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Systems Biology from Chemical Combinations

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Abstract

Systematic testing of chemical combinations in cell-based disease models can yield novel information on how proteins interact in a biological system, and thus can make important contributions to biological models of those diseases. Such combination screens can also preferentially discover synergies with beneficial therapeutic selectivity, especially when used in high-order mixtures of more than two agents. These studies demonstrate the value obtainable from combination chemical genetics, and reinforce the growing realization that the most useful paradigm for a drug target is no longer a single molecule in a relevant pathway, but instead the set of targets that can cooperate to produce a therapeutic response with reduced side effects.

Introduction

Living organisms can be thought of as systems of interacting molecules, whose function and dysfunction would be better understood using a functional wiring diagram [1]. Such network models have already been used to predict the development of complex biological phenotypes [2], and will eventually help researchers gain a better understanding of multi-factorial diseases, identify novel therapeutic targets, and develop personalized treatments [3].

The staggering complexity of biological systems requires large and diverse sets of data on component connectivity and responses to system perturbations. The core topology of the network is determined using direct interactions between genes, proteins, and metabolites, and the models can then be refined using a biological system's responses to perturbations, such as drugs, mutations, or environmental changes. All of these approaches can associate network components with functional roles, even without direct interactions connecting them.

Combined perturbations shift the focus of genetic and chemical genetic studies from the functions of individual genes or proteins to interactions of those components within a biological system [4]. Large efforts to test the viability of double mutants are underway in bacteria [5], yeast [6], and nematodes [7]. Chemicals provide information that is distinct from and complementary to genetic perturbations, given the differences between how they modulate protein functions [8]. The advantages of chemical perturbations are that they can target a single domain of a multi-domain protein, allow precise temporal control that is critical for rapid-acting processes, can target orthologous or paralogous proteins enabling comparisons between species or redundant functions, and do not directly alter the concentrations of a targeted protein, thus avoiding indirect effects upon multi-protein complexes. Small molecules also lend themselves more readily to combination interventions, making them especially useful for integrating systems and chemical biology [9, 10]. Most combined perturbation studies with combinations have focused on exploring drug sensitivities across large sets of knockouts are also being undertaken in various model organisms [11, 12] and human cells [13]. However, these approaches are increasingly being extended to systematic testing of purely chemical combinations [10, 14]. Here we discuss our work that explores how combination studies can discover novel therapeutic treatments for complex disease systems, as well as to obtain biological information on the organization of functional components in those systems.

Discovering Combination Drugs

One of the most powerful resources for discovering novel therapies is the set of existing drugs chemical probes with known biological activity. Over the past century, the pharmaceutical industry has had great success developing drugs with the following essential qualities: selective therapeutic activity with tolerable side effects; desirable absorption, distribution, metabolism, and excretion properties; and stable chemistry to facilitate storage, manufacturing, and distribution. Molecules with these properties are rare, and many disease relevant targets cannot be addressed by chemical inhibitors, so there are currently only ~3,000 approved drug ingredients and research probes which modulate 200 – 500 molecular targets within human biology [15]. Moreover, even the most specific small-molecule drugs can affect many different proteins, leading to off-target effects either through direct binding to secondary targets or through the downstream responses to these binding events.

Such secondary activities can occasionally be beneficial towards other diseases for which the drug was not originally developed, and indeed many successful drugs result from such repurposing efforts.

Phenotypic screening is a very effective method for discovering new opportunities provided by existing drugs [16]. Since the 1970s, the prevailing drug discovery paradigms have been target-based drug design and phenotypic screening. The target-based approach involves identifying a molecular target that is responsible for a disease phenotype and then systematically testing a variety of chemical agents to see if they bind selectively to that target. By contrast, phenotypic screening tests chemical agents against a measurable quality of a whole organism's function, to identify those with a desirable response profile without necessarily having a detailed understanding of the drug's mechanism. While the target-based approach can discover primary target activities, secondary targets are almost exclusively found by phenotypic testing. Systematically testing many agents against phenotypes allows the biology to reveal which agents are likely to yield beneficial activities.

