depend on how synergy was calculated—a notable omission given the historical conflicts in the field. The first DREAM challenge [42] used Bliss while the most recent challenge [41] was based on Loewe (calculated by Combenefit). When we predicted Bliss, Loewe, or HSA synergy of anti-cancer combinations using DeepSynergy [46] – a neural network algorithm which uses drug and gene expression information as input – we found a 12% overlap in the top 1,000 predicted combinations with no overlap in the top 5 (Figure 4A). The first overlap between all three occurred at the 263rd combination by Loewe ranking (Figure 4A). The physicochemical drug features (Figure S5A) and expressed genes (Figure S5B) with the largest influence on synergy/antagonism (as assessed by mean SHAP value [47] over 1,000 combinations), features which could be used to guide mechanistic insights into the basis of synergy, had a rank correlation between the different synergy metrics of <0.04 for all comparisons (Figure S5). This potentially stems from the different sensitivity of Bliss, Loewe, and HSA to different mechanisms of joint action as described by Gilvary and colleagues [48]. Given the historical discrepancy between these metrics, a more customized and rigorous approach is warranted. In contrast to statistical models, mechanistic approaches to predict synergy are based on models of biological processes. An early example of this, coPIA, used a system of ordinary differential equations (ODEs) to predict combination effects in breast cancer cells [49]. A more recent ODE model of the HGF/Met signaling pathway was used to identify personalized combinations in hepatocellular carcinoma based

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on MuSyC synergy calculations [50]. Another example used dynamic logic models of phosphorylation cascades to predict colorectal cancer response [51].

Even in the absence of kinetic parameters necessary for an ODE model, purely topological models have been used to predict with ~80% accuracy perturbation impacts on gene expression in a chemotaxis model of bacteria [52]. Such topological models have also been applied in cancer to identify the synergistic interaction of Aurora B and ZAK in triple-negative breast cancer [53]. The predictivity of such approaches tends to leverage the sparsity of biological networks. Finally, a hybrid approach constraining the architecture of an artificial neural network to match biological networks has been shown to be predictive of growth dynamics in budding yeast [54] and could be used to predict collateral dependencies in biological networks.

Understanding the mechanistic basis of synergy is a common goal; however, no unifying theme from these studies has emerged. This may stem from relying on different synergy models coupled with the mechanistic models or statistical models.

Higher-order interactions.

There has been substantial interest in predicting the effects for mixtures of three or more drugs based on pair-wise effects [19,34,55–57] (See [58] for a more in-depth review of recent work). In a seminal paper on this topic, Wood *et al.* did not find evidence for higher-order interactions in 3-drug combinations in *Escherichia coli* [55]. Specifically, they could predict the effects of 3-drug combinations using an MSP-based, Isserlis-like formula that depended only on pair-wise and single drug effects. A series of follow-up comparisons were done between EDM, the Isserlis-like formula, and Bliss to predict multi-drug effects for combinations up to 10 drugs [19,32,59]. These studies reinforced the absence of higher-order interactions as first postulated by Wood *et al.*

However, these findings were challenged by two studies that found emergent synergy for multi-drug combinations in *E. coli* based on Bliss [34,60]. This trend toward synergy was also observed by Russ and Kishony [56], though less pronounced, in a study that directly compared the scaling of Loewe and Bliss. They found the null models for Loewe and Bliss diverge for increasingly higher-order combinations with Bliss becoming more synergistic (in agreement with [34,60]) and Loewe becoming more antagonistic. Cokol and colleagues also found evidence for higher-order interactions according to Loewe for combinations of anti-tuberculosis compounds, though no trend as a function of the number of drugs was observed [33]. In our own comparison of the trends in the three datasets [34,56,59], we find a trend toward antagonism as the number of drugs increases for Loewe in all datasets (Figure 5A). The trend for Bliss, however, is contradictory between the three datasets (Figure 5B). Given recent work demonstrating bias in Bliss toward antagonism for drugs with maximal effects >0% (Figure S2) [5], we corrected the Katzir *et al.* [59] data to account for this bias and found the trend toward antagonism is attenuated (Figure S6A). We find a higher proportion of drugs with maximal effects >0% in the Katzir *et al.* data than the Russ *et al.* [56] data (Figure S6B) explaining why this bias was more prevalent in the former data.

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In the end, the presence or absence of higher-order synergy does not preclude the deployment of mixtures of 3 or more drugs, as gains in efficacy can still be achieved, as shown in a study in colorectal cancer [61]. Indeed, it has been argued that curative drug combinations in cancer should be constructed from non-synergistic drugs with independent mechanisms of resistance [62].

In summary, the existence and nature of higher-order interactions remain controversial. Clearly delineated studies on the role of assay, metric, drug selection, and model system are needed to better address this question. Further, the recently proposed existence of multiple types of synergy [5,16] opens a new avenue of investigation into how different types of synergistic interactions (*e.g.* synergistic potency and efficacy) scale for increasing numbers of drugs. Nevertheless, what is clear is the discrepancies between the MSP and DEP are further exacerbated by the scaling to higher numbers of drugs amplifying the need for a consensus approach synergy before addressing this problem.

