INNOVATION

Multicomponent therapeutics for networked systems

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Abstract | Therapeutic regimens that comprise more than one active ingredient are commonly used in clinical medicine. Despite this, most drug discovery efforts search for drugs that are composed of a single chemical entity. A focus in the early drug discovery process on identifying and optimizing the activity of combinations of molecules can result in the identification of more effective drug regimens. A systems perspective facilitates an understanding of the mechanism of action of such drug combinations.

For most human diseases, there are no magic bullets. The more we learn about the genomic and molecular underpinnings of disease processes, the more apparent this conclusion becomes. Many diseases with a high incidence in the population, such as diabetes, heart disease, cancer, arthritis, asthma and depression, have a multifactorial basis that involves both genetic and environmental risk factors[1–3]. Yet most modern searches for new drugs take place within the terrain of the one-target, one-drug paradigm, in which efforts are focused on identifying a single new chemical entity that inhibits one well-defined molecular target.

As industry and academia acquaint themselves with the genomics-derived parts list of nearly 30,000 genes, these groups have predominantly focused on determining the function of each part in isolation. Such a reductionist approach, which is undeniably fruitful in some cases, does not exploit the network complexity and pathway redundancy that is engineered into living systems through evolutionary processes. As a consequence, those who limit their search for new drugs to compounds that affect one gene or protein might overlook significant therapeutic opportunities.

We define a multicomponent drug as a therapeutic regimen that, rather than consisting of a single compound that interacts with a single target, is a concerted pharmacological intervention of several compounds that interact with multiple targets. Systems biology provides a perspective from which to understand at a molecular level the basis for the superior efficacy and reduced toxicity of some multicomponent drugs. Systems biology is the large-scale study of the functional and physical relationships between the molecules that make up life[4,5]. Numerous studies have supported the notion that living systems are interconnected networks of molecular components. Young and colleagues, for example, revealed the wiring diagram of the *Saccharomyces cerevisiae* cell cycle, in which transcriptional activators that function in one stage of the cell cycle regulate the activators that function in the next stage, forming a fully connected periodic machine[6]. The circuitry that governs the mammalian cell cycle is likely to be much more complicated than that of *S. cerevisiae* in terms of interconnectedness and the need for complex representations[7].

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Mammalian cellular growth-factor signalling pathways have a similar degree of complexity. For example, AKT (the v-akt murine thymoma viral oncogene homologue 1)-regulated pathways show divergent signalling, in which AKT regulates a host of downstream targets and outcomes (FIG. 1a)[8,9]. Selectively inhibiting AKT would probably result in side effects, as all downstream processes would be inhibited. Multicomponent drugs, on the other hand, would be much more effective in such systems in which there are divergent pathways. For example, inhibition of the downstream pathways of AKT with separate inhibitors could yield blockage of cell survival and cell proliferation without affecting glycogen metabolism (FIG. 1a).

Many signalling networks in mammalian cells are also likely to be wired with redundant pathways, such that optimal therapeutic interventions can be achieved through perturbing several different points of the network[4,10,11]. Redundant pathways, such as transforming growth factor-β (TGF-β)-induced SMAD2- and SMAD3-mediated transcriptional activation, can compensate for each other if either one is inhibited. Such pathways are therefore optimally modulated with multicomponent drugs that block both SMAD pathways in this case, because this ensures that the other pathway(s) do not partially or completely compensate for the inhibition (FIG. 1b). Blocking the upstream signal (for example, TGF-β receptor in this case) would not be appropriate because although this would shut down both SMAD pathways, it would also block other TGF-β-receptor-initiated signalling pathways, which could potentially lead to undesired effects.

A realistic assessment of existing pharmaceutical agents reveals that many effective treatments are drug combinations and that the much sought-after, highly specific small

