PERSPECTIVE

Systems biology and combination therapy in the quest for clinical efficacy

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Combinatorial control of biological processes, in which redundancy and multifunctionality are the norm, fundamentally limits the therapeutic index that can be achieved by even the most potent and highly selective drugs. Thus, it will almost certainly be necessary to use new 'targeted' pharmaceuticals in combinations. Multicomponent drugs are standard in cytotoxic chemotherapy, but their development has required arduous empirical testing. However, experimentally validated numerical models should greatly aid in the formulation of new combination therapies, particularly those tailored to the needs of specific patients. This perspective focuses on opportunities and challenges inherent in the application of mathematical modeling and systems approaches to pharmacology, specifically with respect to the idea of achieving combinatorial selectivity through use of multicomponent drugs.

Contemporary mechanism-based drug discovery aims to identify agents that activate or inhibit specific disease targets (usually proteins) while having as few side effects as possible. The therapeutic index of these agents (the ratio of therapeutic to toxic dose) is typically a function of target affinity, off-target binding (particularly in the case of multiprotein target families) and toxicity in sensitive and metabolically active tissues such as the liver. Molecular and genomic studies that pinpoint disease targets, high-throughput screens that rapidly identify inhibitors and a growing understanding of the chemistry of good drugs (for example, Lipinski's 'rule of five;'[1]) have raised the possibility of a golden era of 'targeted' therapies for serious diseases such as cancer, autoimmunity and diabetes. Imatinib (Gleevec), an inhibitor of the *bcr-abl* oncogene activated in chronic myeloid leukemia (CML), is an outstanding example of a targeted therapeutic[2–4].

However, most diseases of interest to contemporary drug discovery involve physiological processes controlled in a combinatorial fashion. These diseases are frequently difficult to treat using the one-gene-one-

drug approach pioneered by Ehrlich nearly 100 years ago[5,6]. From a top-down perspective, redundancy and homeostasis[7,8] are the most obvious features of combinatorial biological control. For example, cell proliferation is under the joint control of multiple growth factor receptor pathways[9–12], and genetic experiments reveal that inhibition of any single receptor is only partially effective at blocking growth. Many growth control circuits also have feedback compensation that makes them self-regulating with respect to inhibition of upstream components[13,14]. From a bottom-up perspective, multifunctionality is an essential combinatorial feature (in this case, with respect to physiological rather than biochemical activity). For example, the phosphatidylinositol-regulated Akt kinase plays a key role in cell survival but also in metabolism and other cellular processes[15]. Experience has shown that many proteins involved in cell fate determination and cell-cell signaling (which dominant disease targets in oncology and immunology) have a multiplicity of physiological functions. Inhibition of these multifunctional targets, even by highly selective drugs, disrupts several cellular processes and is therefore associated with mechanism-based toxicity. Redundancy and multifunctionality are two fundamental challenges facing targeted therapy. Patient-specific variation in drug response and temporal changes accompanying disease progression are others.

One way to address these challenges is through 'combinatorial selectivity', in which drugs are combined so as to coordinately inhibit several disease processes—thereby achieving efficacy—while nonetheless leaving each target sufficiently active in normal tissues that toxicity is minimized. This is precisely the logic underlying sensitized genetic screens, such as those used to investigate the functions of ubiquitous signaling proteins in specific receptor pathways (*Drosophila melanogaster* Ras or SOS in *Sevenless* signaling for example[6]). In the ideal case, a multicomponent drug would act selectively on a specific combination of target activities found in diseased but not normal cells. Formulating effective multicomponent drugs that have combinatorial specificity will, in our opinion, become increasingly important for drug discovery and development.

Combination therapy

Combination or multicomponent therapy, in which two or more drugs are used together, typically has one or more of the following goals: (i) reducing the frequency at which acquired resistance arises by combining drugs with minimal cross-resistance, such that emergence of resistance requires acquisition of multiple mutations in rapid succession—an unlikely event; (ii) lowering the doses of drugs with nonoverlapping toxicity and similar therapeutic profile so as to achieve efficacy with fewer side effects; (iii) sensitizing cells to the action of a drug through the use of another drug (chemosenstization) or radiation

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(radiosenstization), often by altering cell-cycle stage or growth properties (cytokinetic optimization); and (iv) achieving enhanced potency by exploiting additivity, or better yet, greater-than-additive effects in the biochemical activities of two drugs. The aims of combination therapy are not mutually exclusive, and good combinations such as ABV (doxorubicin, bleomycin, vinblastine) or BEP (bleomycin, etoposide, cisplatin) achieve several, including positive cytokinetic and biological interaction (with and without surgery), and reduced toxicity.

Evaluating combination therapy

Two methods are in common use for calculating the expected dose-response relationship for combination therapy as compared to monotherapy (for example, based on IC_{50}, the dose of drug needed to achieve 50% target inhibition and equal to K_i in the simplest case): Loewe additivity[16–19] and Bliss independence[20,21] (**Box 1**). For simplicity, we limit our discussion of these methods to two inhibitors acting alone or in combination, but similar reasoning applies to three or more agents and to drugs that are agonists. Loewe additivity assumes that two inhibitors act through a similar mechanism, leading to the concept of dose substitution, in which the effects of each inhibitor and the inhibitor combination are related through equipotent dose ratios (equations (1) and (4), **Box 1; Fig. 1a**). Bliss independence, in contrast, assumes that the two inhibitors act through independent mechanisms, leading to the concept of effect multiplication[21], in which combination therapy is represented as the union of two probabilistically independent events (**Fig. 1a**). These two methods yield different outcomes, and only Loewe additivity correctly predicts the trivial case in which the two inhibitors are actually the same compound. Debate continues, however, as to which method performs better with noisy clinical data and uncertainty as to therapeutic mechanism[21–23].

