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An overview of the state-of-the-art in predictive modelling of compound combination activity and the value and significance of systems informatics in identifying combinations for therapeutic purposes.

Modelling of compound combination effects and applications to efficacy and toxicity: state-of-the-art, challenges and perspectives

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The development of treatments involving combinations of drugs is a promising approach towards combating complex or multifactorial disorders. However, the large number of compound combinations that can be generated, even from small compound collections, means that exhaustive experimental testing is infeasible. The ability to predict the behaviour of compound combinations in biological systems, whittling down the number of combinations to be tested, is therefore crucial. Here, we review the current state-of-the-art in the field of compound combination modelling, with the aim to support the development of approaches that, as we hope, will finally lead to an integration of chemical with systems-level biological information for predicting the effect of chemical mixtures.

Introduction and background

In the 1989 movie directed by Tim Burton, Batman describes the Joker's strategy to bring doom to Gotham's people: ''Each product only contains one component. The poison only works when they're mixed. Hair spray won't do it alone. But... hair spray and perfume and lipstick will be toxic''. The possibility that compounds modulate each other's effect(s) is a well known and frequent phenomenon, be it a desired positive effect in the case of drug combinations or an undesirable toxic effect, as in the case of Joker's devious plot. Compound combinations have been a popular approach in interfering with erroneous and undesirable activity in biological systems,

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Dr Krishna C. Bulusu is a Postdoctoral Research Associate in the Bender group with the Centre for Molecular Informatics at the Department of Chemistry, University of Cambridge, where he leads the subgroup: Compound Combination Modelling, comprising two postdocs and four PhD students. The aim of this group is to exploit the wealth of biological and chemical knowledge available in the public domain to generate biologically meaningful mode-of-action hypotheses for combinations of chemicals, which will facilitate efficient modelling and prediction of the additive, synergistic or antagonistic behaviour of compound combinations. Krishna received his PhD from the Institute of Cancer Research, University of London, in 2014 and holds a Masters degree in bioinformatics from the University of Edinburgh.

Dr Andreas Bender is a lecturer for molecular informatics with the Centre for Molecular Informatics at the Department of Chemistry of the University of Cambridge, leading a group of about 20 postdocs, PhD and graduate students and academic visitors. In his work, Andreas is involved with the integration and analysis of chemical and biological data, aimed at understanding phenotypic compound action (such as cellular readouts and also organism-level effects) on a mechanistic level, ranging from compound efficacy to toxicity. He received his PhD from the University of Cambridge as a Cambridge Gates Scholar in 2005 and worked in the Lead Discovery Informatics group at Novartis in Cambridge, MA, as well as at Leiden University in The Netherlands before his current post.

Dr Rajarshi Guha is a research scientist at NIH NCATS where he has developed storage, analytic and visualisation infrastructures for RNAi and small molecule combination screening. His research interests include network analysis and machine learning applied to chemical and biological systems. Over the past 10 years he has worked in a variety of areas related to computational drug discovery including the development of novel algorithms to characterise various aspects of SAR, building predictive models of bioactivity for a variety of targets and implementing software tools and platforms that make these methods and models available to fellow chemists and biologists. Before joining the NIH he was a visiting Assistant Professor in the School of Informatics, Indiana University (where he currently holds an Adjunct Professorship). He is an active member of the open source cheminformatics and computational chemistry community and was the Chair of the ACS Division of Chemical Information (2012).

http://dx.doi.org/10.1016/j.drudis.2015.09.003

be it drug combination therapy for treating complex network-driven diseases such as cancer [1,2] or antifungals and antibiotic combinations targeting infectious diseases [3,4].

This popularity can be attributed to multiple factors, which include overcoming drug resistance [5,6] and multitargeted therapies for perturbing multiple nodes of pathway(s) of interest for better efficacy [7]. Synergistic drug combinations aside, there is also a crucial need to study compound combinations towards understanding the toxic effects of chemical mixtures, either in a drug–drug combination, for example carbamazepine toxicity in combination with several drugs and inhibitors [8,9], or a drug–natural-product combination, for example the well-studied impact of grapefruit juice on the bioavailability of certain drugs [10,11]. Combination therapy has also been extensively studied in traditional Indian [12] and Chinese medicine [13], as has the impact of these traditional medicines when administered in combination with Western medicine [14,15].

Compound combination behaviour can be broadly classified as synergistic, antagonistic or additive. Synergy, in this context, is the result of combining two or more chemical compounds to produce an effect that is greater than additive effects (where additive effects are computed from the individual effects based on specific mathematical models) [16]. The use of compound combinations can be either beneficial to the biological system these are intended towards, as in the case of combination therapy [1], or produce an intended harmful effect, as found for antifungals [17], or an unintended harmful effect, such as for synergistic toxicity [18]. By contrast, antagonism is the phenomenon when a compound combination produces an overall effect that is less than the additive effects of the individual compounds.

Despite the significance of compound combinations in therapeutic and toxicity studies, the ability mechanistically to explain and model compound combinations in a systematic fashion is currently limited. Published reviews discuss the urgent need for multitarget therapeutics and systematic approaches to identify communication hubs between pathways that can be targeted by drugs [6,19]. However, the approach taken to map and understand the systems level view of the organism or disease comprehensively is expensive, time consuming and not necessarily feasible. Although there have been several reports that elucidate the mechanism of action (MOA) of a compound combination [20–22], most reports focus on observational studies of a limited number of combination effects in specific organisms and diseases. Table 1 provides a list of studies that have followed gene-expression-, pathway-annotations/network- and modelling-based approaches towards assessing compound combinations across different disease areas, as well as generalised studies. A similar table listing complementary studies can be accessed in a recent publication by Ryall and Tan [23]. The dynamics of networks of pathways can be investigated through the use of mathematical network models, and the outcome of potential target inhibition within the model can be compared to assay readouts to allow MOA hypothesis generation of a combination [24,25]. These models could make use of large-scale datasets of compound combination responses. Even though limited in terms of availability, opportunities to train and test predictive models can be provided. Table 2 provides a list of publicly available combination data resources or datasets. This information, along with available large-scale chemical and biological resources in the public domain (Table 3), could be used to construct an integrated pipeline to assess compound combination behaviour. Combining the chemical and biological fingerprints mentioned above, along with gene expression profiles in disease cell lines, wherever available, could add further weight to such analysis. However, there are still certain aspects of data missing that are crucial to assessing combinations. For example, many datasets only consider single doses, and thus could prevent appropriate quantification of synergistic (or antagonistic) behaviour using classical methods. In addition, if the dosage is not therapeutically relevant, it might not be suitable for translational development.