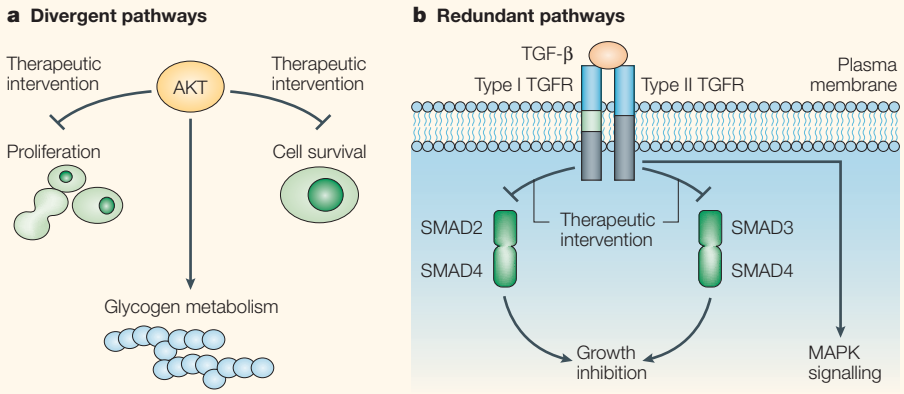

Figure 1 | **Networked systems might require multicomponent interventions to modulate signalling outputs. a** | Targets at divergent pathway nodes might cause undesired side effects when acted on in isolation. For example, AKT regulates several downstream outputs, so inhibiting this protein on its own is not likely to achieve a separation of desired and undesired effects. If we want to inhibit cell-proliferation and cell-survival pathways, for example, without affecting glycogen metabolism, we would need multicomponent drugs to specifically inhibit these two downstream pathways rather than using a single AKT inhibitor. **b** | Redundant pathways can compensate for inhibition of another pathway. For example, SMAD2 and SMAD3 perform largely similar functions in tissue culture experiments. A small-molecule inhibitor of either SMAD2 or SMAD3 alone would therefore not be effective at blocking transforming growth factor-β (TGF-β) signalling if cells responded by upregulating a redundant SMAD. TGF-β regulates several downstream outputs, so inhibiting this protein on its own could cause undesired effects by inhibiting SMAD-independent TGF-β effects, such as activation of mitogen-activated protein kinase (MAPK) signalling. Using multicomponent interventions to simultaneously inhibit SMAD2 and SMAD3 would overcome both these problems by blocking SMAD-dependent TGF-β effects without inhibiting SMAD-independent TGF-β effects. TGFR, TGF-β receptor.

molecules that target single proteins are rarely observed entities[12–20]. As we describe here, a focus on multicomponent therapeutics might lead to new insights and medicines; such drugs comprise several biologically active compounds with mutually interdependent activities that are required for an optimal effect. Ideally, when these components interact in a biological system, they yield a significant and desired pharmacological effect. Preferably, such a drug is administered as a single pill that contains several active components and the necessary excipients and stabilizers. Therefore, from the perspective of a patient or physician, a multicomponent drug might be perceived as a single therapeutic intervention. We posit that multicomponent drugs are particularly effective interventions in networked systems. Below, we review evidence that supports this position on the basis of both existing effective drug combinations and theoretical considerations of networked systems.

History of multicomponent drugs

Drug regimens that contain several active components have, in one form or another, been in use for many years. Traditional Chinese medicine and other historical and traditional approaches to medicine have used mixtures of naturally occurring herbs and herbal extracts[21], and such mixtures are considered integral to the therapy. Many of the natural product extracts that have been tested have yielded activities that later disappeared when the extracts were fractionated into individual chemical components[22–24]. Numerous combinations of active compounds have been found to be produced by natural sources (BOX 1).

In Western medicine, some investigators were interested in the interactions between purified single compounds as early as the late-nineteenth century. Thomas Richard Fraser investigated the interaction between physostigma and atropia[25], and, in 1928, Loewe and others studied the interaction between other defined drug combinations[26,27]. At the beginning of the twentieth century, most therapeutic regimens were composed of cocktails or complex extracts. Examples include serum therapy, for which Emil Adolf von Behring was awarded the Nobel Prize in Physiology or Medicine in 1901, and the application of polyclonal antibody therapy.

However, in 1908, Paul Ehrlich was awarded the same Nobel Prize for his pioneering studies on the search for 'magic bullets' that selectively target the constituents of infectious organisms relative to the host's constituents[28]. This award heralded the gradual shift from the use of complex extracts to the use of defined small molecules[29]. In 1912, Sir Leonard Rogers showed that emetine was the principal active component of ipecacuanha for the treatment of amoebiasis; Gaspar de Oliveira Vianna demonstrated that compounds based on antimony were useful for treating leishmaniasis; and Carl Browning showed that acriflavine and proflavine were effective antibacterials. Compounds were subsequently found that were effective against sleeping sickness, malaria, trypanosomes, syphilis, pneumonia, sepsis, schistosomiasis and babesiasis. These findings were followed in subsequent years by the monumental discoveries of the sulphonamides and penicillin[29].

Therefore, in the early part of the twentieth century, a significant shift occurred from the use of complex extracts to the use of purified single compounds for the treatment of disease. In the latter half of the twentieth century, this evolution reached its logical zenith in the search for single compounds that affect single targets. It is now time to revisit past experiences to identify multicomponent therapeutics for the treatment of complex diseases.

Clinical rationales

There is widespread evidence that combinations of compounds can be more effective than the sum of the effectiveness of the individual agents themselves, a result that can be rationalized using principles derived from modern systems and molecular biology. In addition to the historical examples discussed above, recent findings support this claim. For example, it is now known that several mutations are required for the devlopment of colorectal and other cancers[30]; the correction of these defective pathways will probably require several interventions. Consonant with this knowledge of the multifactorial mechanistic basis of cancer is the observation that oncological chemotherapeutic regimens most often involve combination therapies, such as doxorubicin, cyclophosphamide, vincristine and prednisone (abbreviated as CHOP)[31]. In noticing the success of such combinations, some companies have converted clinically used drug combinations into single-pill formulations.