Synergy in New Contexts

Despite the persistent historical fissures in the field, there are exciting developments that continue to add new dimensions to the quantification of combination pharmacology. One interesting development is the study of synergy in temporally staggered treatments [63], which are known to impact *in vivo* efficacy [64]. These dosing programs better mimic patient combinations which are not commonly given simultaneously—an often-ignored assumption of all Hill-equation based frameworks (Table 1, Hill Approx.). Koplev *et al.* [63] take a geometric approach, similar to the MSP [56], and find several anti-cancer combinations with temporally dependent synergy in pancreatic cancer. Related to this, a recent study by Dean *et al.* has investigated how synergy is related to the short-term development of resistance [65]. They found the rate of adaptation in *E. faecalis* is not related to the synergy of the combination, but rather is a function of the overlap in mechanism between the drugs. In related work, Maltas *et al.* [66] found the collateral effects of a single drug are pervasive but drug-specific. Therefore, the optimal combinations to reduce the development of resistance are drug-dependent, though the connection between collateral effects of a single drug and synergy is not well understood.

Another rapidly evolving concept is the translation of synergy between different scales [67,68]. Palmer *et al.* used a Bliss model to show the gain in survival time for most clinical combinations can be explained by variable sensitivity in a population rather than true pharmacologic interaction. A conceptually similar idea was the basis of DRUG-NEM [69], an MSP-based framework for identifying combinations that maximize coverage of a heterogeneous cell population measured by **CYTOF**. Finally, the impact of cell-to-cell variability on the development of drug resistance has been modeled using a probabilistic, state-transition framework that found drug interactions between cytostatic and cytotoxic drugs to vary in time [70]. Overall, these studies highlight the role of heterogeneity at every scale on our understanding of drug synergy.

Another developing area of research is the use of multi-objective functions to prioritize drug combinations. For example, SynToxProfiler [71] and CSS [31] both calculate synergy as

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well as total efficacy. The possible existence of a synergy-efficacy trade-off [72,73] emphasizes the search for synergistic combinations should not be solely focused on optimizing synergy, but also high efficacy at tolerable doses. One tool for simultaneously considering synergy and efficacy is the S-S plot [31], a scatter plot of the efficacy (related to the area under the surface of the CSS sampling design) and synergy (Bliss, Loewe, or ZIP calculated at the IC50 of both drugs). Toxmatrix instead quantifies toxicity, or protection against toxicity, due to a combination [74]. MuSyC was proposed to decouple different types of synergies: efficacy, potency, and cooperativity [5,16] where the relative merits of each synergy are disease-specific [16]. Such a multi-parametric approach to drug synergy better captures the clinical trade-offs between therapeutic efficacy and tolerable dose. However, it is unclear how much the *in vitro* measures of efficacy, toxicity, and synergy correlate to clinical axes of interest (*e.g.* side effects and clinical efficacy). Furthermore, the optimal trade-off between these different parameters is unknown, though Pareto optimization would appear appropriate.

Finally, the rapid evolution of chemical-genomics screens is only beginning to impact the search for drug combinations. Examples include recent studies in *M. tuberculosis* [75,76] and *E. coli* [77] which identified mutant-specific classes of inhibitors and suggest mutation mimicking drug combinations as a promising path forward. Another study used a CRISPR-based double knockout screen to identify synergistic combinations against leukemia cells [78]. However, as gene knock-outs and mutations are fundamentally different than molecular inhibition, the relationship between synthetic lethal and drug synergy is likely case dependent. Notably, Cokol *et al.* did not find an increase in Loewe synergy in targeting synthetic lethal genes compared to random [79]. While the historical overlap between functional genomics and drug synergy has been small, these recent efforts are beginning to bridge these disciplines.

Conclusions and Future Perspectives

Herein we have highlighted recent advances in the field of drug synergy as well as provide a template for future comparisons (Table 1). Twenty-five years on from the Saariselkä conference, consensus seems even further away; however, recent efforts have reinvigorated the conversation of unifying the MSP and DEP fostering the hope unity is possible [5,17,18,21]. We have compiled key areas in need of consensus (see Outstanding Questions). The notable development of a drug combination database [45] should facilitate more comprehensive comparisons in the future. To this end, we recommend standardizing the format of experimental data (Table S1) and the annotation of the pharmacologic parameters (Table S2) to assist subsequent systematic comparisons between drug synergy experiments or frameworks, respectively.

Quantitative models of drug-drug interactions are critical for rationally guiding drug combination discovery and translation in the treatment of complex, multi-factorial diseases. However, the clinical translation of such discovery efforts depends on the reproducibility of synergy studies – reproducibility hampered by the persistence of historical fissures. A collective effort toward consensus on the models and methods to measure drug synergy is therefore urgently needed.