Experimental approaches to characterizing combination therapy typically involve determining dose-response curves for inhibitors individually and in combination. In most cases, inhibitors are mixed at constant-molar ratios at varying overall concentrations. When experimental dose-response data match the predictions of Loewe additivity or Bliss independence, the inhibitors are said to be additive (corresponding to the zero-interaction case); greater than predicted potency indicates synergism (positive interaction); and lower potency argues for antagonism

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BOX 1 A BRIEF HISTORY OF ADDITIVITY

Loewe additivity[16,19,62] assumes that two inhibitors act on a target through a similar mechanism. Given the concentration of two inhibitors that individually achieve $X\%$ target inhibition ([I_1], [I_2]), the concentration of inhibitors theoretically required to produce the same $X\%$ effect when used in combination ([CI_1], [CI_2]) can be calculated by taking into account the potency of the inhibitors.

$$1 = \frac{[CI_1]_{X\%}}{[I_1]_{X\%}} + \frac{[CI_2]_{X\%}}{[I_2]_{X\%}} \tag{1}$$

Loewe additivity involves dose-ratio addition and is therefore also called dose additivity. The approach was popularized for enzyme inhibitor studies by Chou and Talalay[17] and shown to be valid for mutually exclusive enzyme inhibitors (**Fig. 1a**), whether the inhibitors acted competitively or noncompetitively toward the substrate[17,18,63]. A combination index was developed to denote whether or not inhibitors interacted with each other:

$$\textit{Combination index} = \frac{[CI_1]}{[I_1]} + \frac{[CI_2]}{[I_2]} = \begin{cases} > 1 \text{ antagonism (negative interaction)} \\ = 1 \text{ additive (no interaction)} \\ < 1 \text{ synergy (positive interaction)} \end{cases} \tag{2}$$

The combination index compares the doses of inhibitors individually and in combination that experimentally produce the same level of inhibition. By ascertaining the dose required for equal effect, it is possible to determine whether the combination is effective at a lower total dose. Moreover, by incorporating kinetic principles of enzyme inhibition, it is possible to calculate the degree of inhibition expected of the combination. For example, a simple equation for mass action kinetic enzyme inhibition with constant substrate is:

$$F_{UA} = \frac{E}{E_{MAX}} = \frac{1}{1 + \left(\frac{[I]}{K_I}\right)^m} \tag{3}$$

where E is the enzyme activity, E_{MAX} is the maximum activity, F_{UA} is the fraction unaffected, m is the hill coefficient and K_I is the concentration of inhibitor I required to decrease enzyme activity by 50%. Substituting equation (3) into equation (1) yields an expression relating the expected combined effect, F_{UA}, to the inhibitor concentrations used in a combination:

$$1 = \frac{[CI_1]}{K_{I_1}\left(\frac{1 - F_{UA}}{F_{UA}}\right)^{\frac{1}{m_1}}} + \frac{[CI_2]}{K_{I_2}\left(\frac{1 - F_{UA}}{F_{UA}}\right)^{\frac{1}{m_2}}} \tag{4}$$

Chou et al.[18] solved equation (4) for F_{UA}, using a line-fitting technique, but modern numerical nonlinear solvers can determine F_{UA} for any combination of inhibitor concentrations[24], making it possible to evaluate clinical dose response curves relative to a simple computed standard of additivity. When the combination is better than additive, a beneficial case of synergism has been identified, although the mechanism usually remains unknown.

BOX 1 (CONTINUED)

Bliss independence[20,21], is the second primary means by which to treat combination therapy; it assumes that inhibitors can bind simultaneously and mutually nonexclusively through distinct mechanisms (**Fig. 1a**). Bliss independence is also called effect multiplication or the fractional product and was popularized by Webb[64]. The combined effect of two inhibitors (F_{UA}) is computed as the product of individual effects of the two inhibitors, F_{UA1} and F_{UA2}

$$F_{UA} = F_{UA_1} \times F_{UA_2} \tag{5}$$

Effect multiplication takes into account the idea that if I_1 has already inhibited a portion of the target, then I_2 has fewer target molecules remaining to be blocked. A helpful analogy suggested by Berenbaum[65] imagines an attempt to break a collection of eggs by throwing rocks or nails. The rocks or nails act independently of each other and each has a certain probability of breaking an egg. The combined effect of the rocks and nails can then be found by correctly combining the individual probabilities that an egg will be hit as the union of independent rock and nail events, which after rearrangement results in equation (5). Enzyme kinetic relations (equation (3)) can be incorporated into the Bliss independence model resulting in an expected combined effect (equation (6)) that is only consistent with Loewe additivity in specific circumstances of nonexclusivity[22]:

$$F_{UA} = \frac{1}{1 + \left(\frac{[CI_1]}{K_{I_1}}\right)} \times \frac{1}{1 + \left(\frac{[CI_2]}{K_{I_2}}\right)} \tag{6}$$

Loewe additivity and Bliss independence are often applied to complex biochemical analysis (**Fig. 1b**) independently and naïve of the network dynamics. Thus they become phenomenological definitions of additivity, treating the system under study as a black box with limited predictive ability as compared with mechanistic network models (**Fig. 1c**).

(negative interaction). Within the last decade or so, three-dimensional dose-response surfaces have been measured experimentally with the aim of identifying regions of robust synergistic behavior[21,23,24]. Generating dose-response surfaces is demanding, however, requiring an extensive checkerboard of inhibitor concentrations. Considerable attention has therefore been devoted to sparse-search strategies that maximize statistical power while minimizing the number of trials[25–28].