Many more multicomponent drugs have been developed from separate single-compound drugs that already exist to treat the target disease on the basis of a clinical rationale. Three examples of such drugs are given here: Advair for asthma (GlaxoSmithKline), Advicor for hypercholesterolaemia (Kos Pharmaceuticals) and Combivir for HIV (GlaxoSmithKline).

Advair was developed on the basis of clinical observations, and combines a steroid, which affects an inflammatory component of asthma, with a long-acting β_2-adrenoceptor

Box 1 | **Combinations of natural products**

Numerous interacting combinations of natural products have been discovered in extracts of material derived from natural sources. For example, Nakayama *et al.* isolated a combination of butterfly oviposition stimulants from the host plant *Toddalia asiatica*[60]. Berberine alkaloids, which are antibacterial agents, have been found to be produced by medicinal plants along with a multidrug resistance (MDR) pump inhibitor. This MDR inhibitor, 5′-methoxyhydnocarpin, has no antibacterial activity on its own, but acts as an enhancer of berberine and other substrates of the NorA MDR pump, which is found in *Staphylococcus aureus*[61]. Honey bee queens have been found to produce a mandibular pheromone that is a combination of nine interacting components that functions to attract worker bees, to attract drones for mating and to prevent workers from reproducing. The nine components of this pheromone have all been shown to be required for maximal activity of the cocktail[62].

Another example can be found in the regulation of the human immune system, which uses dozens of cytokines and chemokines[63,64]. The specific response of the immune system to a perturbation depends on the concerted effects of these multiple cytokines. A classic example is the requirement for co-stimulation with both an antigen, which activates the T-cell receptor, and B7, which activates CD28, to achieve full activation of T cells.

These examples illustrate that interacting combinations of molecules are bountiful in nature. One hypothesis to explain this phenomenon of naturally occurring multicomponent drugs is that there are significant benefits for the producing organism, such as the ability to induce effects that cannot be obtained with a single compound or the ability to elicit fewer undesired side effects. It might be that nature has learned a lesson that could be of value to the pharmaceutical industry — namely that there can be strength in numbers.

agonist, which acts as a bronchodilator to relax constriction of the airways. The combination therefore provides greater benefit to the patient than either agent alone[32]. In one clinical trial involving 356 patients, Advair significantly improved baseline 1-second forced expiratory volume (FEV_1) by 25%, which is greater than that achieved with either of the individual components fluticasone (15%) or salmeterol (5%), or a placebo (1%)[33]. Such products had previously been administered separately and had been shown to provide superior clinical results but required the use of two inhalers, which limited patient compliance and convenience. GlaxoSmithKline created a single combination product that combines these two compounds, which resulted in a multibillion-dollar product. Advair is an example of a combination therapeutic product that comprises compounds that act through distinct, but complementary, pathways and mechanisms. It is useful to distinguish between products such as Advair that offer the benefit of the combined activity of the two drugs and those that merely provide convenience of packaging (such as Tylenol Flu). Although both types of combination can be commercially successful, discovering interacting pathways and mechanisms is significantly more difficult than co-prescribing for convenience.

Advicor was developed on the basis of clinical observations that indicated the benefits of taking both niacin and a statin, which inhibits 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase[34]. This combination provides a greater decrease in low-density lipoprotein (LDL) concentration and a greater increase in high-density lipoprotein (HDL) concentration, both of which are desired and beneficial effects[35]. Many physicians had previously prescribed both niacin and a statin for patients with hypercholesterolaemia. Kos pharmaceuticals and DuPont, building on this experience, combined an extended-release version of niacin and a statin into a combination product. The clinical data on the combination product demonstrated the benefit of using the two agents together: whereas Advicor decreases LDL by 42% and increases HDL by 30%, extended-release niacin on its own decreases LDL by 14% and increases HDL by 24%; lovastatin on its own decreases LDL by 32% and increases HDL by 6%[36]. Kos now has one of the fastest market-share growth rates in its class.

Combivir, a combination of azidothymidine (AZT) and lamivudine (3TC) (and Trizivir, a three-way combination with abacavir), was developed after disappointing effects were observed for the first generation of AIDS drugs, as well as to address the fear of increasing resistance[37]. As with cancer, in which progression of the disease can quickly lead to death, physicians tested combinations of the few agents that they had at their disposal. Some laboratory work had indicated that although it might not have been obvious in advance, interactions could be observed with combinations of reverse transcriptase inhibitors[37]. This observation was then tested in the clinic on a pilot basis with pairs of existing agents. AZT and 3TC seemed to be the most promising combination, and in Phase II and III trials showed a substantial combination benefit when the agents were given as separate pills[38]. After these combined benefits were demonstrated in the clinic, GlaxoSmithKline developed the combination single pill that is now known as Combivir, which has since become a commercial success.