Following such an integrated approach, as described in this section, Fig. 1 suggests a modelling pipeline towards predicting the synergistic and/or antagonistic behaviour of compound combinations. The aim of this pipeline is to integrate and explain the observations from combination assays. For a suggested compound combination, the model will be able to search bioactivity space and integrate available chemical and biological information that includes network and pathway annotations, gene expression profiles and chemical fingerprint similarities. This could help identify patterns that contribute towards synergy predictions for the compound pair, as well as develop a MOA hypothesis for the combination. These predictions could then be further validated by *in vitro* and/or *in vivo* experiments. This review explores the challenges, limitations and, more importantly, the value and perspectives of predictive modelling of compound combination effects in therapeutic development and toxicological studies.

Applications and impact of drug combinations

The applications of studying and analysing the synergistic, additive or antagonistic behaviour of compound combinations can be manifold. These range from therapeutic applications, such as drug combinations, to counter selectivity and resistance, to assessing safety of household chemical combinations through toxicity studies. Drug–target selectivity has long been a high priority, yet not always achievable, part of the drug discovery pipeline [26]. However, many kinase inhibitors and central nervous system (CNS)-active drugs exhibit promiscuity that is often crucial to achieve better efficacy [27,28]. In a study by Lehár *et al.*, the authors performed large-scale simulations of bacterial metabolism and ~94,000 multidose experiments across multiple diseases to show that synergistic drug combinations display higher specificity to certain cellular contexts than single agent activities [29]. Furthermore, results validated in a rat model showed that the anti-inflammatory drug prednisolone and the antidepressant nortriptyline display therapeutic synergy, but not toxicity. Selectivity in this case was achieved through the differential expression of the proteins targeted by these drugs in stimulated peripheral blood mononuclear cells (PBMCs). This evidence could have broad implications in identifying and studying therapeutically relevant selectivity for drug combinations.

Combination therapeutics have also been utilised as an approach to overcome drug resistance of pathogens [21]. This strategy has been popular in antimalarial and antituberculosis drug discovery and usually involves the first drug acting on mutants resistant to the second drug when administered together [30,31]. Drug combinations are a standard-of-care in many cancers, by

TABLE 1

Published studies assessing or predicting compound combinations: separated into gene-expression-based, pathways/networks-based and mathematical approaches

Study	Context	Technique	Limitations
Gene expression and cell-line-sensitivity-based approaches for compound combination modelling			
Prediction of drug combination chemosensitivity in human bladder cancer [70]	Prediction of growth response of human bladder cancer cell lines to chemotherapeutic agents using expression profiles	A predictive model utilising misclassification-penalised posterior (MiPP), for single and combination drug sensitivity	Comprehensive expression profiles required for extrapolation, model less successful in predicting synergy of cytotoxic effects of combinations
Predicting cooperative drug effects through the quantitative cellular profiling of response to individual drugs [67]	Predicting combinations based on differential expression profiles from a β-cell lymphoma cell line in response to single drugs	Integrated expression profiles and human protein interaction network approach. Ranked combination predictions using probabilistic c-index	Single agent expression profiles required, along with confidence in network annotations
An integrated approach to anticancer drug sensitivity prediction [112]	Sensitivity prediction of targeted drugs using cell line sensitivity and target inhibition profiles illustrated on erlotinib and AZD0530	Integrated sensitivity prediction (ISP), Integrated regression modelling (IRM) and constrained tumour proliferation model (CTPM)	Comprehensive pipeline that requires functional and genomic data of drugs of interest, which is often not available
An enhanced Petri-net model to predict synergistic effects of pairwise drug combinations from gene microarray data [69]	Mechanism of synergy of compound combinations from individual compound treatment transcriptional responses	EPN model built on expression profiles across different doses of single compounds, subsequently suggesting mechanism of synergy for pairs	Model validated on only one case of combinations predicted, requires knowledge of downstream targets and signalling pathways to be efficient
Utilisation of translational bioinformatics to identify novel biomarkers of bortezomib resistance in multiple myeloma [113]	*In silico* prediction of novel drug combinations to bortezomib-resistant multiple myeloma	Correlation of drug signatures to experimentally derived gene expression profiles for bortezomib	Requires drug-induced differential expression signatures. No explicit combination modelling was done
Pathways/network-based approaches for compound combination modelling			
DrugComboRanker: drug combination discovery based on target network analysis [114]	Prioritised synergistic drug combinations using disease and drug genomic profiles, evaluated on lung adenocarcinoma and oestrogen receptor (ER)-positive breast cancer	Drug functional network communities using a Bayesian approach	Dependent on accuracy of functional network annotations, tool available on request
Systems-pharmacology dissection of a drug synergy in imatinib-resistant chronic myeloid leukaemia (CML) [115]	Elucidate mechanism-of-action of kinase inhibitor combinations in Bcr-Abl T315I gatekeeper mutation	Integrated phosphoproteomics, transcriptomics and chemical proteomics techniques	–
Chemical combination effects predict connectivity in biological systems [24]	Cellular responses to combinations reveal target connectivity	Models for combination effect morphology (highest single agent, Loewe additivity, Bliss boosting and potentiation)	Complete dose matrices required, expensive to generate. Models cover only a subset of observed responses
Pathway-based screening strategy for multitarget inhibitors of diverse proteins in metabolic pathways [116]	Pathway-based screening to identify multitarget inhibitors to modulate shikimate pathway in *Helicobacter pylori*	Virtual screening and docking to identify binding site moieties, enzyme inhibition assays as validation	Restricted to proteins in the same pathway, and requires protein structures for determination of which exhibit conserved binding sites
Target inhibition networks: predicting selective combinations of druggable targets to block cancer survival pathways [34]	Functional systems pharmacology approach to predict combinations in breast and pancreatic cancer cell lines	Target inhibition model (TIMMA) to predict drug efficacy, validation using single and combination siRNA screens	Requires drug sensitivity readouts for specific cell lines of interest
Computational analyses of synergism in small molecular network motifs [25]	Feedback loops governing the synthesis of CREB1 and CREB2 transcription factors	Ordinary Differential Equation (ODE) models based upon Michaelis–Menten kinetics	Requires knowledge of underlying network connectivity and implication in signalling pathways
Mathematical approaches primarily utilising chemical information for compound combination modelling			
Systems toxicology: from basic research to risk assessment [117]	The integration of classical toxicology with multiple levels of biological network information	Perspective of the systems toxicology field	–
Chemical mixture toxicology: from descriptive to mechanistic, and going on to *in silico* toxicology [118]	Improving on conventional toxicological approaches to modelling the toxic response to mixtures	Reaction network modelling, analysing metabolism networks with computational models	–
A model-based approach for assessing *in vivo* combination therapy interactions [119]	Approach for evaluating the efficacy of combination antitumor agent	Tumour growth/drug effect model fitted to single agent data, then used to quantify the deviation from additive of the combination	Classifying subadditivity could be difficult

TABLE 1 (*Continued*)