Loewe additivity and Bliss independence were originally developed to describe simple enzyme reactions, and both can be justified theoretically, but it is not necessarily true that either adequately represents the biochemistry of complex cell-signaling networks (**Fig. 1b**). In many combination therapies, drugs act in a nonexclusive manner on one or more targets, arguing for use of Bliss independence. However, when drugs affect processes that feed into common pathways (for example, growth factor receptors and MAP kinase cascades)[29], the combination may act according to Loewe additivity with respect to physiological effect. In actuality, both Loewe additivity and Bliss independence constitute black-box approaches rather than realistic mechanistic approximations (**Fig. 1c**)[30]. However, black-box approaches are very valuable in clinical trials because it is impractical for a measure of success (such as synergism between two drugs) to change with every biochemical advance.

Oncology is one therapeutic area in which combination therapy is ubiquitous, perhaps because clinical experiments are easier to justify when the disease is less serious and existing treatments are better. The constituents of some multicomponent chemotherapies target related biochemical processes, such as DNA repair and synthesis (cisplatin and 5-fluorouracil) and others target distinct processes such as DNA repair and microtubule dynamics (cisplatin and paclitaxel)[31]. Of greatest value are combinations in which synergy between agents is observed: gemcitabine (a pyrimidine analog) and platinum in the treatment of ovarian cancer, for example[32–34], or cytotoxic chemotherapy, radiotherapy and 'targeted' inhibitors of the epidermal growth factor receptor (EGFR) such as gefitinib, erlotinib or certuximab[35]. To date, the success of multicomponent drugs has relied on clinical evaluation of drug dose and

order of administration[33,36], with biochemical insight into mechanism playing a limited role. High-throughput screening of drug pairs has sought to systematize and extend the empirical approach as a means of identifying new combination drugs[37]. However, given the likelihood that many new multicomponent drugs must be developed and that screening is restricted to a few cell types, it would be very valuable to develop predictive models of pharmacology in which at least part of the work can be done *in silico*.

Predicting the effects of combination therapy *in silico*

A systems or network biology approach to analyzing combination therapy relies on the use of numerical models to simulate the effects of drugs individually and as mixtures. Mathematical analysis is potentially powerful because many pairwise drug combinations can be explored computationally at much lower cost than in preclinical or clinical experiments. What are the prospects for development of suitable network models? Preliminary success has been achieved in formulating mathematical models of signaling pathways[38–41] and oncogenic processes[42] relevant to human disease. Numerical analysis has also been used to identify critical network nodes[43] and model drug action[44]. It is very important to note, however, that computational approaches to pharmacology require models that accurately recapitulate biochemical events in normal and diseased cells. These models will be larger than the mechanistic models constructed to date; they will be more sophisticated with respect to morphology, post-translational modification, and cell-to-cell variation; and they will rest on a solid foundation of empirical data and experimental validation.

Notwithstanding the admonishment above against purely theoretical approaches to pharmacology, in this paper we explore 'toy' models of cell signaling networks as a means of illustrating how models can be used to evaluate combination drugs. The ODE-based toy models contain one or more cell surface receptors and a downstream signaling cascade but make no claim to represent real biological networks except insofar as they show amplification, ultrasensitivity and feedback control (see
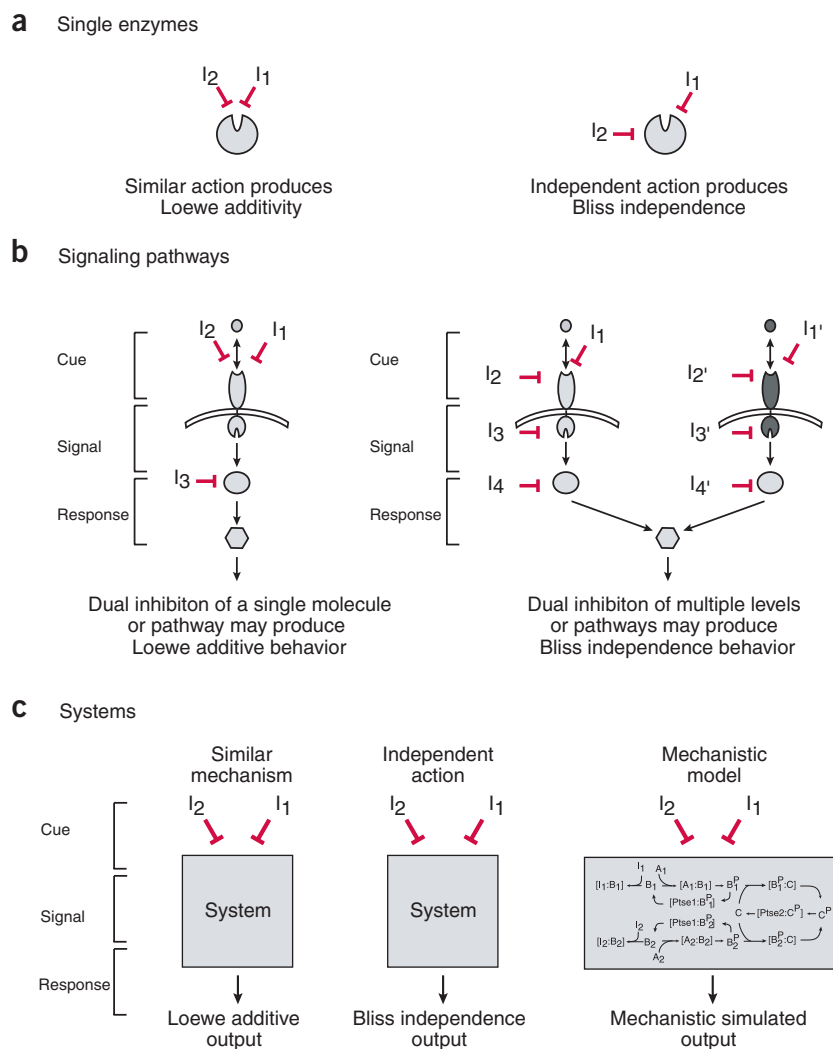
Figure 1 Application of traditional definitions of additivity to signaling networks. (**a**) Single enzymes: (left) combinations of enzyme inhibitors that have overlapping binding sites are inhibited according to Loewe additivity (see **Box 1** for details) and (right) combinations of enzyme inhibitors that have nonexclusive (independent) binding sites are inhibited according to Bliss independence. (**b**) Signaling pathways: applying Loewe additivity and Bliss independence to signaling networks is not straightforward. (left) Inhibitor combinations in a simple network that could be classified as similar action, and may generate Loewe additive behavior: same site on target molecule ($I_1 + I_2$) and same pathway ($I_1 + I_3$). (right) Inhibitor combinations that could be classified as independent action and may generate Bliss independence behavior: inhibition at different sites on the same target molecule ($I_1 + I_2$); inhibition at different levels on the same pathway ($I_1 + I_3$ or I_4); inhibition of separate pathways ($I_{1–4}$ with $I_{1'–4'}$). (**c**) Systems: Loewe additivity and Bliss independence do not capture the mechanisms of inhibitor interactions in complex systems and instead serve as black box models in which Loewe and Bliss criteria represent phenomenological additive standards. Mechanistic models can capture complex signaling dynamics and thereby be used to compute how inhibitors will perform in combination.