The opportunity for discovering useful off-target effects increases dramatically when drugs are used in combination [17]. Combinations are traditionally used to reduce toxicity, by sparing doses between two different drugs affecting the same disease function, to take advantage of complementary activities that independently improve the disease condition, and as the most effective response to drug-resistant pathogens, notably for viral and bacterial infections. Biological systems are robust, exhibiting a high degree of resilience that can compensate for a variety of attacks [18]. For example, mutation studies in many organisms [19, 20] find that only ~10 – 20% of genes affect viability when deleted from the genome. Combinations can overcome this robustness by targeting compensatory pathways [21], and many such cooperative relationships could work through known or yet undiscovered secondary drug targets.

Chemical combinations need to be tested at varying drug doses, in order to find synergistic effects at unknown concentrations. This can be done with a ''dose matrix'', where a combination is tested in all possible permutations of serially-diluted single agent doses (Fig. 1), or a fixed dose-ratio series, where component drugs are mixed at a high concentration and the mixture is tested in serial dilutions. Synergy is calculated by comparing a combination's response to those of its single agents [21], usually against the drug-with-itself dose-additive reference model [22]. Deviations from dose additivity can be assessed visually on an Isobologram (Fig. 1) or numerically with a Combination Index [23]. To capture synergies that can occur anywhere on full dose-matrix experiments, it is also useful to calculate a volume $\boldsymbol{V}_{\mathrm{HSA}} = \Sigma_{\mathrm{X,Y}}\, \ln \boldsymbol{f}_{\mathrm{X}}\, \ln \boldsymbol{f}_{\mathrm{Y}}\, (\boldsymbol{I}_{\mathrm{data}} - \boldsymbol{I}_{\mathrm{HSA}})$ between the data and the highest-single-agent surface, normalized for single agent dilution factors $\boldsymbol{f}_{\mathrm{X}}$,$\boldsymbol{f}_{\mathrm{Y}}$, to quantify the strength of combination effects [21]. Both synergy scoring methods generalize readily to mixtures of three or more chemicals.

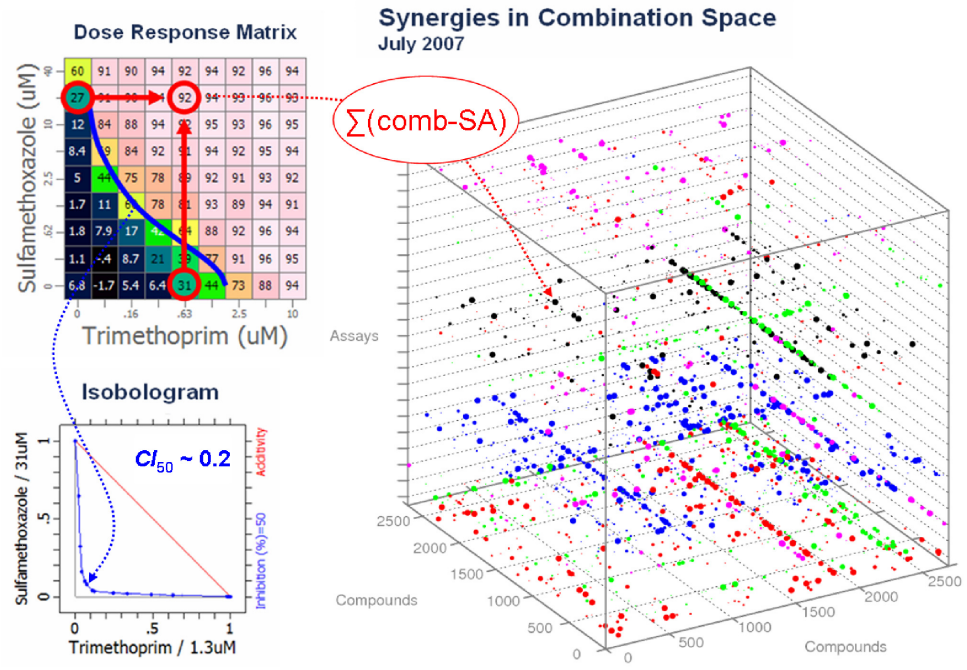

Figure 1. Dose matrix and synergy. Phenotypes (e.g., inhibition of *S. aureus* growth by Bactrim®) are measured for each drug, dosed in serial dilutions (bottom, left edges) and in all paired mixtures. Synergy is scored on differences between combined and single agent effects, while the isobologram shows the dose-sparing achieved over drug-with-itself dose-additivity. Using combination screening, we have discovered thousands of synergies in many disease areas. A cube showing 22 assays, each with colored symbols scaled to synergy, shows that despite some prolific drugs, strong synergies are rare.