Study	Context	Technique	Limitations
Two-stage model-free tests of synergy in drug combinations [120]	A technique to detect synergy in two-drug combination	No underlying assumption about the models for dose–response curves are made	Requires an estimate of the potency ratio. Low performance for poor estimates
Biogeographical analysis of chemical co-occurrence data to identify priorities for mixtures research [121]	Chemical risk assessment must consider joint behaviour of compounds, but usually only binary combinations are considered	The paper showed that pesticides usually have strong co-occurrence geographically, which should guide risk assessment	The approach might not be appropriate for low-order combinations
Characterisation of mixtures part 1: prediction of infinite activity coefficients using neural network-based QSPR models [122]	Prediction of infinite dilution activity coefficients using DECHEMA Chemistry Data Series	Usage of neural network models to overcome limitations of linear models	Physical properties modelled, unclear how well the method would extrapolate to biological systems
Existing and developing approaches for QSAR analysis of mixtures [99]	Review of mixture descriptors and their usage in various QSAR tasks	Review of multiple QSAR modelling techniques and descriptors	No novel techniques suggested
QSPR approach to predict non-additive properties of mixtures [123]	Prediction of vapour–liquid equilibrium data using a dataset of 167 mixtures of combinations of 67 liquid compounds	Usage of a consensus of nonlinear predictors (Support Vector Machine, Associative Neural Network, Random Forest)	Physical properties modelled, unclear how well the method would extrapolate to biological systems

TABLE 2

A list of combination data sources available in the public domain

Data source	Combination data	Publication
Drug Combination Database	1363 drug combinations	[124]
NCATS DLBCL Dataset	459 compounds in combination with ibrutinib in DLBCL	[20]
Antifungal Synergistic Drug Combination Database	210 combinations, 1225 drug–target interactions	[125]
Therapeutic Targets Database	97 drug combinations	[126]
TWOSIDES	59,220 combinations, 1301 adverse events	[127]
NCATS Malaria Dataset	206 compounds, 14,810 combinations in *Plasmodium falciparum*	[50]
Antifungal drug combinations	34 drugs, 200 combinations in *Saccharomyces cerevisiae*	[44]
Antibiotic combinations	21 drugs, 210 combinations in *Escherichia coli*	[128]
DREAM Drug Sensitivity Challenge	14 drugs, 91 combinations on the DLBCL cell line LY-3	[68]
European Chemicals Agency	Repository of chemicals manufactured and imported in Europe. Consists of a significant number of mixtures	http://echa.europa.eu/ http://www.echemportal.org/

TABLE 3

Large-scale chemical and biological data resources that could be utilised for compound combination modelling

Bioactivity resources	
ChEMBL: a large-scale bioactivity database for drug discovery	[102]
DrugBank: a knowledgebase for drugs, drug action and drug targets	[129]
WOMBAT: World of Molecular Bioactivity	[130]
Network and pathway resources	
SignaLink: a signalling pathway resource with multilayered regulatory networks	[80]
STRING: a database of known and predicted protein–protein interactions	[103]
STITCH: known and predicted protein–chemical interactions resource	[131]
Gene expression resources	
Gene Expression Omnibus: archive for functional genomics datasets	[132]
Connectivity Map: a collection of genome-wide transcriptional expression data	[73]
LINCS: perturbational profiles across multiple cell and perturbation types	http://www.lincsproject.org/ http://www.lincscloud.org/
Toxicity/off-target effects resources	
SIDER2: side-effect resource	[133]
OFFSIDES: resource of data-driven predicted drug effects and interactions	[127]
ToxCast™: a toxicity forecaster resource	[134]

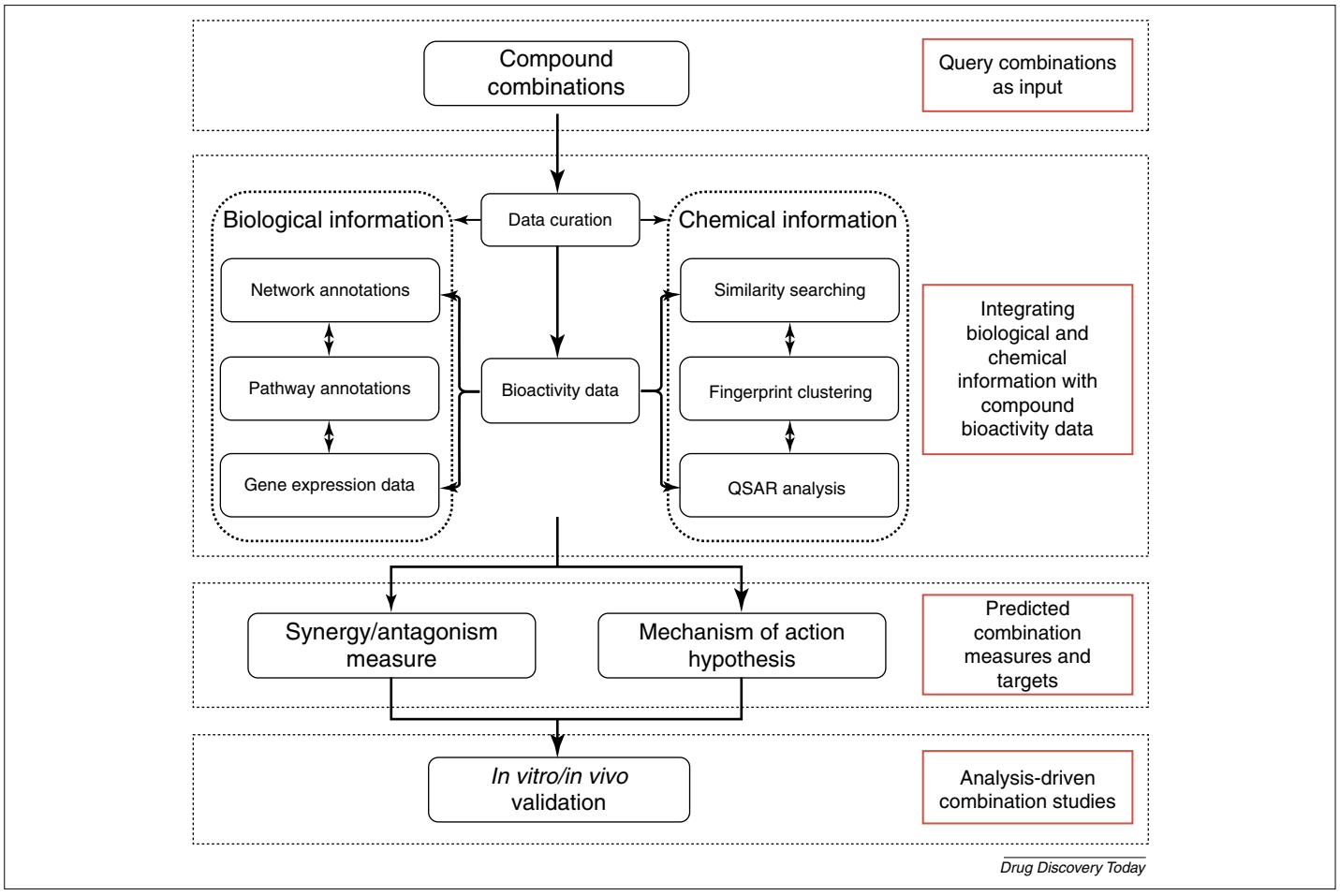

FIGURE 1

An integrated approach for analysing compound combinations using chemical and biological fingerprints. For compound combinations, the model will be able to search the bioactivity space along with integrating network and pathway annotations, gene expression profiles and chemical fingerprint similarities. This will lead to synergy predictions and developing a mechanism of action hypothesis for the combination that could be further validated by *in vitro* and/or *in vivo* experiments.