with greater efficacy), and horizontal synergy if the concentrations of inhibitors are adjusted downward so as to achieve a constant level of combined efficacy. In addition to illustrating a rather obvious example of synergy, this model also highlights just how abstract Loewe additivity and Bliss independence are when applied to cell signaling networks. Inhibitors I_1 and I_2 are nonexclusive inhibitors, but neither Loewe additivity nor Bliss independence correctly predicts their combined effect. Synergy, often treated as an unexpected experimental discovery, can actually arise as a straightforward manifestation of network topology.

The model in **Figure 2** represents, in a highly simplified fashion, the apparent interaction of insulin-like growth factor 1 (IGF-1) and ErbB signaling in breast cancer. Upregulation of IGF-1 signaling is observed in cells from a subset of patients whose tumors have become resistant to anti-EGFR drugs[9], and cross-talk between IGF-1 and EGF signaling is apparent in glioblastoma lines[10]. Only modest inhibition of growth is observed with trastuzumab (Herceptin) in ErbB2-overexpressing cell lines[11], but this effect is greatly potentiated by simultaneous addition of anti-IGF-1R. A similar effect may occur with drugs targeting ErbB2 and VEGF, as inhibition of both receptors is necessary to prevent the emergence of resistance in xenograft studies[12]. As noted above, however, only experimentally validated models that accurately describe actual signaling systems can be used to explore the mechanisms of action of real combination drugs; toy models only illustrate possibilities.
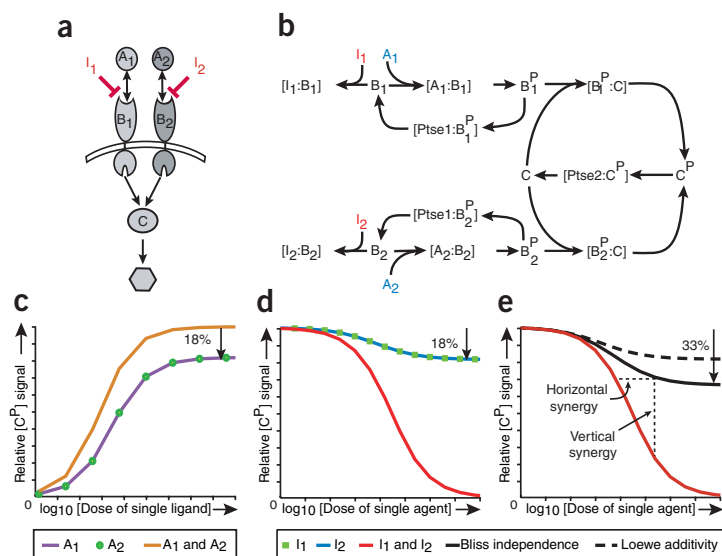
Inhibition of a single target by two inhibitors

A somewhat less obvious situation is encountered when a single receptor is targeted by two inhibitors (**Fig. 3a**). In our model we assume that inhibitors I_1, I_2 and I_3 block binding of ligand A to receptor B

Figs. 2–6)[45]. In the spirit of Jackson's[46] examination of metabolic inhibitors in a simple amphibolic network, our goal is illustrative and pictorial rather than formal and algebraic. Unlike Jackson, we concentrated primarily on network topologies rather than parameter values (V_{max}, K_m, and others) although both are important. In each case, we calculated dose-response curves for a single downstream signaling protein when inhibitors are used individually and in combination, and we also computed curves corresponding to Loewe additivity and Bliss independence. Simulations were performed using Matlab SimBiology, and reactions and parameter values are provided in **Supplementary Tables 1** and **2**.