CombinatoRx has been using a high throughput screening platform to explore combination therapies [24], by systematically testing combinations of ~3,000 agents in cell-based assays that preserve disease-relevant pathway complexity yet which are efficient enough to explore the vast space of combinations [21]. Using this platform, we have conducted dose-matrix screens in over ten disease areas to discover thousands of synergies (Fig. 1), many of which we have advanced into clinical trials for inflammation, diabetes and cancer indications. In addition, we have funded research programs utilizing our cHTS platform for drug discovery in neurodegenerative disease, infectious diseases, cystic fibrosis, muscular dystrophy, drug-device combinations and ophthalmology indications.

Combinations for Systems Biology

Dose matrix responses to combinations of chemical probes also can be used to determine mechanistic relationships between their targets [10]. Our therapeutic screens produce a wide variety of response surfaces, with distinct shapes for combinations having different known combination mechanisms. Many of these responses can be described by simple models that use the single agent curves to predict the combination effect (Fig. 2), and whose parameters provide quantitative measures of combination effects.

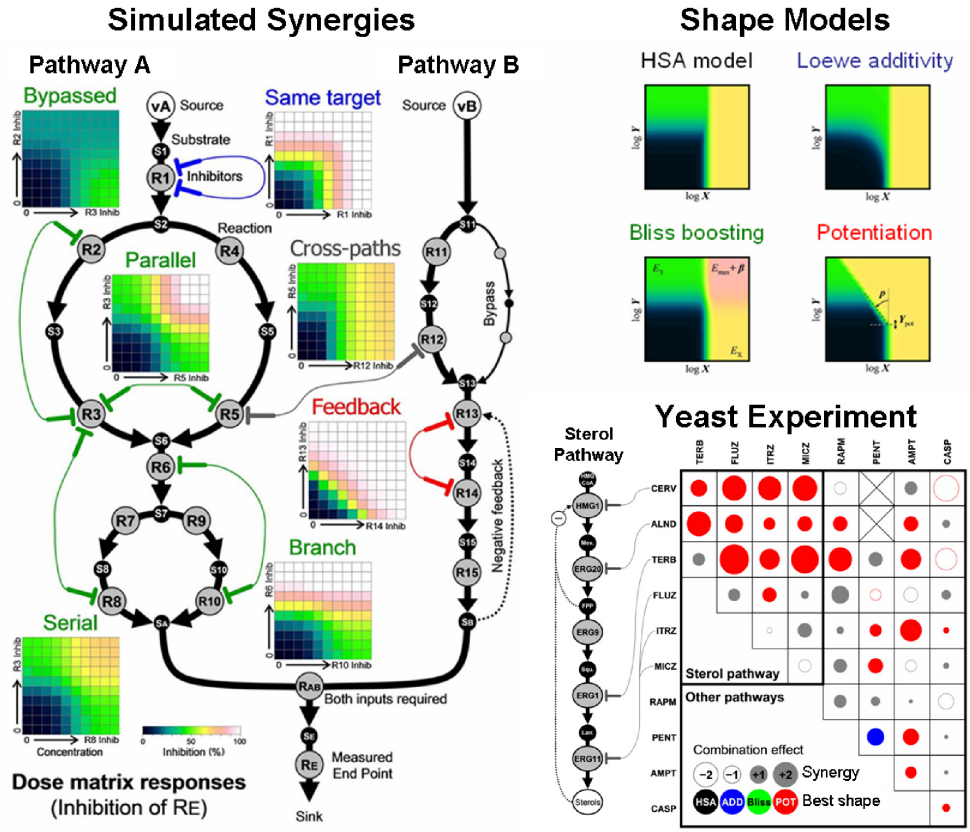

Figure 2. Synergy and connectivity. Simulated combination effects in a pathway of enzymatic reactions (grey enzymes, black substrates) with paired inhibitors (joined markers) depend on how the targets are connected. The effects can be described in terms of simple response shape models based on the single agent curves. A yeast experiment using sterol pathway inhibitors confirmed the expected response shapes in the pathway (symbol size scales with synergy, color shows the response model), and effects across pathways were weaker. Figure adapted from Lehár *et al.* 2007.