combining drugs that act on pathways essential for the survival of the cancer cell but not the normal cell [32–34]. Recent publications on tumour heterogeneity and clonal evolution in cancer further support such practice [35,36]. Genetic diversification as the tumour evolves pointed to distinct and multiple mutations across the same tumour specimen. Identifying and administering drugs that could bypass the resistance mechanism of all such identified mutations could not be realistic, because patient tolerability to the large number of drug combinations would have to be very high. However, such studies provide an opportunity to identify oncogenic pathways that contribute most towards the tumour progression. This could help prescribe a carefully selected drug cocktail at tolerable doses, which would be a significant step towards tackling tumour heterogeneity.

Compound combinations are also a popular approach for antibacterial and antifungal therapy [4,37,38]. Hill *et al.* evolved experimental populations of *Candida albicans* and *Saccharomyces cerevisiae* with a combination of heat shock protein Hsp90 inhibitors and widely used antifungals: the azoles [38]. This helped the authors identify mutations that evolved as a consequence of the combinations, understand resistance mechanisms as well as suggest strategies that could bypass the resistance. Apart from bypassing drug resistance, the aim is also to achieve greater efficacy utilising lower-dose combinations compared with higher-dose monotherapies. Numerous studies that show the greater efficacy displayed by compound combinations compared with monotherapy have been published [3,6,39]. This could potentially result in lower risk of side-effects and, hence, better quality of life [40–42].

Tolerability is of prime concern to the medical and scientific communities. A recent review discusses the advantages of fixed-dose combination therapies relative to monotherapy in the context of type-2 diabetes treatment [43]. These include greater efficacy, reduced risk of adverse reactions and lower overall costs. There are several contributors to determine whether drug combinations do indeed reduce the risk of adverse reactions; one of which is if a pathway targeted by a drug also contains off-targets that would cause an adverse reaction.

The rational discovery of novel drug combinations can be expedited by predictions of combination effects based on data generated from these experimental studies. Such predictive models could utilise combination data across disease areas, which could be another key step towards efficacious multitargeted therapies. This will not only provide an opportunity to exploit the unexplored and available bioactivity space but could also help identify novel and unexpected synergistic drug combinations [19,39].

Identifying synergistic compound combinations

Recent developments in high-throughput approaches to combination screening have enabled scientists to explore large collections of compound combinations experimentally. However, even with sophisticated automation systems, the number of combinations screened is a small fraction of the possible number of combinations. Strategies for experimentally determining the possible effect of drug combinations can range from screening several compounds at a time from a large database to exhaustive pairwise screening [44–46] such that an effective combination can be found. There have been several reports of large-scale combination screening campaigns. Borisy *et al.* [47] provided the earliest report of a systematic, large-scale screen of ~120,000 compound combinations using a proprietary platform. More recently, Mathews Griner *et al.* [20] have described the National Centre for Advancing Translational Sciences (NCATS) high-throughput platform for combination screening which screened thousands to tens of thousands of combinations in a checkerboard format (i.e. all combinations of two drugs at *n* doses each). Subsets of the data generated by this platform are publicly available (https://tripod.nih.gov/matrix-client/). Although such high-throughput platforms are crucial for running large numbers of combination experiments in a reasonable timeframe, they are expensive, especially if one considers replicate data and high-resolution checkerboard patterns (i.e. ten or more doses per drug resulting in 100 or more individual dose combinations). As a result, there are two roles for computational approaches in this area. Firstly, when faced with a large collection of combination responses, we wish to have computational methods that can characterise the combinations quantitatively, thus enabling ranking and filtering. Secondly, we desire to identify useful drug combinations prospectively, initially focusing on pairs of drugs but also higher-order combinations.

Approaches to the characterisation of combination responses

When characterising combination responses we propose and employ a two-step process. First, given the large number of combinations tested in a high-throughput setting, we must employ quality control (QC) metrics to ensure that only robust combination responses are considered for downstream analysis. Dispense errors, batch effects and edge effects can introduce noise and artefacts on screening plates, which result in noisy or incomplete responses [48]. In a high-throughput setting each plate includes positive and negative controls, and these can be used to derive plate-level QC metrics. Examples of such metrics include the Z', signal:background ratio and coefficient of variation [49]. Such metrics can indicate that all combinations on a plate are to be rejected. However, there are cases where the plate controls perform well but a few combinations on the plate are affected by a screening artefact (such as a localised well dispense error or problems at high concentrations). As a result, it is useful to employ combination-level QC metrics. Currently, there are no published guidelines for such metrics. The NCATS platform has implemented a heuristic QC score [50] that is useful for identifying poor-quality combination responses. This score takes into account the quality of the single agent dose responses, the absence of randomness in the dose combination sub matrix (as measured by spatial autocorrelation) and thresholds for no activity and variance of combination response (Fig. 2). Whereas the method employs several empirically selected thresholds, its use allows one to filter out severely compromised combination responses rapidly.

Having performed screening QC on the set of combination responses, the next step is to characterise the combination effect as potentially synergistic or antagonistic. There is rich literature on the topic of such quantification starting with Loewe, Bliss and Berenbaum [51–53]. Underlying all these models is the assumption