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Supplementary Material

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Glossary:

Cooperativity:
Also known as the Hill slope, this is a measure of the steepness of the dose-response curve (Figure S1A).

Curse of dimensionality:
The name given to a large class of problems arising from the combinatorial expansion of considering higher dimensions.

CyTOF:
Mass Cytometry uses heavy-metal tagged antibodies to quantify single-cell expression of up to 50 target proteins.

Dose-response curve:
To assay drug effect, drugs are commonly titrated across several concentrations and the resulting effect measured. Commonly the dose-response is sigmoidal well fit by a Hill equation.

Dose-response surface:
The measured effect of a combination of two drugs over a range of different concentrations. Commonly plotted as either a heatmap (Figure 2A) or 3D surface plot where the X-Y axes are drug concentration and the color or Z-axis is the measured effect, respectively.

Higher-order combinations:
Combinations of three or more drugs.

Isoboles:
Contours of equal effect. For all pairs of drug 1 and drug 2 concentrations along an isobole, the resulting effect (*e.g.* percent of viable cells) is the same.

Mutually Exclusive:
Two inhibitors are mutually exclusive if binding to one precludes the binding of the other.

Parametric equations:

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Equations with parameters which are fit to the data. An example is the Hill equation (Figure S1A) which varies E0, Em, h, and C to fit dose-response data. An example of a non-parametric equation is the Bliss equation (*U1,2=U1*U2*) as there are no values to fit.

Percent Affect vs. Percent Effect:
For historical reasons, these are often used interchangeably, but they are fundamentally different. Affect measures whether there is a discrete change in phenotype (live vs. dead). The percent of affected cells, eggs, etc. can never exceed 100%. Percent effect is the relative change compared to control and is commonly measured in phenotypic assays. In contrast to percent affect, it can exceed 100% (Figure 2, S2A,C).

Potency:
The concentration of drug required to achieve a particular effect. Commonly quantified by the EC50. the drug concentration is required to achieve a half-maximal effect (Figure S1A).

Sham Experiment:
A thought experiment in which a combination of drugs is tested for synergy; however, the combination is actually two of the same drug. The sham principle states no combination should be synergistic with itself. Classically, DEP frameworks are sham compliant while MSP frameworks are not.

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Outstanding Questions:

- What is the relationship between and applicable domain of the Dose Equivalence Principle (DEP) and Multiplicative Survival Principle (MSP)?
- Is synergy a property of drug combinations or is it dose-dependent?
- Do higher-order drug interactions exist? If so, how prevalent are they?
- Should sham compliance be included as a measure of a new synergy framework's validity?
- What are common mechanisms which underlie a successful clinical combination?

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Highlights:

- Historical rifts between drug synergy paradigms have persisted into the modern era with modern frameworks further dividing the field.
- These divisions impact the reproducibility of synergy calculations limiting the translational potential of combination studies.
- Additionally, these divisions undermine the utility of computational approaches to predict synergy.
- The properties of different synergy metrics have led to contradictory conclusions regarding the existence and nature of higher-order synergistic interactions for increasing numbers of drugs.
- Unifying the field necessarily precedes a broader acceptance and utilization of synergy calculations for discovering clinically-impactful therapeutic mixtures.