These examples show how successful multicomponent products can be. Such combinations, built on the basis of clinical observations, account for a significant share of the pharmaceutical market[39]. They are examples of what we call 'congruous' combinations, or combinations that would be logical to test because both of the component drugs are already being used to treat the target disease (BOX 2). As such, they are not of the same class as drugs that comprise at least one component that is by itself not used for treating the target disease. We refer to such a drug as a 'syncretic' drug; it represents the combination of two or more chemical entities with discrete mechanisms of action into a single, effective therapeutic intervention (BOX 2). It is worthwhile to note that in the case of Combivir, even though the combination

Box 2 | **Proposed terminology for combination drugs**

Syncretic combination drug

The term 'syncretism' derives from the Greek term for the federation of Cretan cities that formed to oppose a common foe. According to the *Merriam-Webster English Dictionary*, it refers to "the combination of different forms of belief or practice", or "the fusion of two or more originally different inflectional forms". Now, it is used to denote a drug that is composed of two or more active ingredients, at least one of which is not used individually to treat the target disease indication.

Congruous combination drug

A drug that is composed of two or more active ingredients, each of which has been individually used to treat the target disease indication.

Multicomponent therapeutic

An optimized combination and formulation of multiple active ingredients. This category includes both syncretic and congruous drugs.

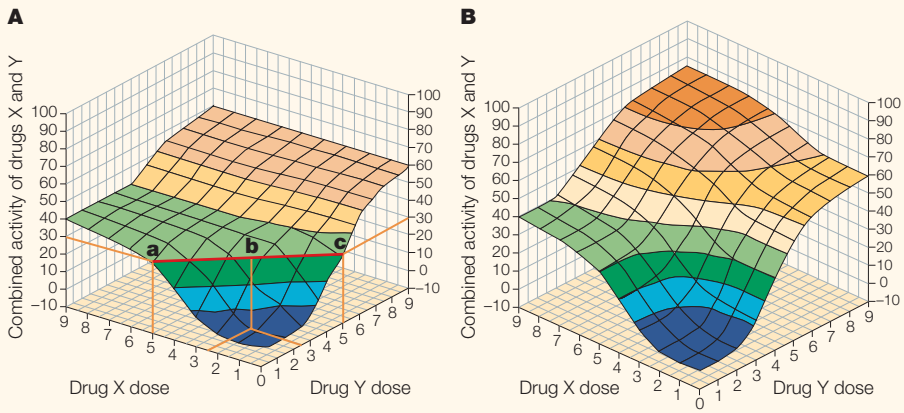

Figure 2 | **Response surfaces for combination effect reference models.** Synergy or antagonism is determined by comparing a measured response to a chosen combination effect reference model. Different reference models are used to represent different types of underlying connectivity, and no one model is preferred in all circumstances. **A** | Loewe additivity is the expected response for compounds that act similarly on the same molecular target. The combined effect is determined by adding specific doses, after correcting for the relative potency of each compound at a particular effect level. So, according to this model, the combined activity of two compounds X and Y at concentrations C_X and C_Y is the activity level $I_{X,Y}$ that satisfies the equation $C_X/C(I_X = I_{X,Y}) + C_Y/C(I_y = I_{X,Y}) = 1$ (where C_X and C_Y are the single-agent concentrations for X and Y that individually produce an activity of $I_{X,Y}$). Therefore, in the example shown, 30% inhibition (shown by the red line) can be achieved with 5 dose units of X (point **a**) or 5 dose units of Y (point **c**), or with 2.5 dose units of X plus 2.5 units of Y (point **b**). Note that the response surface has linear iso-effect contours for effect levels that both single agents can achieve, and that the combined activity never exceeds that of the most effective single agent. **B** | Bliss Independence is the expected combination response when both single agents act on targets interacting through independent probability events, like bullets emanating from separate guns in target practice. Here, the combined activity $I_{X,Y}$ at concentrations C_X and C_Y satisfies the equation $I_{X,Y} = I_X + I_Y - I_X I_Y$ (where I_x and I_y are the single-agent activity levels at C_X and C_Y). This much simpler expression depends only on the single-agent effect levels at corresponding concentrations, so Bliss Independence can be experimentally determined even if the single-agent curves have not been well sampled. In the example, note that the same single-agent curves as in (**A**) produce nonlinear iso-effect contours, and the combination reaches higher effect levels than the single agents ever do.

itself might have been rational to test, the level of clinical interaction that was seen between the two agents was not predictable *a priori*.