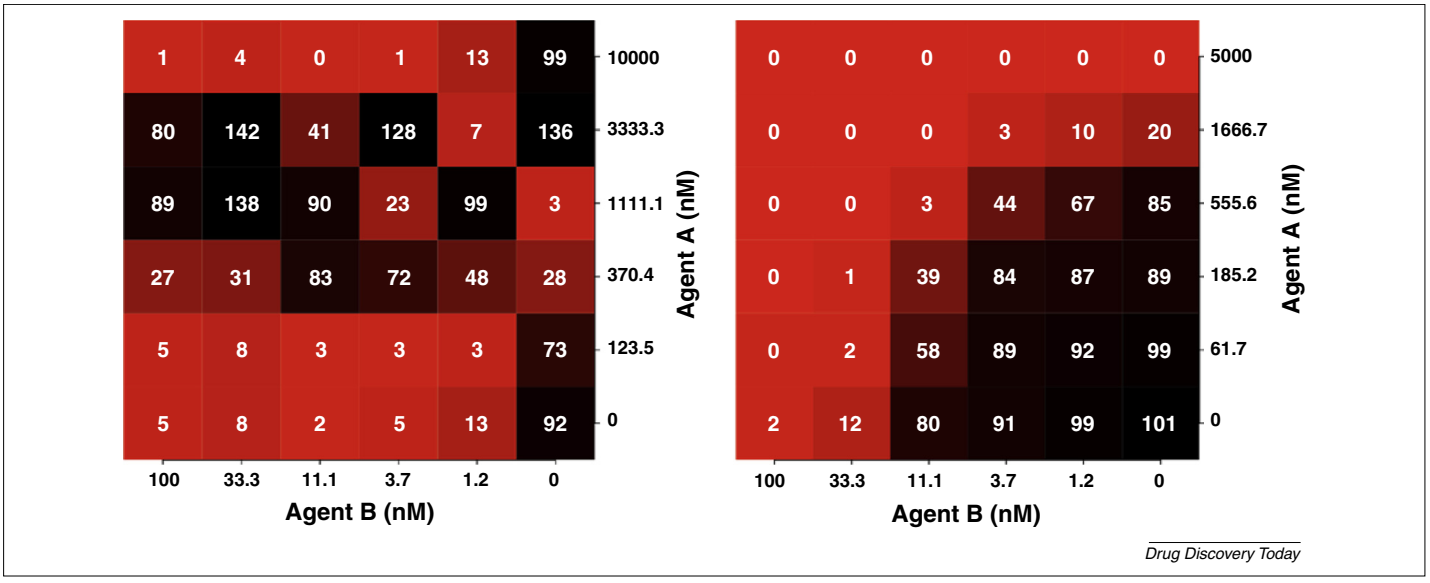

FIGURE 2

Examples of noisy (left) and well-defined (right) response surfaces that are characterised by the QCScore heuristic [50]. This score is useful for identifying and filtering poor-quality combination responses. The colour of each cell represents the assay response at that dose combination where black is no cell death through to red being full cell death.

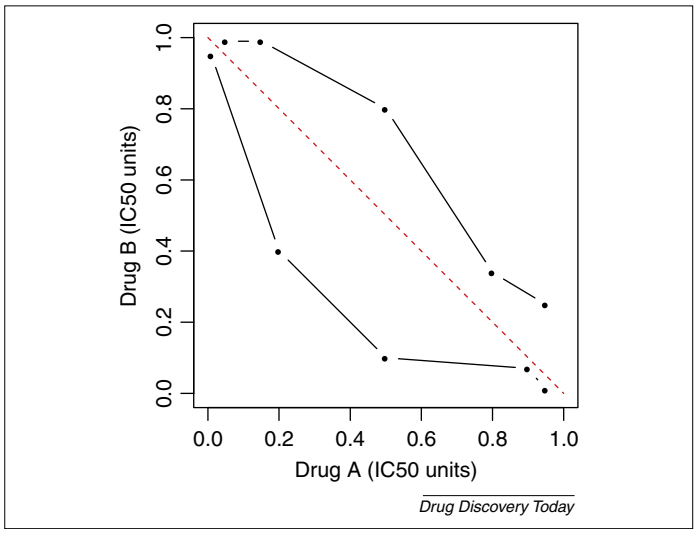

FIGURE 3

Synergy can be visualised using an isobologram, which displays the dose (in IC_{50} units) of drugs A and B required to achieve a specified effect level. The red dashed line indicates additivity, the line below the diagonal is indicative of a synergistic combination and that above the diagonal is an antagonistic combination.

that the combination effect of two drugs is purely additive – with the actual definition of additivity being model-dependent. Synergy (or antagonism) is then characterised as a deviation from additivity.

The Loewe additivity model is mathematically characterised by the isobole equation. It can be interpreted as modelling synergy as a departure from dilution effects [53]. Assume for instance that specific doses of drugs A and B produce a given effect (e.g. 50% inhibition). According to the isobole equation, if drugs A and B are additive then there exist several combinations of drugs A and B at reduced doses that should produce the same effect. For instance, half the dose of A combined with half the dose of B or one-third of the dose of A combined with two-thirds of the dose of B should produce the same effect as A or B alone. Synergy with respect to this model can be visualised using an isobologram (Fig. 3), which displays the dose (in IC_{50} units) of drug A and B required to achieve a specified effect level. The red dashed line indicates additivity, the line below the diagonal is indicative of a synergistic combination and the line above the diagonal is that of an antagonistic combination. Berenbaum [51] and Chou [54] have defined the combination index (CI) as a way to quantify synergy based on the Loewe model. The CI corresponds to the sum of the ratio of the amount of drug A used in combination to the amount of drug A required as a single agent for a specific effect (usually 50%) plus the corresponding ratio for drug B for the same effect. A CI <1 indicates synergy and CI >1 indicates antagonism. This model has been used by Chou (the Chou-Talalay method) [54] to define several metrics and graphical representations such as the median effect curves. It is important to note that the Loewe model is not really applicable when the two compounds do not produce a dose–response with the same maximum effect or even when the shapes of the dose–response curves differ [55].

The Bliss model defines synergy as a departure from independent compound effects [52]. Importantly, the model states that the drugs act in an independent fashion and that there are no effects because of drug–drug interactions (DDIs). Thus, according to Bliss independence the fractional response of the combination is the sum of the fractional responses of the individual drugs minus their product (Equation 1). Although simple in nature, the model is not considered to be robust in many situations, because it often characterises combinations of a compound with itself as synergistic.

$$PE_{A,B} = E_A + E_B - E_A E_B \quad (1)$$

where $PE_{A,B}$ is the predicted combined effect of compounds A and B, E_A is the known effect of compound A and E_B is the known effect of compound B.

Finally, the Gaddum non-interaction reference (or highest single agent) model simply defines additivity as the larger of the two single-agent responses. Although a very simplistic model it is applicable when the targets of the two compounds have no functional relation, so that compounds elicit their effects entirely independently of one another. See [56] for a detailed discussion of these and other models.

In general, given a combination screen, one selects a model and for each tested dose-combination the model is used to predict a combination response. If the observed response is less than or greater than the predicted response one makes a call of synergistic or antagonistic, respectively. For checkerboard style screens, the differences from the model can be visualised in a heatmap, or otherwise summarised by a scalar value [44]. However, with multiple models available, it is not always clear *a priori* which model should be used to quantify a particular experiment. In some cases, authors simply report the results from multiple models; whereas Lehár *et al.* [24] employ multiple models and use a χ^2 test to select the best-fitting model. The definition of a generalisable, robust model of additivity is therefore still an open problem; however the latter approach appears to tackle the problem in the most reasonable manner.