Consider a signaling system in which two receptors (B_1 and B_2) converge on a single downstream target C whose activity is a measure of therapeutic effect (**Fig. 2**). As represented in the biochemical reaction scheme of **Figure 2b**, activation of B_1 by ligand A_1 and of B_2 by A_2 contributes equally to the activity of C and is subject to negative regulation by phosphatases. Because the two receptor systems are independent, using either I_1 or I_2 alone is relatively ineffective at blocking activation of C, whereas the combination of I_1 and I_2 is very potent (**Fig. 2d**). The combination dose-response curve computed from the network and the computed Bliss independence and Loewe additivity curves differ substantially (**Fig. 2e**), indicating the existence of synergy between I_1 and I_2. This synergy can be viewed in two ways: vertical synergy if combination therapy involves the same doses as monotherapy (but

Figure 2 Inhibitor combinations targeting two converging pathways. (**a**) Regulatory scheme for two distinct ligands (A_1 and A_2) and their cognate receptors (B_1 and B_2) that converge on a downstream signaling kinase (C) leading to its activation by phosphorylation (C^P). Inhibitor I_1 targets B_1 and I_2 targets B_2. (**b**) A reaction scheme for the pathway in **a**. Phosphatases (Ptse1 and Ptse2) are present to ensure recycling of activated C and B. (**c**) Simulated dose-response curves showing that A_1 and A_2 identically activate C to submaximal levels. A_1 and A_2 together maximally activate C. (**d**) Simulated dose-response curves in which A_1 and A_2 activate C in the presence of increasing amounts of inhibitors I_1, I_2 or their combination. I_1 and I_2 have identical effects when used separately, completely blocking signaling through their respective receptors but only partially inhibiting activation of C. In contrast, I_1 + I_2 fully blocked activation of C. (**e**) Comparison of simulated I_1 + I_2 combinations with the Loewe additivity curve (calculated by fitting the simulated single inhibitor dose response curves using equation (3) and iteratively solving equation (4) and the Bliss independence curve (generated using the same fitted dose-response curves and applying the effect multiplication formula, equation (6)). The simulated combination produced inhibition that was greater than the naïve additive models (vertical synergy), requiring a lower dose to achieve the same level of inhibition (horizontal synergy).
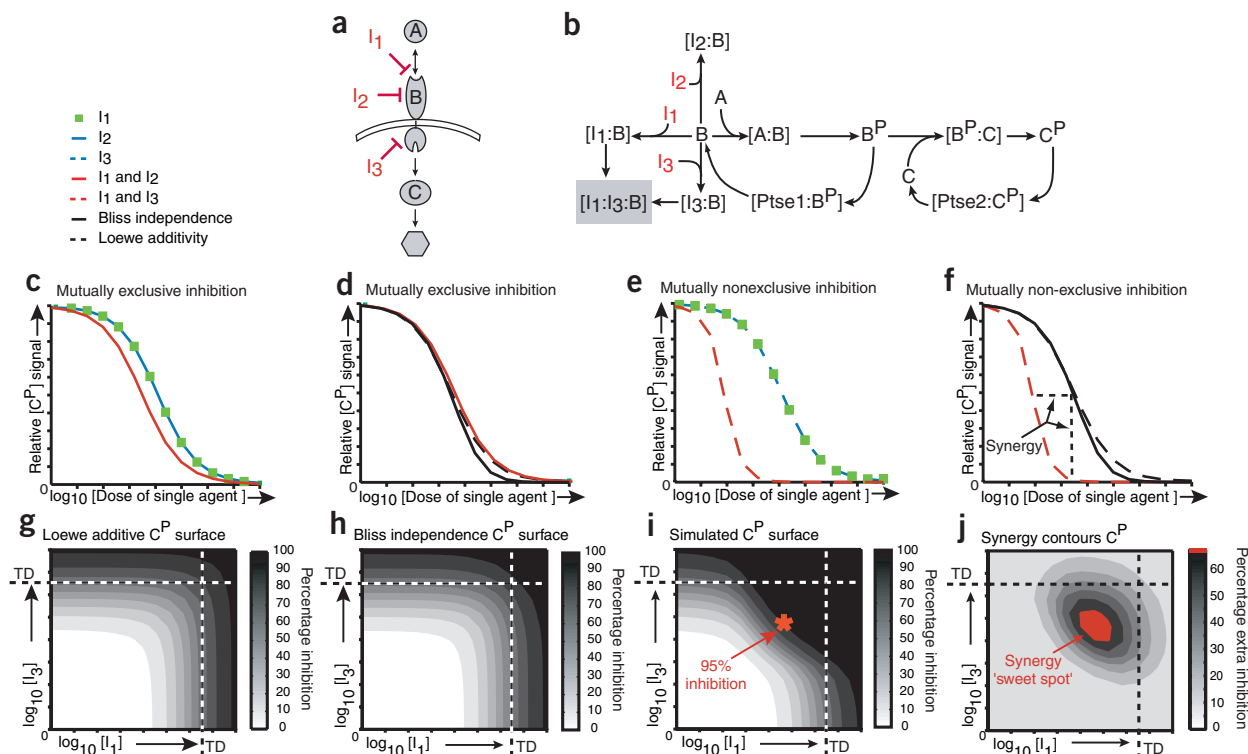
Figure 3 Simulation of dual inhibition of a single target examined with mutually exclusive or nonexclusive inhibitors. (**a**) A regulatory scheme of a single linear pathway. Inhibitors I_1 and I_2 or I_1 and I_3 act on the same target molecule, the receptor B. (**b**) A simplified reaction scheme for **a**. Binding of the second inhibitor either prevents inhibitor I_1 from binding (I_2, mutually exclusive model) or does not affect binding of I_1 (I_3, mutually nonexclusive model). (**c,d**) Simulated dose-response curves, in which C is activated by A in the presence of inhibitor I_1, I_2 or the combination. The mutually exclusive combination follows the Loewe additivity model, confirming the assumption of a similar mode of action. (**e,f**) Simulated dose-response curves, in which C is activated by A in the presence of inhibitor I_1, I_3 or the combination. The mutually nonexclusive combination inhibited activation of C to a greater extent than the expected Bliss independence curve. The enhanced sequestering of B into a long-lasting nonactive triplex [I_1:I_3:B] was responsible for the synergistic effect on activation of C. (**g–j**) Response surfaces for the mutually nonexclusive inhibitors from a checkerboard of 400 dose pairs: Loewe additivity (**g**), Bliss independence (**h**) and the simulated combination (**i**). Hypothetical toxic dose (TD) thresholds limit the amount of inhibition the inhibitors can achieve individually. In combination, the inhibitors achieve hypothetical therapeutic efficacy (95% inhibition, marked as *) without reaching the TD. The region of synergistic inhibition was highlighted by subtracting the inhibition in **i** from the Bliss independence surface, identifying a sweet spot where the extra inhibition exceeded 50% (**j**).