There have been attempts to systematize a set of principles that can account for combination drugs that exist today[26,27,40–42]. In some cases, researchers have attempted to understand successful drug combinations by categorizing relationships between molecular targets that might lead to combined effects. In other cases, organismal-level effects can be used to produce effective syncretic combinations. Such efforts might facilitate the discovery of future effective combination drugs.

What is synergy?

It is worth briefly considering the concept of synergy in the context of combination drug discovery. What is its meaning and relevance for multicomponent therapeutics, be they syncretic or congruous? Synergy has its origin in the Greek words *sunergia*, meaning 'cooperation', and *sunergos*, meaning 'working together'. Modern usage in pharmacology has evolved to emphasize the idea that a synergistic combination provides greater effect than would be predicted by simply adding together the effects of the components. Numerous researchers have attempted to provide an unambiguous framework for the calculation of additive effects between compounds; these approaches are reviewed comprehensively elsewhere[26,27,40–44]. Here, we consider two of the most useful reference models of synergy.

Loewe additivism: agent against itself. For many, the preferred additive reference model has been that devised by Loewe[26,27], which in its broader sense encompasses not only isobolographic analyses (a graphical method of detecting synergy by plotting iso-effect curves of varying ratios of two compounds), but also the Combination Index method of Chou and Talalay, which is a generalized method for analysing combination effects on the basis of the principle of mass action (that is, on the basis of equilibrium binding kinetics)[41,42]. The central assertion of these models is that a compound, when combined with itself, must by definition be additive. A theoretical Loewe additive response surface for a combination of two agents can be calculated from the fitted dose–response curves of the individual compounds as shown in FIG. 2A. An experimentally determined dose–response surface is synergistic when its level of effect exceeds Loewe additivism, and antagonistic when it falls below the model surface; in the context of the Chou and Talalay method, these cases yield combination indices of less than and greater than 1, respectively.

Bliss Independence: independent competing agents. Another reference model for combinations of compounds is Bliss Independence[43], which describes the case for two active agents that, when combined, do not directly interfere with each other, yet can both contribute to a common result. A practical advantage of the Bliss Independence approach is that, unlike the Loewe model, it does not require the determination of dose–response curves for the individual compounds to generate the theoretical reference case. Rather, the Bliss Independence expectation for a combination is simply the product of the activity ratios of the individual agents at the same compound concentrations. For example, two non-interacting agents that each inhibit a process by 50% have a combined inhibition of 75% according to Bliss Independence (FIG. 2B).

A crucial consideration when using the Bliss model is that a compound that is tested in combination with itself will not generally seem to be 'independent', so the term should not be thought of as a substitute for 'additive'. The model is theoretically appropriate for pairs of agents with different targets that have no mechanistic connection other than the outcome. In phenotypic screening, compounds can exert their effects through any expressed protein or biochemical pathway that ultimately contributes to the phenotype of the system. Because the expected connection between compounds with randomly selected targets becomes weaker in complex systems, Bliss Independence will be relevant to many biological applications.

It should also be noted that synergy determination depends on testing specific doses, as compound combinations are frequently found to be synergistic over one range of doses and antagonistic over another. Therefore, rather than simply asking whether a particular combination is synergistic, we might do better to consider what dose range optimizes the synergy of this combination.

A definition of synergy relevant for combination drugs. What definition of synergy is most relevant for syncretic drug discovery? In one sense, we must attempt to answer the pragmatic question of the physician: should I use more of the first drug, or is it advantageous to combine with a second drug? In the case of

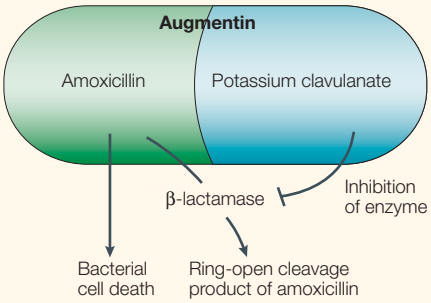

Figure 3 | **The antibiotic Augmentin is a mechanism-based combination drug.** Amoxicillin is a β-lactam antibiotic that acts by inhibiting biosynthesis of bacterial cell-wall mucopeptide. However, amoxicillin is degraded by the enzyme β-lactamase, which hydrolyses the central β-lactam ring of amoxicillin. Potassium clavulanate inhibits β-lactamase, thereby preventing the degradation of amoxicillin. In combination, these two compounds show significant synergy and powerful antibacterial activity.

truly syncretic drugs, the answer will often be that no increase in the amount of the first drug can produce the effect that is desired by the physician, and that it can only be achieved by combination (for example, see FIG. 2B). This is the clinical analogue of 'coalism' described for *in vitro* effects, although it should be noted that this is not strictly considered to be synergy according to many quantitative definitions, such as that formulated by Loewe[40].