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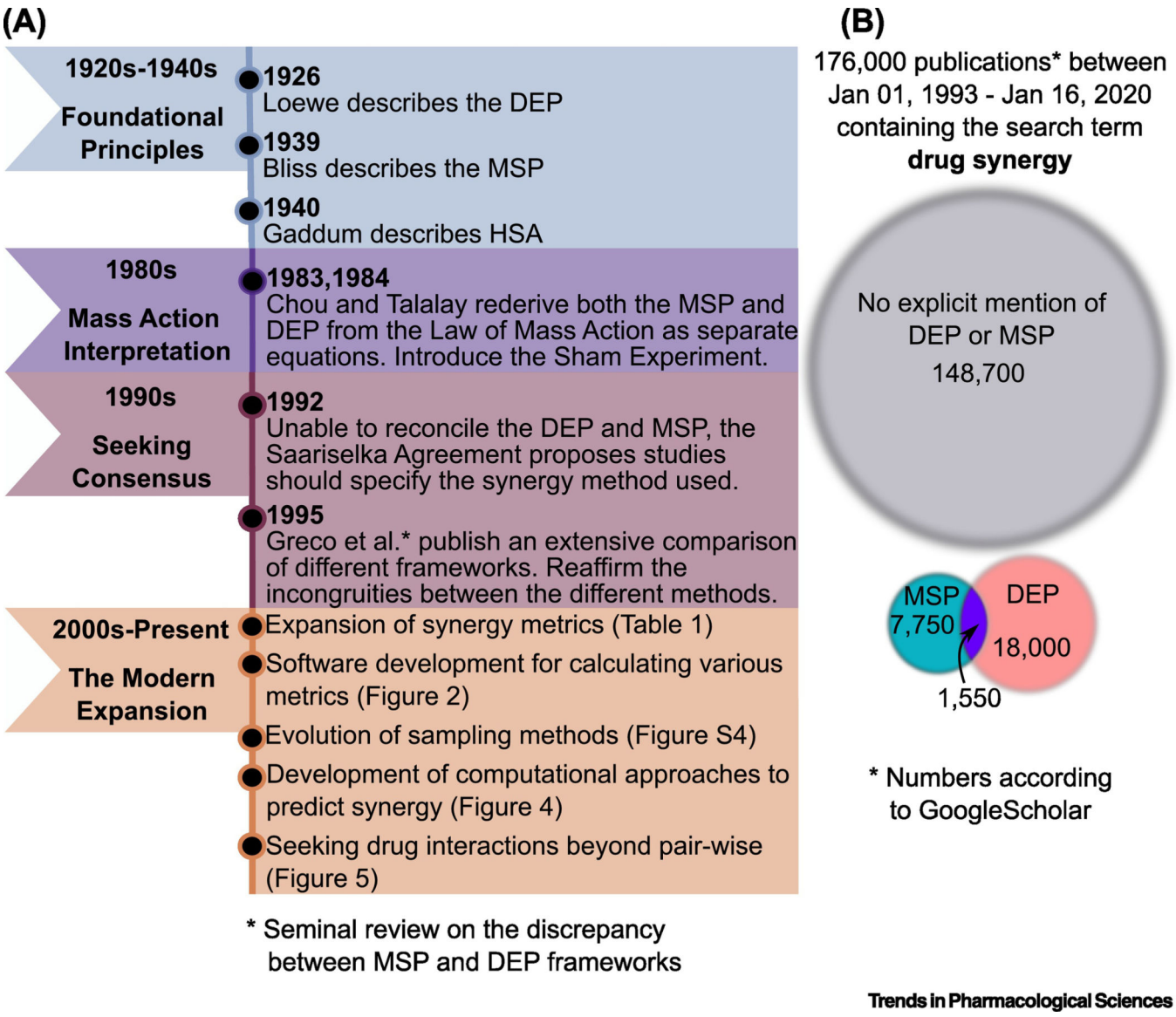

Figure 1: Timeline of developments in drug synergy highlights the persistence of historical rifts in the field.

A) Developments in drug synergy span four distinct epochs. During the Foundational Principles period, the major drug synergy principles were first described. Subsequent work in the 1980s rederived the DEP and MSP based on a mass-action model of drug effect resulting in two equations. The DEP-based equation became known as the Combination Index (CI). A series of studies during a critical period in the 1990s highlighted the incongruence of the DEP and MSP culminating in the recommendation studies should explicitly state how synergy was calculated. The modern era is characterized by an expansion of synergy models that seek to extend the DEP and MSP (Table 1). Substantial developments have also occurred in related subfields such as software, sampling methods, computational prediction, and higher-order interactions. B) Publications (in Google Scholar) which include the term “drug synergy.” Publications are grouped by those mentioning MSP (blue), DEP (red), both (purple), or neither (gray). Papers the terms “dose equivalence

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principle” or “isobologram” or “loewe” or “combination index” or “Chou and Talalay” or “isobole” containing the terms “dose equivalence principle” or “isobole” or “Loewe” or “combination index” were considered to reference the DEP. Papers containing the terms “multiplicative survival principle” or “bliss” or “fractional product method” were considered to reference the MSP. The time span included was: 1993–2019. The search date was 01/16/2020. Over 84% of publications continue to use the term synergy without referencing a particular model. Of those that do, < 6% acknowledge the existence of multiple frameworks.