and thereby prevent activation of C (**Fig. 3b**). The kinetic parameters of I_1, I_2 and I_3 are equal, hence they produce the same dose response (**Fig. 3c,e**). If the inhibitors are mutually exclusive (that is, I_1 and I_2), it is reasonable to assume they act through a similar mechanism and that Loewe additivity correctly describes their combined effect on C. Indeed, the dose-response curve for $I_1 + I_2$ is simply a leftward-shifted version of curves for I_1 or I_2 and matches precisely the predictions of Loewe additivity (**Fig. 3c,d**). If, however, simultaneous binding is possible, representing a case of mutual nonexclusivity (that is, I_1 and I_3), it is reasonable to expect Bliss independence. This is represented in the toy model by inclusion of a triplex species [I_1:I_3:B] (**Fig. 3b**). The effectiveness of the individual inhibitors is unchanged, but the IC_{50} value for $I_1 + I_3$ is now much lower and synergy is observed (**Fig. 3e**). Synergy arises because Bliss independence treats binding of I_1 and I_3 as redundant, whereas in reality, independent binding results in cooperative suppression of B and thus C: That is, 'double inhibition' of B causes it to spend a greater fraction of its time inhibitor-bound even under conditions in which I_1 or I_3 have appreciable dissociation rates. A similar situation appears to exist with gefitinib (Iressa), a small-molecule inhibitor of the ErbB1 tyrosine kinase, and certuximab, (Erbitux), a monoclonal antibody that generates inactive ErbB1-ErbB1 and ErbB1-ErbB2 dimers by associating with ErbB1 extracellular domains[47–49]. The synergism observed in **Figure 3f** corresponds qualitatively to observations in xenografts for certuximab and gefitinib in combination[50].

The potential value of computing 3D dose-response surfaces is clear when the Loewe and Bliss additive surfaces, the simulated activity of C and a 'synergy' surface (C activity subtracted from the Bliss surface) are compared over a range of I_1 and I_3 concentrations (**Fig. 3g–j**). Synergy is observed only over a relatively narrow range of inhibitor concentrations (**Fig. 3j**). Finding this region, which would be laborious experimentally, is especially important if the hypothetical toxic dose (TD) for I_1 and I_3 is near the effective dose (that is necessary to achieve 95% inhibition). When used in combination, $I_1 + I_3$ achieves 95% inhibition well below TD, whereas neither inhibitor achieves a useful therapeutic index on its own.

Targeting different levels of a single pathway

Another case of interaction arises when two inhibitors act on different components of a linear pathway (**Fig. 4a**). The regulatory scheme in **Figure 4** is the same as in **Figure 3** except that I_2 now binds to C (**Fig. 4b**). Simulation shows that I_2 is more effective than I_1, in large measure because signals at B are amplified ~10-fold at C, so B must be nearly completely inactivated to inhibit C. Although I_1 and I_2 are mutually nonexclusive, the potency of the $I_1 + I_2$ combination closely matches Loewe and Bliss predictions and therefore fails to show the synergy arising when I_1 and I_2 bind the same target (compare **Fig. 3f** and **Fig. 4d**). This counterintuitive result arises because no beneficial effect on the half-life of inhibitor-bound complexes occurs when two different targets are inhibited as opposed to one target double-inhibited, a potentially important consideration when designing combination therapies. However, the details of the reaction scheme are also important. If we alter the model so as to include ultrasensitivity similar to that shown by the MAP kinase cascade (in which two independent phosphorylation events are required to activate MAPKK, **Fig. 4e**) the IC_{50} for either I_1 or I_2 alone is higher than in the absence of ultrasensitivity (**Fig. 4c,f**), but the $I_1 + I_2$ combination is now synergistic (**Fig. 4g**). Thus, analyzing real biological networks for ultrasensitivity and similar nonlinear effects is likely to be helpful in predicting the consequences of using combinations of drugs with different mechanisms of action.