In some cases, adding more of the same drug will incur unacceptable negative consequences. For example, when the dose of the first drug is just below a threshold of toxicity, even a small amount more might be unacceptable, whereas combining it with a drug that possesses non-overlapping (orthogonal) toxicities might provide enormous benefit, even if the combined effect on efficacy is only Loewe additive. For example, an increase in tumour-cell killing from 50% to 75% without additional side effects might be clinically meaningful, but not mathematically synergistic.

Ultimately, the best definition of synergy for multicomponent drugs must reflect the physician's requirement that, when the drugs are used in combination, a benefit is observed that could not be achieved by the constituents on their own. Rather than specifically focusing on exceeding additive mathematical models, we would do well to remember an aspect of synergy that is reflected in the organic language of earlier definitions — that of elements acting together and cooperating to achieve completeness of effect.

De novo discovery of syncretic drugs

We propose that there is a largely untapped source of novel drugs for the pharmaceutical industry in the form of syncretic combination drugs. However, despite such precedence from the development of congruous drugs, there has not yet been a concerted effort to develop *de novo* multicomponent drugs. This might be because the industry has not sought such therapies, and because by standard drug discovery approaches they are difficult to find.

One noteworthy example of a commercially successful syncretic drug is Augmentin (GlaxoSmithKline), a combination of amoxicillin and clavulanic acid[45]. Clavulanic acid inhibits one of the mechanisms for degrading amoxicillin, and therefore increases the potency of amoxicillin by overcoming resistance (that is, the ability of bacteria to grow in the presence of amoxicillin by expressing β-lactamase) (FIG. 3). This is an example in which a simplistic aspect of the connectivity of a system was understood and targeted with a combination therapy, and yielded a successful antibiotic. A genuinely systematic approach to the discovery of multicomponent syncretic drug regimens must allow for those cases in which, unlike Augmentin, a beneficial relationship or connectivity cannot be anticipated in advance. Such examples of conditional activity are the pharmacological equivalents of synthetic lethal genetic effects that are found in model organisms such as *Saccharomyces cerevisiae*[46–48]. Screening for such synthetic (and syncretic) combinations allows us to move beyond our current set of target proteins to discover drugs that act through novel mechanisms of action.

Cell modelling and computational approaches. One approach to discovering syncretic drugs is to use a detailed understanding of a system to enable *a priori* design. A growing number of scientists in drug discovery are embracing efforts to understand biology and disease through the *in silico* modelling of cellular systems[49]. Modelling efforts, although representing an intriguing approach to drug discovery, face significant challenges, particularly in the pragmatic world of drug discovery. These approaches require intimate knowledge of the systems being modelled to a level at which it becomes possible to model the actions of perturbations in a predictable way. The reliable prediction of complex interactions, such as the synergistic or antagonistic effects that combinations of compounds might exert through cross-pathway wiring, is not yet feasible. Although modelling approaches are currently in their infancy, in 20–30 years, when