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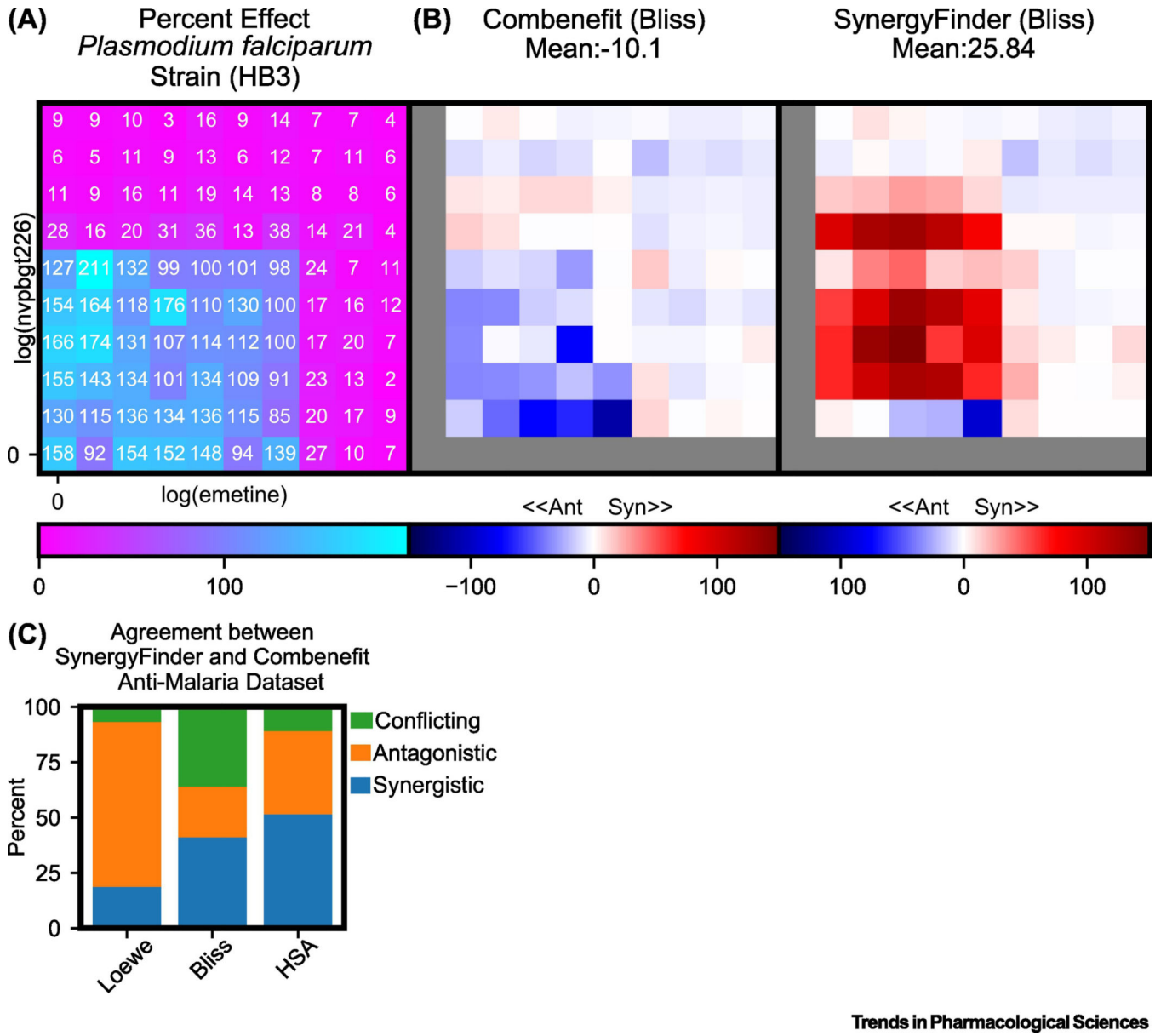

Figure 2: Conflicting results between synergy software packages impairs reproducibility.

A) Percent effect (color-bar bottom, numbers in boxes) of nvpbgl226 (PI3K/mTor inhibitor) and emetine (anti-protozoal) against HB3 strain of malaria. Data from Mott *et al.* [25]. The percent effect can be greater than 100% because static endpoint measures of drug effect rely on normalization to untreated controls. If the treated condition has more cells than the control, the normalized percent effect is >100%. This highlights the difference between percent *affect* (which can never exceed 100%) and percent *effect*. B) Combenefit and SynergyFinder calculate Bliss (color scale bottom) for this combination differently resulting in conflicting synergy classification based on the mean across the surface. Gray boxes are undefined as synergy is only calculated for combination conditions. Syn=synergy, Ant=Antagonism. The color scale is the same for both heatmaps. This disparity may arise from different approaches to deal with effects >100%. C) The frequency of agreement

between SynergyFinder and Combenefit in the anti-malarial dataset is synergy metric dependent.