We established the relationship between synergy and connectivity by simulating a metabolic pathway as a series of linked Michaelis-Menten reactions with pairs of competitive inhibitors aimed at different targets with varying doses. We found that the shape and amount of synergy of each combination response depended on how the inhibitor pair's targets were connected in the pathway (Fig. 2). The predicted response shapes were robust to kinetic assumptions, parameter values, and nonlinear response functions, but were very sensitive to topological alterations in the target connectivity, such as branching, feedback regulation, or changing the type of junction at a branch point. The predicted shapes from these simulations were confirmed in a yeast proliferation experiment using drug combinations targeting sterol biosynthesis (Fig. 2), with further support from experiments on human cancer cells.

To explore the relationship between synergy and connectivity on a larger scale than can be addressed with dynamic pathway modeling, we are using flux-balance analysis (FBA) simulations of *E. coli* metabolism[25], with "minimization of metabolic adjustment" (MOMA) [26] to determine the impact of the perturbation upon bacterial growth. A variety of combination effects are observed, including many synergies in excess of the single agents, and a considerable variety of shapes that would have been indistinguishable with only knockouts. We estimated the total level of synergy by integrating the volume between the simulated data and the HSA model, $V_{HSA} = \Sigma_{X,Y} \ln f_X \ln f_Y (I_{data} - I_{HSA})$, summed over all positive concentrations X,Y and corrected for the dilution factors f_X and f_Y.

When the single agents are organized by their target location in the network, clear patterns emerge (Fig. 3). Most combinations produce no synergy, but there are strong synergies between adjacent enzymes within a pathway, and clear patterns of synergy between pathways, showing distinctions between the upper and lower ends of the pathway in some cases. These simulations demonstrate that genome-scale simulations of partially inhibited networks are tractable, and confirm that combination effects are strongly dependent on the connectivity of the perturbers' targets. Some of the response shapes clearly do not fit the shape models shown in Figure 2, so it is clear that we will need to broaden the reference set in order to capture the increased complexity of the system. Measuring connectivity between targets is considerably more challenging for a complex network like the *E. coli* FBA network,

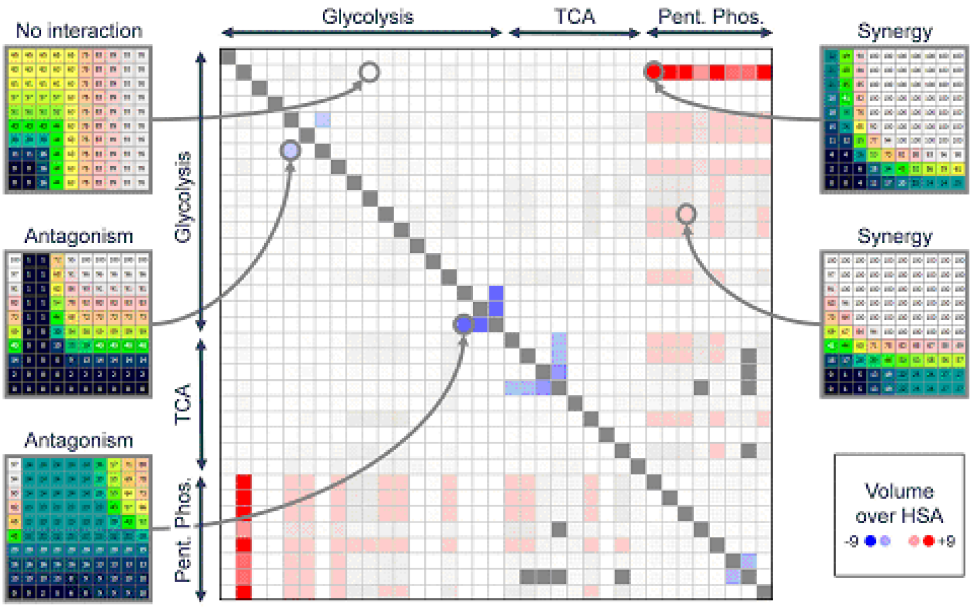

Figure 3. Simulated synergy. FBA/MOMA simulations of inhibited *E. coli* metabolism (central metabolism detail). The matrix shows synergy (V_{HSA}) for all pairs of targets sorted by pathway location. Most inhibitor pairs don't interact, but synergies and antagonisms show clear mechanistic patterns. The response surface shapes are more diverse than those shown in Figure 3.