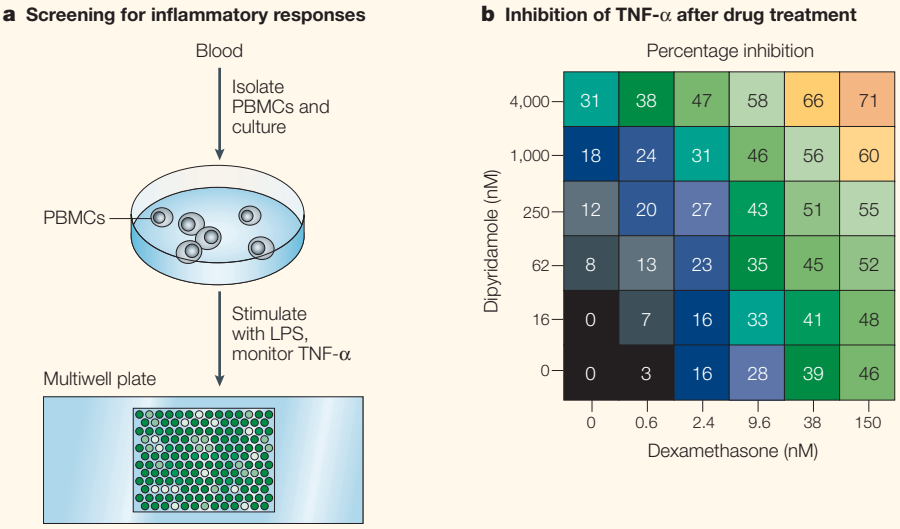

Figure 4 | **A phenotypic assay for combination screening. a** | Stimulation of primary human peripheral blood mononuclear cells (PBMCs) with lipopolysaccharide (LPS) results in the activation of a complex immunological response involving several cell types. This signalling cascade causes upregulation of several pro-inflammatory cytokines, including tumour-necrosis factor-α (TNF-α), which therefore provides a marker of inflammatory responses. By monitoring the production of TNF-α using an enzyme-linked immunosorbent assay (ELISA), it is possible to screen in 384-well format for combinations of compounds that inhibit this inflammatory response. Such compounds might be candidate therapeutics for treating inflammatory disorders such as psoriasis, rheumatoid arthritis and asthma. **b** | Percentage levels of inhibition of TNF-α production from primary human PBMCs after treatment with the indicated doses of dexamethasone and dipyridamole are shown. Part **b** reproduced with permission from REF. 55 © (2003) National Academy of Sciences, USA.

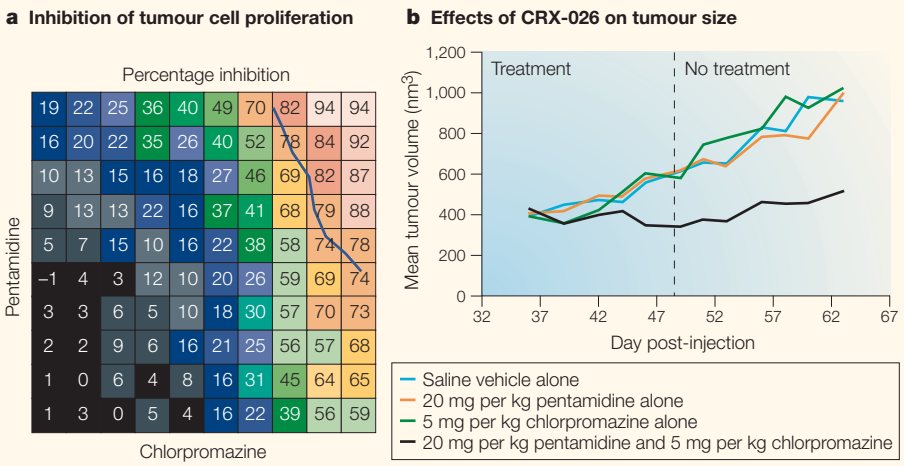

Figure 5 | ***De novo* discovery of syncretic drugs: CRx-026 as a case study. a** | CRx-026 was discovered in a high-throughput combination screen of tens of thousands of combinations of existing drugs for those that synergize in killing human tumour cell lines. CRx-026 is a combination of chlorpromazine, a phenothiazine sedative, and pentamidine, an anti-infective agent. Neither compound is approved for use as an anticancer drug. The colour scale indicates the percentage level of inhibition of viability of A549 lung carcinoma cells by chlorpromazine and pentamidine at a range of concentrations. **b** | CRx-026 effectively inhibits tumour formation in a nude mouse xenograft model, whereas the individual compounds are by themselves less effective[55]. The *x*-axis shows time (in days) post-injection of tumour cells (treatment with compounds begins at 35 days to create a more realistic model of treating existing human tumours). The *y*-axis shows mean tumour volume (in mm^3). Tumour sizes in mice treated with saline vehicle alone (blue line), with 20 mg per kg pentamidine alone (orange line), with 5 mg per kg chlorpromazine (green line) or with both 20 mg per kg pentamidine and 5 mg per kg chlorpromazine (black line) are shown. Only the combination of these two compounds effectively halted tumour growth. Figure reproduced with permission from REF. 55 © (2003) National Academy of Sciences, USA.

cellular circuitry has become more precisely defined, *in silico* approaches could become the foundation of syncretic drug discovery.

Empirical discovery. A more pragmatic approach to systematic, systems-based syncretic drug discovery uses empirical discovery methods that are based on the high-throughput screening (HTS) of compound combinations in phenotypic models of disease. This strategy benefits from, but does not require, understanding disease systems at the level of their molecular circuitry. Moreover, some issues of target druggability[50] and cell permeability are circumvented by discovering combinations that are effective in cells.

Phenotypic disease assays. Although it is possible to conceive of opportunities for discovering desirable combination effects in cell-free assays or with isolated molecular targets, the main benefits of the combination approach are to be found in complex assay systems that contain many networked elements. Such screens can use isolated primary cells, cell lines or mixtures of cells that allow inter- and intracellular interactions (FIG. 4). In recent years, there has been a great improvement in the availability and creation of disease-relevant cell-based assays for phenotypic screening[51–53]. For example, cell-based assays can be used to identify antibacterial[54], antifungal[55], antiviral[56], antitumour[55,57,58], anti-inflammatory[55] or anti-neurodegenerative agents[59].