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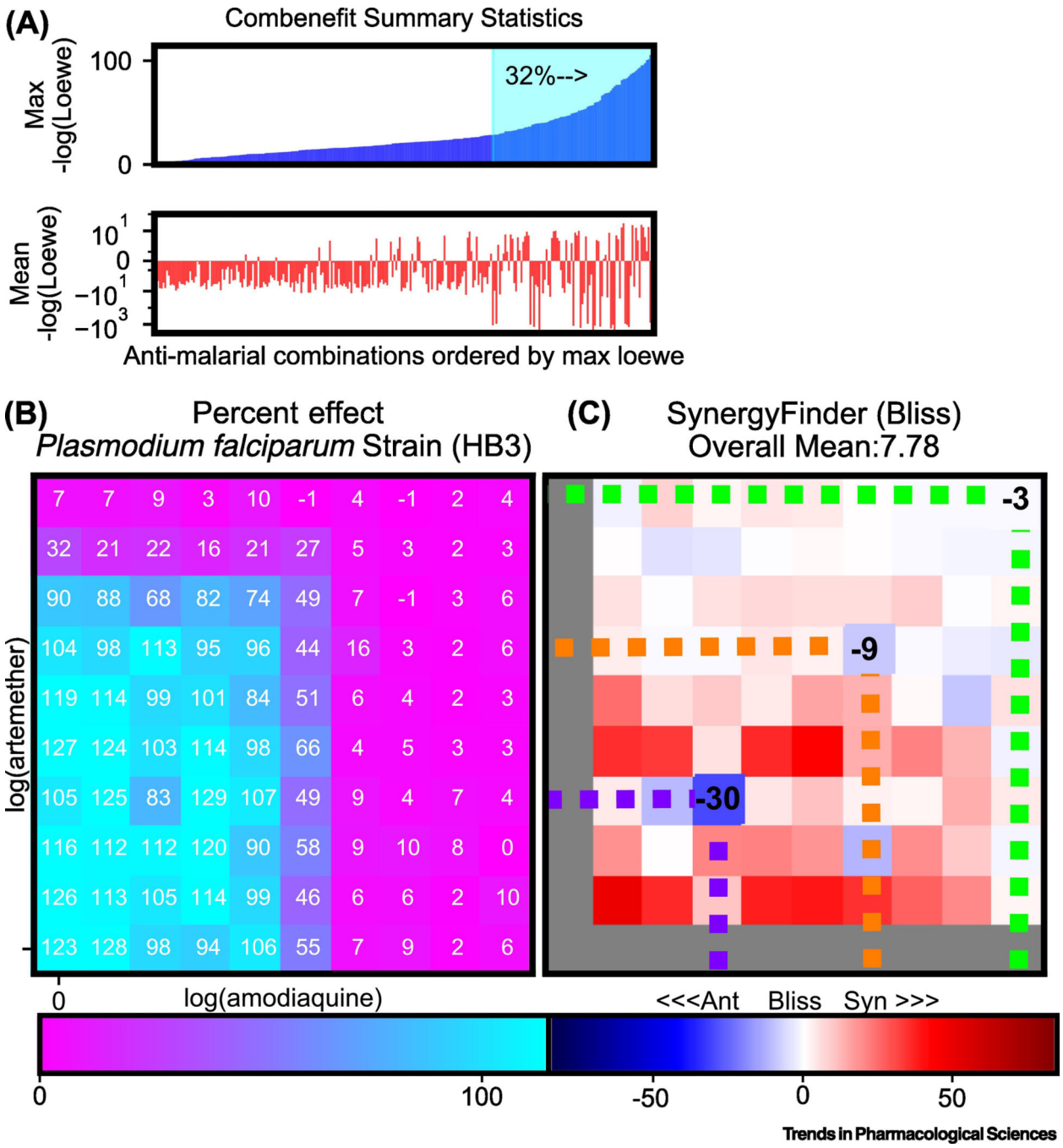

Figure 3: Dose-dependent synergy frameworks perform inconsistently when using summary statistics.

A) Different summary statistics for dose-dependent synergy metrics (Table 1, Dose-Independent) prioritize different combinations for follow-up. Anti-malarial combinations are ranked from left to right in order of increasing maximum observed Loewe synergy (calculation by Combenefit). The rank-order correlation between the maximum Loewe and mean Loewe is 0.13 (Figure S3A) and combinations with high maximum synergy are often antagonistic by mean (red lines bottom panel). The top 5% of antagonistic combinations by the mean fall in the top 32% of synergistic combinations by the max. -log(Loewe) <0 is

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antagonistic and >0 is synergistic. B) Percent effect (color scale bottom, numbers in boxes) of amodiaquine (polymerase inhibitor) and artemether (proposed to function by inhibiting anti-oxidant enzymes) on the HB3 strain of malaria. C) The combination is antagonistic (Ant) by Bliss (color scale bottom) according to SynergyFinder at three different combination doses (purple, orange, green lines); however, the combination is synergistic (Syn) by mean over the surface. Gray boxes are undefined as synergy is only calculated at combination conditions.