Evaluating and analysing combination data

Methods to predict combination effects can easily identify hundreds of drug combinations that are predicted to be efficacious or synergistic. Table 4 provides a list of well-known, publicly available software (free as well as commercial) for analysing combination data. After appropriate *in silico* validation techniques one would, ideally, perform experimental validation of the compound combination model that has been generated. There are a multitude of techniques to evaluate combination effects. Some of these approaches can be performed in high throughput, such as checkerboard approaches described by Mathews Griner *et al.* [20] and Borisy *et al.* [47], whereas other methods such as fixed-dose ratio [54] methods are generally more time consuming (but can be accelerated using robotic automation systems or miniaturisation technologies) [57,58].

Several *in silico* studies have performed validation of retrospective or prospective predictions. A matrix imputation approach was employed to predict synergistic target pairs that were then perturbed using small molecules [59]. A key feature of this approach is that it estimated the propensity of two targets to exhibit synergism and, in combination with the imputation framework, selected a subset of the entire collection of targets that, when screened, will identify a significant fraction of synergistic pairs. Importantly, the

TABLE 4

A list of commercial and freely available software to analyse combination datasets

Combination software	URL	Licensing
Chalice	http://cwr.horizondiscovery.com/	Commercial
Genedata Screener®	https://www.genedata.com/products/screener/combinations/	Commercial
SynergyFinder™	http://www.ntrc.nl/services/synergyfindertm/	Commercial
CalcuSyn	http://www.biosoft.com/w/calcusyn.htm	Commercial
CompuSyn	http://www.combosyn.com/	Free
Combenefit	http://www.cruk.cam.ac.uk/research-groups/jodrell-group/combenefit	Free

formulation of this approach is sufficiently general so that one can replace targets with compounds. The results of the algorithm were validated using published data as well as on a set of five *in vitro* glioblastoma cell lines.

By contrast to purely computational approaches, the combination of an evolutionary algorithm (EA) and iterative testing was used to identify combinations of anti-inflammatory molecules [60]. In this work, the top-ranked computational predictions (made by the EA) were experimentally tested and those results were then fed back into the EA. As a result, the authors were able to find combinations with significantly improved anti-inflammatory activities after testing just 550 combinations (out of a possible nine-billion combinations).

Metabolic network analysis of gene targets in *Leishmania major* has been employed to identify combinations of FDA-approved drugs [61]. This approach also identified synthetic lethal targets and validated four combinations against these target pairs in an *in vitro* model of *L. major*. Another network-based approach [34] used drug inhibition and target binding data to identify relevant targets for combination therapy. The computational results were validated using siRNA knockdown of single targets and target pairs. An approach that quantified crosstalk in target networks of drug pairs [62] was then used experimentally to verify the synergistic effects of curcumin when combined with multiple drugs, including capsaicin and celecoxib, in a rat model of myocardial ischaemia.

Miller *et al.* [63] employed a drug combination screen along with phosphoproteomics measurements to develop a computational model of the signalling network that explained the observed synergy (epistasis) between insulin-like growth factor 1 receptor (IGF1R) and cyclin-dependent kinase (CDK)4. The model was then used to predict that combined inhibition of these targets would reduce activity in the Akt pathway, which was subsequently experimentally confirmed. This is also an example where network models could help explain unexpected synergistic observations, as well as identify new perturbation points in a network that could be targeted by a combination of inhibitors.

Finally, high-content approaches have also been used to identify candidate drug combinations. An example is the construction of multivariate profiles of compound activity in triple-negative breast cancer (TNBC) cell lines using high-content imaging [64]. These profiles were then used to compute profile similarity scores between small molecules, which were subsequently used to predict five candidate combinations in multiple TNBC cell lines. Of these, vinblastine and ispinesib showed synergism in three TNBC cell lines but not in normal fibroblasts. A mouse model confirmed their antitumour activity, with the finding that the combination reduced tumour growth significantly when compared with monotherapy.

Utilising gene expression and pathway annotations in predictive models

Microarrays are an accessible, cost effective and fast way of measuring cellular responses and gene expression profiles [65], which facilitates research with the aim to understand regulatory networks on the basic level, as well as when applied in a drug discovery context [66]. In recent years, this approach has been utilised to predict synergistic effects of drug combinations on cancer cell lines, given gene expression profiles of individual drug treatments. In the following case studies we point out a functional enrichment analysis method and an enhanced Petri-net (EPN) model for assessing synergistic effects of compound combinations. The former study utilises cellular profiling of 14 individual drug responses for predicting synergy or antagonism of drug combinations [67]. In this study, differentially expressed genes after treatment of each of the 14 drugs on human β-cell lymphoma (DLBCL) cell line (LY3) from the NCI-DREAM Drug Sensitivity Prediction Challenge [68] represented the drug signature. It was hypothesised that correlated drug signatures should predict synergistic effects, whereas anticorrelation of those signatures indicates antagonism. The rank-ordered list of predicted synergistic drug combinations was compared to a gold standard experimental (*in vitro*) ranking of compound combinations provided in the challenge, and a measure of concordance (concordance index) was used to score the validity of prediction. In an effort to improve the method, functional enrichment analysis was applied on the overlapping gene signature of each paired drug mapped to protein interaction network to check interaction of the proteins produced by those genes. The network approach improved the performance of the model significantly. In the second case study, an EPN model was designed to explain mechanism of synergy of compound combinations from individual compound treatment transcriptional responses [69]. The EPN was applied on gefitinib and docetaxel and was capable of explaining mechanism of observed synergistic effects to a good extent. For this purpose, the effect of each drug on gene expression was depicted in the EPN with tokens and transitions in different colours, which illustrated the mechanism of synergy. Also, gene expression data of treatment of different doses of each of the two compounds (in a total of 16 different concentrations) was provided to the model. The model successfully predicted the effective dose, and predicted genes involved in the mechanism of synergy using each concentration. However, as a major limitation the model has been validated on only one particular drug combination studied here. Also, the authors acknowledge that, for the EPN model to work efficiently, knowledge of the targets and downstream signalling pathway annotations are necessary. Irrespective of the fact that this study utilises large-scale resources populated with network biology

annotations, these annotations are not always available in certain cases.