We are also using flux balance analysis to model metabolism-dependent growth in *Saccaromyces cerevisiae* (yeast). This model organism has a fully sequenced genome, richly annotated functional pathways, and some of the most advanced network models both for metabolism and protein interactions [27, 28]. To provide an experimental basis for validation, we have screened all pairwise combinations of 60 chemical probes known to target metabolic enzymes in the FBA model, using an Alamar Blue metabolic readout as a proxy for cell growth (Fig. 4). Preliminary results from the combination screen are very encouraging. The observed responses are diverse, roughly ~4% of the tested combinations showed synergy (V_{HSA}) that were significantly different from the agent-with-self score distribution. The detected synergies are scattered throughout the combination space, but there are regions of consistent synergy between some pathways. In our preliminary studies, both individual combinations and synergy profiles confirm the results seen in our initial exploration of *Candida glabrata* [10]. We have performed FBA simulations of *S. cerevisiae* metabolism, which we will use for comparisons with our bacterial FBA models as well as with the yeast experimental results.

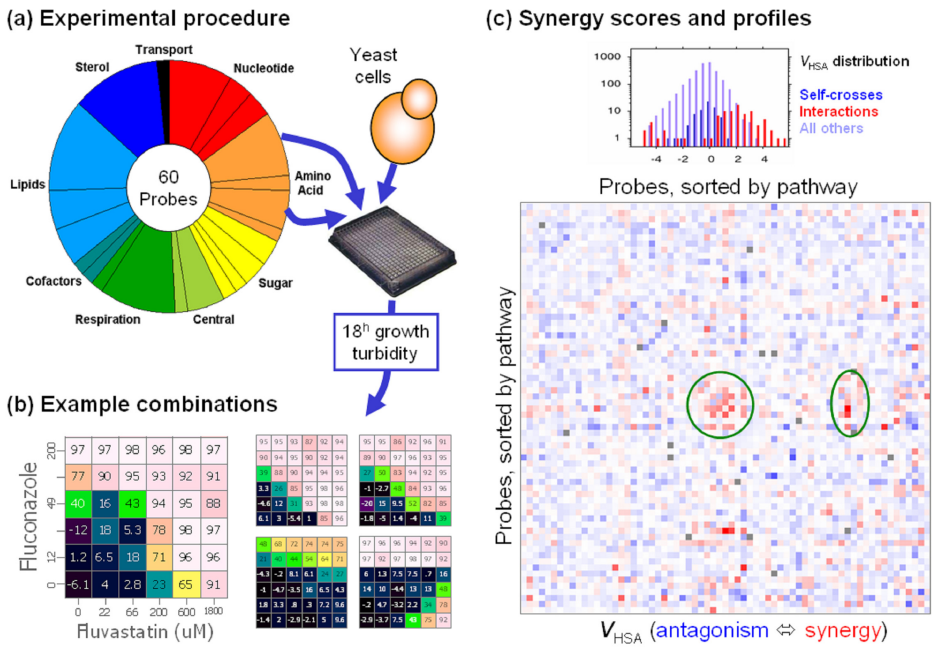

Figure 4. Yeast experiment. (**a**) Yeast cells were grown in pairwise combinations of 60 probes targeting metabolism. (**b**) Resulting responses confirm sterol synergies and produce diverse responses. (**c**) Analysis shows a 4% rate of interaction with synergies and antagonisms. Synergy profiles show both isolated synergies and coherent pathway interactions.

Higher order combinations (of three or more agents) can provide useful information about the overall complexity of a biological system. Randomly connected networks become increasingly sensitive to perturbations as the combination order is increased [29], and this trend is confirmed in more realistic network simulations using FBA approaches [30]. In principle, one would expect a trend towards more greater fragility to perturbation as the system is subjected to perturbations of increasing combination orders, due to their overwhelming any functional redundancy in the system (Fig. 5). From this it follows that by determining the "combination order of fragility" (COF), high-order perturbation experiments can be used to probe the robustness of a biological system [31]. We are experimenting with systematic high-order perturbation screens on bacterial proliferation assays, and preliminary results show that *Escherichia coli* survival networks have finite complexity that can be overcome with combinations at ~4th order (Fig. 5). This study also has identified a number of surprising synergies and antagonisms that arise at 3rd or 4th order without any suggestion at lower order. Such studies can be used to identify useful high-order synergies, to dissect a network in terms of its functional complexity, and to compare two systems (e. g., different bacterial species) in terms of their functional robustness.