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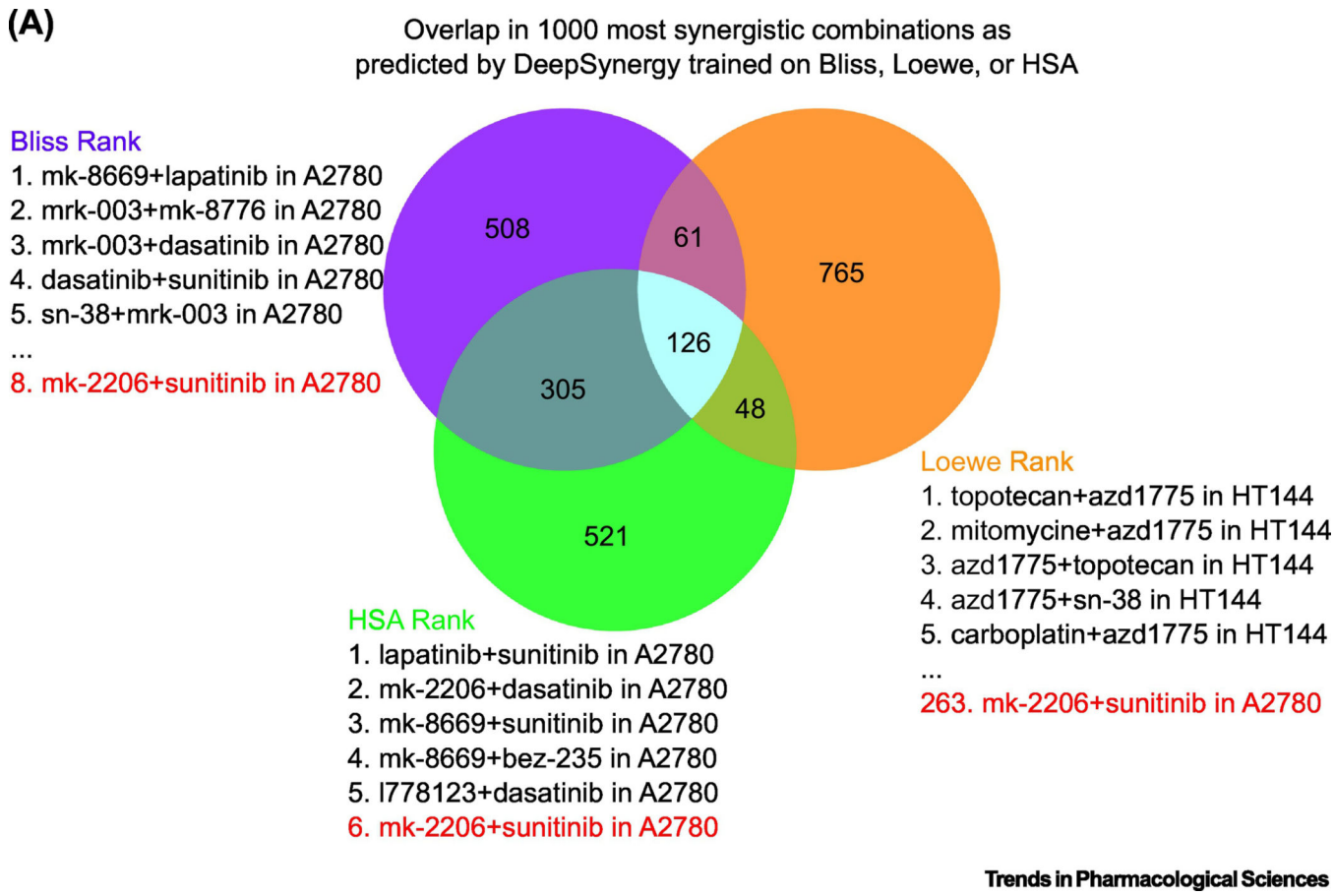

Figure 4: Top, machine learning-predicted, combinations depend on the synergy metric.

A) We trained DeepSynergy [46], a machine-learning algorithm to predict drug synergy from gene expression and drug physicochemical properties, on the O'Neil *et al.* dataset [24] with Bliss (purple), HSA (green), or Loewe (orange) as the measure of synergy. The top-predicted synergistic combinations were different depending on which metric was used. The mixture of mk-2206 (AKT inhibitor) plus sunitinib (tyrosine kinase inhibitor) in A2780 cells was the top combination present in the rank-orderings of all three metrics at the 6th, 8th, and 263rd position for HSA, Bliss, and Loewe respectively (red highlight). The top 5 predicted synergistic interactions in Loewe are all in HT144 cells (malignant melanoma) while in Bliss and HSA all are in A2780 (ovarian carcinoma).