Gene expression data have also been beneficial in designing models for predicting chemosensitivity of three widely used drugs as a single agent or in combination [70]. Cisplatin, paclitaxel and gemcitabine were tested on 40 human urothelial cancer cell lines *in vitro* and cell lines were labelled resistant, sensitive or intermediate for each of the drugs. Differentially expressed genes were derived after application of each drug on each cell line. Among them, genes that were able to differentiate between resistant and sensitive cell lines were identified for each of the three drugs. The accuracy of sensitivity prediction was estimated between 93% and 96% for each of the drugs across cell lines. The probability of sensitivity of drug combination was estimated by multiplying probabilities calculated for each individual drug, and the accuracy of synergistic drug combination predictions was hence assumed to be around 80%. Even though the probability of sensitivity of drug combinations was predicted robustly, the model is less successful in predicting the synergy of cytotoxic effects of combinations. Moreover, extrapolation of the method to other cell lines or drug combinations requires generation of extensive *in vitro* drug response data for each combination. In a similar study, kinases that drive tyrosine kinase inhibitor (TKI) resistance in non-small-cell lung cancer (NSCLC) cell lines were identified by integrating data from genetic screens and RNAseq analysis [71]. This analysis led to the identification of the chronic myelogenous leukaemia (CML) drug bosutinib as an efficient inhibitor that could induce apoptosis in TKI-resistant cell lines. Further analysis led to the identification and validation of the synergistic effects of the combination of bosutinib and gefitinib in gefitinib-resistant NSCLC cell lines and suggests a good opportunity for drug repurposing. These studies suggest that gene expression data of individual compound treatments have potential for predicting synergism and sensitivity of compound combinations. An algorithm called TIMMA provides an integrated workflow that employs chemical bioactivity and cell line sensitivity data, towards predicting synergy for drug combinations and the proteins that these drugs could target [34,72]. However, predicting synergism and sensitivity of drug combinations on a large scale still remains a challenge. Large-scale databases of gene expression data on individual compound treatments are publicly available such as the Connectivity Map [73] (1309 compounds) and LINCS (20,413 compounds) (http://www.lincscloud.org/). This could also be an interesting opportunity to benefit from individual compound treatment databases for predicting synergism and sensitivity of compound combinations on a much larger scale.

Genetic and RNA interactions to guide the design of compound combinations

The effect of a single compound on a biological system can be thought of as an amalgamation of its effects on different (protein) targets. The effect of a combination is therefore a complex function of the protein interactions of each individual compound. In a localised example, it was shown that the protein dynamics of a system can be described simply by the linear superposition of the perturbations of the individual compounds [74]; this result is intriguing yet the phenomenon of synergy and antagonism suggests that any combination effects are unlikely to be linear in general. However, chemical perturbation is by no means the sole process by which a biological system can be modulated. Genetic perturbations in the form of gene knockout often have similar effects to the application of targeted drugs (although the behaviour of drugs is much more complex owing to dose and binding-site elements) and have been used as a useful biological probe in classical genetics. Yet, because gene interaction networks can be used to predict the effect of a combination of knockouts on the resulting phenotype, for example epistasis, they could also provide insight into synergy, and have been used as a context for exploring drug combinations [75]. Chemical–gene interactions (from single compound screening against a library of mutants) have been carried out in *S. cerevisiae*, and lead to several MOA discoveries [76]. These could potentially be exploited through the replacement of the genetic deletion with a targeted compound [37].

The development of RNAi yields another biological perturbation technique, because knockdown of a gene also often has a similar effect to a drug targeting the product of the same gene (although variation in time and dosage elements can confound this picture). Double or even higher-order knockdown experiments, known as combinatorial RNAi (coRNAi) [77], are an RNAi equivalent to compound combinations. These have been investigated to the third order by Sahin *et al.* [78] with no unintended silencing or cytotoxicity supporting the viability of the technique. coRNAi treatments might be deployed directly (they are currently being investigated as a treatment for hepatitis C) [79], but can prove even more powerful by providing an added insight into the effect of compound combinations, by matching their underlying biological perturbations; it is possible a combination of the two approaches could prove even more potent [75].

To summarise, whereas there are important differences, mRNA knockdown and gene knockout are related biological perturbation methods to chemical–protein interaction. According to this notion of potential equivalence, it might be possible to consider a broader 'biological combination', allowing data from these different biological facets to be integrated into a single, more descriptive model. Data integration of this nature has proved fruitful in other data-driven fields such as network biology, for example the integration of gene regulatory and protein–protein interaction networks [80], and thus might also be conducive to progress in modelling the effect of compound combinations.

Combinations as an investigative tool

Although the main interest in compound combinations is as a prospective avenue for therapeutics, there is also strong potential in research as an investigative probe of biological network structure. The concept is similar to synthetic genetic array analysis [81], which employed combinations of double genetic knockouts to locate genes that interact nonadditively with regard to phenotype. This observed phenomenon, termed synthetic lethality [82], shares certain parallels with chemical synergy. Mapping these interactions led to the production of gene-interaction networks [83] as a useful representation of biological space [84]. Taking advantage of the relatedness of inhibition of a gene product, and the knockout of the gene itself, the methodology was adapted by replacing one knockout with a panel of drugs with known protein targets, known as combination chemical genetics [75], to test for 'synergy' in knockout–compound pairs. The techniques

have been used to derive MOA hypotheses in yeast: for example, the antianginal drug molsidomine was found to target lanosterol synthase [85]. The use of chemical perturbation is (idealistically) preferable to genetic perturbation; whereas library design is difficult because of ADMET, selectivity and many other problems, in that in many cases chemical perturbation studies are faster, simpler and easier, allow for the perturbation of essential genes and, finally, a dose–response curve can be obtained, which describes the response of the biological system during gradual modulation of a target – a crucial distinction to most genetic approaches. The natural progression was then the replacement of the second knockout with another drug panel, essentially now a pairwise screen of drug–drug combinations. A synergistic drug–drug surface not only encodes a genetic interaction but a study found the surface itself provided deeper insights into the relationship [86]. In fact, it was found that it was possible to classify the local network topology of the drug targets according to the relative shape of the response curve [24], paving the way for more-detailed interaction networks.

Modelling drug–drug interactions

Up to 30% of adverse drug reactions are caused by DDIs. The ability to model DDIs could play a significant part in understanding the behaviour of compound combinations, as well as contributing to the identification of novel synergistic chemical pairs. When administered together, DDIs can result in a reduction in efficacy (antagonism), an increase in toxicity of either or both drugs (synergistic toxicity) or a previously unobserved effect that is unrelated to either drug taken by itself (coalistic) [87]. Patients who take multiple drugs are often afflicted with multiple comorbidities, and it is difficult to determine whether adverse events are the result of side-effects from a single drug, interactions between two or more drugs or exacerbations of the patient's underlying disease(s) [88]. DDIs could have a direct impact on the bioavailability of drugs, as well as their ADMET and drug metabolism and pharmacokinetic (DMPK) properties. Hence, understanding the PK and pharmacodynamics (PD) of a drug is crucial to assessing its (potential) DDIs [88]. This is mechanistically often explained by the fact that drugs share metabolic pathways, as has recently been discussed for the case of co-administering antituberculosis and antimalarial drugs, as is frequently the case in the developing world [89].

The approaches taken to model DDIs have been presented in multiple recent studies. One such approach considers the structural similarity between query drugs and those involved in established DDIs to infer the possibility of adverse drug reactions (ADRs), and requires access to databases of known DDIs with similarity profiles that include 2D and/or 3D chemical structures, together with known interactions, targets and side-effects [87]. Similarly, text mining has also been utilised for prediction of DDIs [90,91], and a recent review summarises these approaches [92]. Network-based approaches for predicting DDIs analyse the common targets and pathways involved with drug action, and correlate these with ADRs [93,94]. Notable examples of this include the annotation and analysis of 45,180 DDIs of 1352 drugs [93], and networks of Protein–Protein Interactions (PPIs) constructed for 1249 FDA-approved drugs that included 4776 associations to 1289 targets [94]. The main drawback of network-based approaches is an excessive number of assumptions that are made, which can ultimately decrease predictive capabilities. A comparison of dynamic and static models for the prediction of DDIs via inhibition mechanisms has shown similar performance between both approaches [95]. The aforementioned studies provide a set of well-annotated datasets for predictive combination models to train on, alongside extensive pathway and ADR annotations.