Feedback-controlled targets

Negative feedback in engineered circuits ensures robustness with respect to parameter variation, an observation that may explain its frequent appearance in biological networks[51–55]. However, the presence in a network of multiple feedback and feedforward loops makes it difficult to intuit the effects of combination therapy. By way of illustration, C in its active form is postulated to negatively regulate activation of B (**Fig. 5a,b**). Intriguingly, in this case, C (the output of the system) is now inhibited more effectively by I_1 than I_2 (**Fig. 5c**), the opposite of what we find in the absence of feedback (**Fig. 4c**). This arises because blocking C directly (with I_2) relieves inhibition on B and therefore promotes activation of C, whereas blocking B (with I_1) suppresses activation of C without affecting feedback control. Careful consideration of reaction pathways is necessary
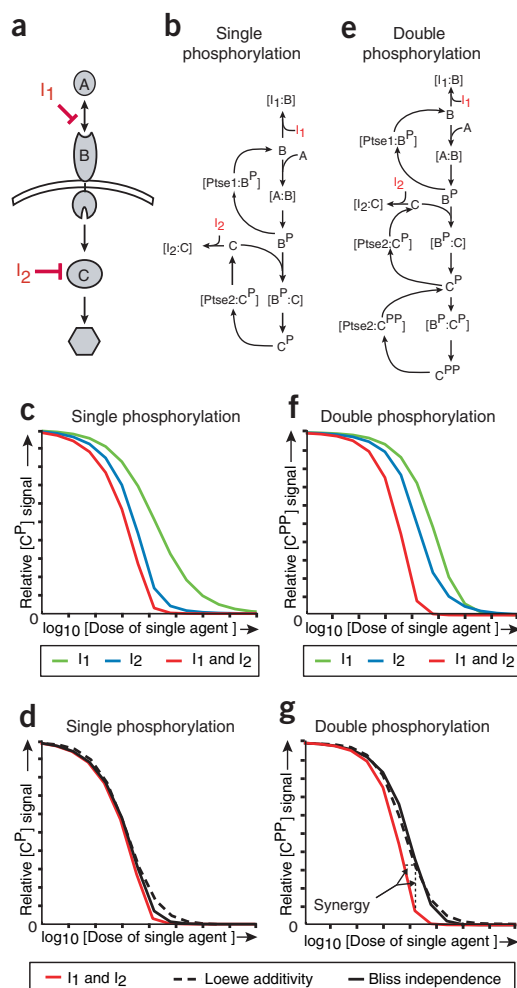
Figure 4 Targeting multiple levels of a simple signaling network leads to additivity or synergy depending on network topology. (**a**) Regulatory scheme of a linear amplification pathway, depicting inhibitors acting at multiple levels. (**b**) The mechanistic model of linear pathway in **Figure 3** is modified so that inhibitor I_2 targets a downstream signaling molecule, C. (**c,d**) Simulated inhibitor dose-response curves, where activation of C requires a single phosphorylation event and produces additive behavior. Inhibition of C directly with I_2 was more effective than inhibiting B because of the amplification present along the cascade. (**e**) A mechanistic model of an ultrasensitive signaling cascade in which activation of C requires two phosphorylation events. (**f,g**) Simulated inhibitor dose-response curves for the ultrasensitive network, showing steeper curves and synergistic inhibition as compared to additive models.
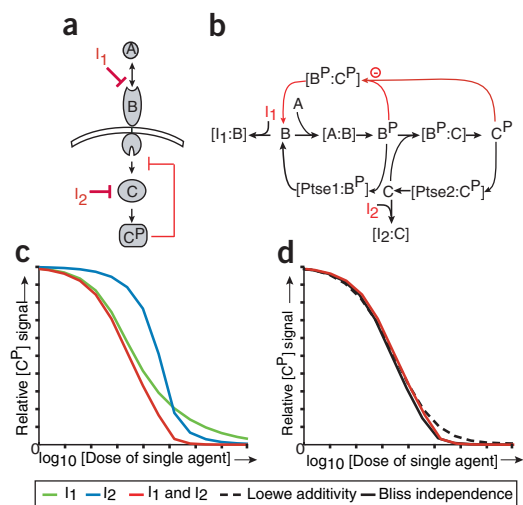
Figure 5 Negative feedback alters inhibitor potency. (**a**) Regulatory scheme in which the target of inhibitor I_2 is within a negative feedback loop. (**b**) A simple mechanistic model in which activated C can deactivate B creating a negative feedback loop. (**c**) Simulated inhibition dose-response curves showing that activated C was less affected by I_2 than by I_1, in contrast to the situation in the absence of **Figure 4c**, indicating the robustness or insensitivity of a target within a negative feedback loop. (**d**) The inhibitor combination was more effective than either inhibitor alone, and produced inhibition comparable with the additive models.

to maximize therapeutic efficacy when feedback regulation is involved.

One recent example of a feedback network targeted by small-molecule inhibitors is the mammalian target of the rapamycin (mTOR) pathway, which mediates cellular response to nutrients and growth factors such as IGF-1 (refs. 14,56). Inhibition of mTOR is promising as an anticancer strategy, as many proteins lying upstream and downstream of mTOR are deregulated in a human cancer[57]. However, growth of cells in the presence of IGF-1 was found to be unaffected when mTOR was inhibited with a rapamycin analog; instead, activity of the upstream regulator Akt increased[58]. An explanation for this paradoxical finding is that mTOR and Akt are components of a negative feedback loop involving

the downstream S6-kinase and upstream adaptor IRS1 (refs. 13,14). mTOR inhibition is counteracted by reduced negative feedback on IRS-1, leading to higher Akt signaling and negligible effect on proliferation. Encouragingly, a rapamycin analog was effective in blocking growth when used in combination with a small-molecule inhibitor to IGF1R, a finding qualitatively similar to observations in the toy model (**Fig. 5c**).