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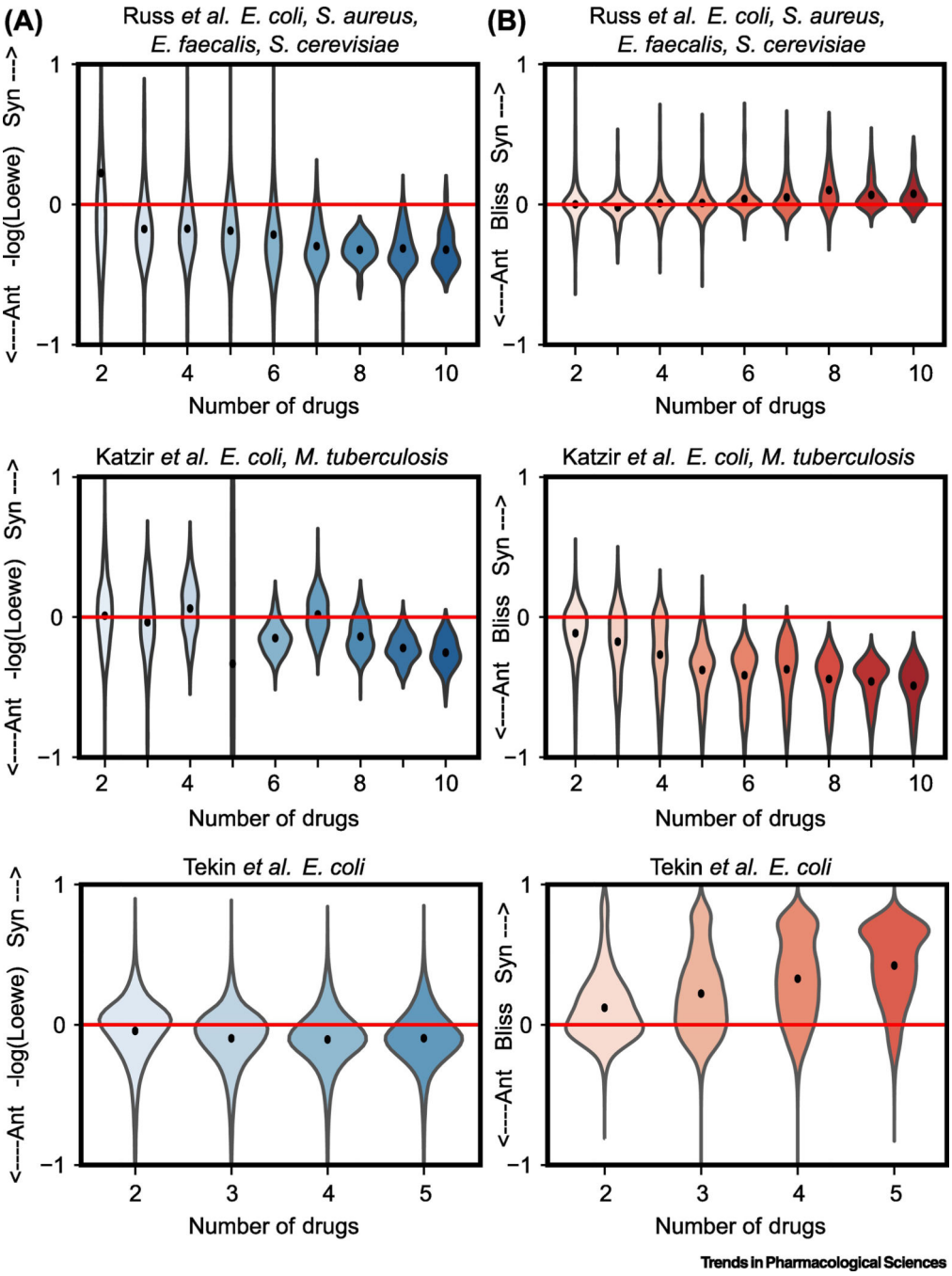

Figure 5: Seeking consensus on the existence and nature of higher-order interactions.

A) Average Loewe synergy decreases for an increasing number of drugs in three datasets (Russ *et al.* [56], Katzir *et al.* [59], and Tekin *et al.* [34]). The black dot is the mean value of the distribution. Red-line demarcates synergy (Syn) from antagonism (Ant). The species tested in each paper are indicated in the panel title. B) Distribution of Bliss synergy as a function of increasing numbers of drugs. Accounting for the difference between percent

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effect and percent affect reduced the bias toward antagonism for ultra-high order combinations in the Katzir *et al.* data (Figure S6A).

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Table 1:

Comparison of drug synergy models.

	Equation Assumptions					Data Assumptions			Availability	
	Dose-Independent	Parametric (#Parameters)	# Synergy Variables	Hill Approx.	Not Sham Compliant	>2 drugs	Non-% data	Unbounded Drug Effect	Software [Citation(s)]	Original Citation
MSP										
Bliss Independence			1		✓	✓			Web,R,Matlab [27,28,45]	[2]
EOB			1		✓		✓	✓		[26]
ZIP		✓ (10)	1	✓	✓		✓	✓	Web,R [27,45]	[17]
EDM	✓	✓ (6)	2	✓	✓	✓				[19]
DEP										
Loewe Additivity			1			✓	✓		Web,R,Matlab [27,28,45]	[1]
CI*		✓ (4)	1	✓					DOS,python [80,81]	[9]
Hill PDE		✓ (6)	1	✓						[20]
Hand			1			✓	✓	✓		[23]
BIGL			1	✓	✓		✓	✓	R [22]	[22]
BRAID	✓	✓ (10)	2	✓			✓	✓	R [18]	[18]
Other										
HSA			1		✓	✓	✓	✓	Web,R,Matlab [27,28,45]	[4]
GDPI_LA	✓	✓ (12)	4	✓			✓	✓		[21]
GPDI_BI	✓	✓ (12)	4	✓	✓			✓		[21]
MuSyC	✓	✓ (10,12)+	3,5	✓	✓	✓	✓	✓		[16]

*CI is the DEP-based equation in [10] which is the most commonly used.

+MuSyC has 2 forms as detailed in [5] including or excluding synergistic cooperativity. Checkmark indicates satisfies the property