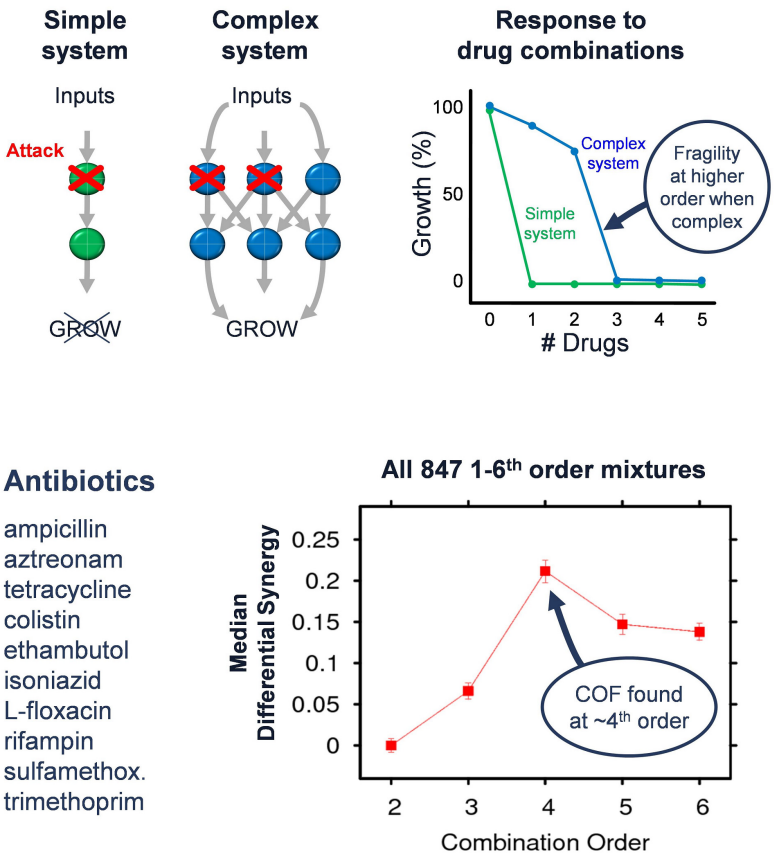

Figure 5. High order combinations. Single-pathway systems can be blocked by only one inhibitor, but redundant systems can function until all alternative routes have been blocked (upper). If perturbations are tested with increasing combination order, there will be incremental synergies until the functional redundancy has been reached, leading to a critical order above which the system is fragile to further attacks. A preliminary experiment testing all $1-6^{th}$ order combinations of ten diverse antibiotics (each with a 12-dose response curve) using an *E. coli* proliferation assay (lower) shows that the median differential synergy [31] across all combinations at each order peaks at ~4^{th} order, suggesting a possible limit of functional redundancy.

Concluding Remarks

Our approach towards chemical combination studies is an efficient way to extract important information on the topology of biological networks. Individual combination responses show how targets are connected in ways that can't be resolved by genetic interactions, and analyzing synergy profiles across large combination experiments can be used to determine drug targets and pathway interactions. It even seems that systematic exploration of high-order combinations can provide information on the overall complexity of a system, in terms of its functional redundancy. Because our approach relies on phenotypic screening, the biases towards known pathways and mechanisms are minimized, increasing the opportunity for discovering totally novel biology in the process. In many ways, this provides an excellent empirical complement to systems biology efforts to generate network models to predict the behavior of complex diseases.

For drug discovery, combination approaches will help reinforce the growing realization that a useful paradigm for a therapeutic or bioengineering target is the set of nodes (e.g., metabolites, genes, proteins, or pathways) in a network that can selectively control the state of a biological system [32, 33]. In principle, the behaviour of biological systems should be controllable by individually adjusting the state of many components, and the precision with which the system can be manipulated should depend on the number of such state settings. Because of this increased precision, high-order perturbations are more likely than single agents to produce a therapeutic outcome without triggering toxic side-effects. In practice, therapeutic selectivity can result both from having more points of control and from the ability to reduce the doses of individual perturbers if they cooperate towards a beneficial endpoint. High order experiments can identify such selective synergies and determine the optimal number of ingredients. Although there will always be some conditions that are best treated by a single drug, high-order multi-target combinations represent a strategy that addresses the very complexity of biological systems.

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