Maximizing therapeutic index
The key to effective therapy is not potency per se but therapeutic index. Diseased cells must be more sensitive to a drug or combination of drugs than normal cells. Consider the linear ultrasensitive network of **Figure 4e**. If the amount of receptor B is 10-fold higher in diseased than normal cells (in this case, B might be an oncogene such as *Her2*), inhibitors I_1 and I_2 show substantial synergy in diseased but not normal cells (compare **Fig. 6b,c**). However, the IC_{50} for $I_1 + I_2$ is higher in diseased than normal cells (**Fig. 6e**), and the therapeutic index is therefore poor, despite the existence of synergy. If, on the other hand, B is underexpressed 10-fold in diseased cells, $I_1 + I_2$ are no better than additive (**Fig. 6d**), but diseased cells are now dramatically more sensitive than normal cells (**Fig. 6e**). Thus, therapeutic index is achieved despite the absence of synergy. These considerations argue that computational analysis of drug combinations should not focus simply on synergy, but rather on maximizing potency in diseased versus normal cells.

Summary and future directions
Redundancy in the control of many disease processes and multiplicity in the physiological functions of important pharmaceutical targets limit the ability of even potent and chemically selective drugs to achieve efficacy with acceptable toxicity. An attractive way to enhance therapeutic index is combinatorial selectivity, in which drugs are mixed so as to preferentially inhibit diseased versus normal processes, ideally in a manner tailored to individual patients. The value of combination therapy is suggested by numerous gene knockout studies, and its practicality is shown by the ubiquity of multicomponent drugs in the treatment of cancer. As more and more potent single-agent inhibitors are developed, the question becomes how to find useful combinations without resorting to large mechanism-blind clinical trials. We suggest here that mathematical analysis of biological circuits holds considerable promise as a means of comparing mono- and combination therapies. Even in the case of very simple networks, the optimal choice of targets and drugs is not
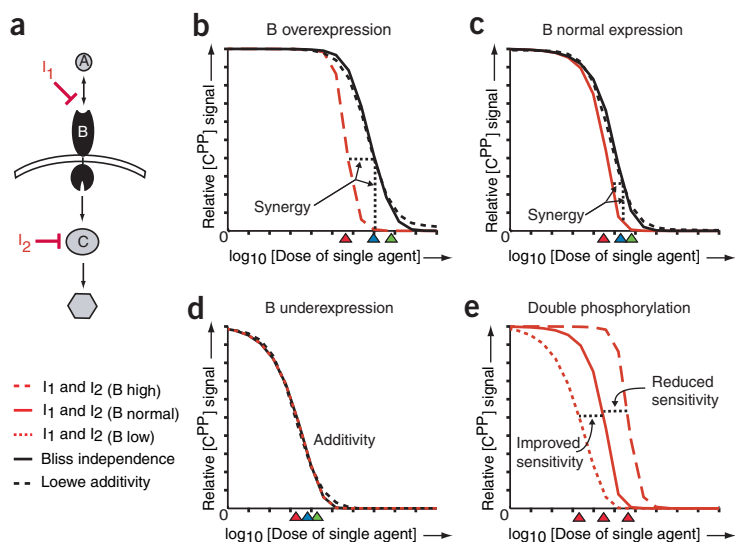
Figure 6 Comparing inhibitors under conditions in which parameter values vary between diseased and normal tissues. (**a**) Regulatory scheme of a linear ultrasensitive pathway in which the concentration of B (the target of I_1) was varied over a 100-fold range among diseased and normal cells. (**b**) Simulated inhibition dose-response behavior when B was 10-fold more abundant in diseased than in normal cells. IC_{50} values for I_1, I_2 and the combination are indicated on the *x*-axis by green, blue and red triangles, respectively. (**c**) Synergy was observed as compared to the normal network. (**d**) Dose-response behavior when the amount of B was reduced 10-fold below normal levels. In this case, additive behavior was observed. (**e**) Measures of synergy do not always correctly predict cases in which therapeutic index is observed with combination therapy: synergy is high with overexpression of B but therapeutic index is low; whereas synergism is absent with underexpression of B but therapeutic index is good.

necessarily obvious, and mathematical modeling should be useful during preclinical screening of lead compounds (to guide identification of compounds that synergize in cells) and also later during clinical testing (to aid in the selection of combination drugs and doses with the highest therapeutic index). It is worth noting, however, that the mechanistic models described here address only the issue of efficacy. Suppression of resistance, reduced toxicity and other aims of combination therapy currently lie outside of their purview, but all should be amenable to quantitative study.

Development of a practical computational and systems approach to pharmacology clearly awaits the development of models of biological networks relevant to human disease that realistically capture, in mathematical form, actual cellular and tissue physiology. We do not yet know precisely what these models will look like, but efforts are under way in several groups to create experimentally verified models of cell signaling, cell differentiation and oncogenic transformation (see http://www.systembiologie.de, for example). Typically these models encompass only a small subset of cellular reactions in relatively simple biological settings. Newly developed software is making it easier to merge small models and perform sophisticated analyses, such as parameter estimation and sensitivity analysis[59], on the combined therapies. However, large models with multiple signaling circuits raise new conceptual issues, such as how best to represent the partitioning of shared components (for example, Ras, MAP kinases and others) among potentially competing pathways[60]. Large models also highlight the data-starved state of most systems biology research.

Lest one be tempted simply to wait for 'final' models to emerge, it is worth remembering that "all models are wrong; some are useful"[61]. We specifically reject the critique that experimentally validated mathematical analysis of cellular networks will be useful only when 'complete' models of cells or tissues are available. Quantitative modeling of bridges and buildings was essential for mechanical engineering long before it was practical to represent every beam and bolt or to understand the atomic properties of steel. Whatever the limitations of emerging mathematical models of cellular physiology, they will almost certainly be better guides for drug development than the prevailing practice of representing biochemistry pictorially and reporting network interactions piecemeal.

Note: Supplementary information is available on the Nature Chemical Biology website.

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