Combination screening through interaction surfaces. The identification of 'hits' in combination screening requires the integration of computational synergy analysis into informatics tools for high-throughput use. In traditional drug screening, the activity of a compound in a primary screen is sufficiently described by a small set of values that measure its maximal effect, potency (for example, the molar concentration that produces 50% of the maximum possible response (EC_{50})) and, optionally, a value for the sigmoidicity or shape of the curve. The basic unit of screening for combinations, by contrast, comprises a dose-ratio matrix of two agents over a broad range of concentrations and ratios. This provides an interaction surface for the pair, including the full dose–response curves of the individual agents on their own. To select hits, the experimentally determined interaction surface is compared with a series of computed 'additive effect' model surfaces (such as Loewe and Bliss) to determine whether the paired compounds are acting synergistically in the assay, and, if so, at what concentrations and ratios. In addition, the overall shape of the interaction surface can provide information about how the compounds act on pathways and, ultimately, even how the targets for the compounds are related to each other in terms of network connectivity.

Searching combination space: experimental design and informatics. For a set of n compounds, binary combination space is described by $n*(n–1)/2$. For example, a set of 2,000 compounds has nearly 2 million possible binary combinations. Therefore, even a relatively small compound library, such as the set of approved drugs, yields a large number of combinations to be tested. An efficient search of this space balances detailed testing with adaptability to HTS. One solution begins by partitioning combination space for a particular assay system in such a way that active (or partially active) compounds are combined separately from those compounds that have no measurable activity even at their highest achievable concentrations. Whereas partially active compounds are tested in primary screening through full dose-ratio interaction surfaces, synergistic pairs of inactive single agents can instead be identified using an orthogonal pooling strategy at a single high concentration[55].

It should be noted that for partially active compounds, Loewe synergy per se in a primary screen is not absolutely required for ultimate clinical utility to be seen when drugs are used in combination. For example, two compounds that interact according to Bliss Independence might together exceed a threshold of efficacy that could not be achieved by either compound on its own at any dose. Furthermore, when screening for therapeutic window broadening, dose-sparing in Loewe additive mode will be desirable if the 'enhancer' compound is relatively innocuous or has toxicities that are orthogonal to those of the other drug. In summary, the multicomponent drug discovery paradigm has distinct analytical requirements for hit-picking, and therefore demands a unique informatics capability and laboratory information management system (LIMS).

Prioritizing and optimizing combinations for development. Secondary assays, database mining and clustering of combinations according to interaction surfaces are invaluable activities for prioritizing combinations, just as they are for single agents. It is also appropriate to evaluate the chemical compatibility of the two compounds as well as the compatibility of their adsorption, metabolism, excretion, toxicity and other pharmacokinetic and pharmacodynamic parameters.

Just as it is possible to determine the structure–activity relationship (SAR) for a single compound, it is possible to determine the combination SAR (CSAR) for two or more compounds. The structure of each component can be varied systematically to determine which structural moieties are necessary for the observed combination effect. The ability to vary the structures of both compounds simultaneously might allow for the discovery of optimized combinations when varying only one of the components is not effective.

It is often possible to determine the mechanism of action of a single agent by testing various compounds with known mechanisms of action. For example, a recent study found that the potassium ionophore valinomycin selectively kills A549 lung carcinoma cells but not normal MRC9 lung fibroblasts[58]. It is possible that the selective activity of valinomycin results from its previously reported potassium ionophore activity or its ability to bind to some other target protein or molecule. To distinguish between these possibilities, five additional ionophores were tested for their ability to kill A549 cells while sparing MRC9 cells. In this case, all five of the additional ionophores showed selective lethality towards A549 cells, indicating that the selective lethality of valinomycin in this system is probably due to its ionophore activity and not an unrelated activity. We refer to the result of this type of analysis as a mechanism–activity relationship (MAR), by way of analogy to SAR. A similar analysis can be performed with compound combinations, in which the components are substituted with putative mechanistic homologues to identify the relevant mechanism of action of each. We refer to the result of this type of analysis as a combination MAR (CMAR). Together, CSAR and CMAR allow for the optimization and mechanistic assessment of newly discovered combinations.

Summary

The empirical, *de novo* discovery of multicomponent syncretic drugs requires an integrated informatics and experimental pipeline approach that is dedicated to such a process. Such a method can be used to screen millions of combinations of already approved drugs (or new chemicl entities) for activities in new disease indications[55]. Scientists at CombinatoRx, Inc. have used this approach to discover numerous syncretic drug candidates that are being tested in early clinical trials (FIG. 5). Such efforts necessarily encompass cross-disciplinary expertise, including mathematics, statistics, physics, chemistry, biology and computer science. With such groups, it is possible to find novel and effective syncretic drugs for a variety of human diseases. Indeed, the systems-based discovery of therapeutics probably represents the next frontier in drug discovery research.

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Competing interests statement

The authors declare competing financial interests: see Web version for details.

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