Traditional QSAR approaches are based on two key assumptions: (i) activity (property) is a function of structure; and (ii) similar structures will have similar properties. These approaches are attractive because after building a model it could be used for prediction of results of DDIs for millions of new combinations created by almost any drugs. QSAR modelling of DDIs using SiRMS [96] and GUSAR [97,98] approaches was performed. To enable this approach, a combination of two drugs participating in DDIs was formally represented as a binary chemical mixture. Predictive models based upon ~25,000 DDIs caused by more than 200 drugs were developed for inhibition of 1A2, 2C9, 2D6 and 3A4 – four major cytochrome P450 (CYP) isoforms. Because the validation of QSAR models for DDIs is more complicated than in traditional QSAR analysis, developed models were validated using a 'compounds out' strategy specially developed for rigorous validation of QSAR models of mixtures [99]. Predictions for more than half of the one million new DDIs were made using developed models.

Despite the plethora of different existing approaches for modelling DDIs, the prediction of new drug interactions, especially for complex medication regimens, remains an extremely challenging task that would still benefit from the development of new modelling approaches. Such models would require high-quality data to be trained upon, which cover multiple aspects of the known interactions between drugs. These datasets would benefit from the inclusion of target proteins for each compound, pathways that these targets are involved in, known resulting side-effects, bioavailability of the compounds and dosage levels. Additional information, such as the health, lifestyle and medication status of the individual from which this information is derived could prove in the future as modelling approaches become more refined. Although many tasks remain to be solved, once and where functioning, DDI modelling will be invaluable in guiding drug choice for combination therapy and hopefully result in fewer adverse effects.

Towards an improved understanding of compound combination effects: data integration, modelling and biological interpretability

A key bottleneck in identifying and understanding the molecular effects of compound mixtures on a systems level is the integration of biological and chemical information. The strengths, weaknesses and the challenges of integrating data from disparate sources have been discussed [100]. Compound activity is detectable on multiple levels in the living system – phenotypically on the gene expression patterns and on the target interaction level as well [101]. Meaningfully combining diverse information requires an understanding of the specific context in which individual datasets have been devised. This diversity could also involve specific procedures followed, experimental platforms, disease area focus and to some extent the cultural differences at the source of this information as

well. This variation becomes even more crucial and challenging to tackle in the case of compound combinations.

In the context of this review, the data that need to be integrated for a comprehensive systems-level analysis to identify compound combinations efficiently include chemical bioactivity data [102], targets and pathway annotations [80], gene expression data [73] and protein interactions [103]. Along with these, annotations regarding negative and positive feedback loops in pathways would be significant in optimising the predictive model. Unfortunately, these annotations are not always available as a part of network biology resources and have to be extracted from publications. The challenge is not only to identify specific data sources that can be integrated but also to have a common identifier(s) that can connect these datasets. Although this can be reasonably achieved with a good level of accuracy and confidence for anecdotal cases, data integration on a global scale, towards obtaining a generalised systems level understanding, deals with a much higher level of complexity. For example, a limited number of select pathways involved in a certain disease type, along with chemical bioactivity data tested against protein targets specific to the involved pathways could be obtained [104], but very rarely is information available on a larger scale. Cross-referencing at this level can be tedious, and in some cases meaningless. To predict compound combinations efficiently, comprehensive and quantitative data are key but often unavailable. At the same time, integrated relational databases are valuable in efficiently making biological and chemical predictions towards answering specific questions, for example generating MOA hypothesis for an observed phenotypic effect as a result of compound activity or identifying perturbation points along a given network(s) for identifying efficient drug combinations [16] and subsequent experimental validation. For this purpose, robust algorithms that consider and address the limitations of the integrated dataset are needed. Hence, careful consideration of the datasets and sources to be integrated is required, working towards answering the scientific questions of interest while enabling hypotheses and knowledge-driven analyses.

Challenges in the identification of useful compound combinations

Drug combinations provide many advantages in the treatment of diseases with complex aetiology, usually involving multiple targets and pathways. Even though drug combinations in some cases do lead to increased specificity [29], concerns regarding the side-effects of drug combinations still remain. Although this concern has been reported [105], and observed in the case of teratogens [106], several studies have not found evidence of such synergistic toxicities and indicate that the benefits of combinations outweigh the potential side-effects [107–109]. A notable concern when considering combination therapies *in vivo* is the role of PD, PK and interactions thereof in leading to unintended side-effects and/or lack of efficacy [110].

Although the reduction of drug-level-related side-effects is a key driver in the move to combination therapies, another limitation, in some cases, is that combination effects are dependent on the biological environment. In particular, synergies observed *in vitro* might not lead to effective combinations *in vivo* [50]. As an illustration, Brandl *et al.* [64] reported that synergism between vinblastine and kinesin spindle protein KSP/Eg5 inhibitors is observed in TNBC cells but not in normal fibroblasts. Even in the same biological setting, different routes of administration might or might not lead to synergistic effects. For example, Kolesnikov *et al.* [111] report that effects of topical combinations of analgesics mimic the effects of same combinations when administered systemically. By contrast, Banner and Press [107] report that oral administration of phosphodiesterase (PDE)3 and PDE4 inhibitors does not lead to synergistic effects, whereas an inhaled combination does. Thus, combinations offer many advantages but at the same time care must be taken to understand the systemic effects of drug combinations, as characterised by the PD and PK of the individual drugs and their effects on each other.

Concluding remarks

Compound combination studies integrating biological and chemical knowledge provide an opportunity for tackling key therapeutic and toxicity issues. Most prominently, these issues involve efficacy, drug resistance, side-effects and dosage tolerability in patients. These are problems that have always dogged the field of drug discovery but for which an efficient solution is yet to be achieved. Combination analyses studying chemical properties and bioactivity data do support and enhance the discovery of drugs that display better selectivity and possibility of overcoming drug resistance. However, the more we understand about this field the more numerous are the potential issues that surface. These include the possibility of undesired drug interactions, side-effects, biological selectivity where the combination works only in specific microenvironment and the differences in efficacy as a consequence of drug administration routes.

In the area of combination therapies, a deeper understanding of the underlying biological impact is required, such as that on gene expression and regulatory pathways. This can be achieved by integrating chemical bioactivity data from different realms of the biological system, *viz.* gene expression, gene–protein interaction networks and pathway annotations, among others. This approach can also assist in generating biologically meaningful MOA hypotheses for combinations of chemicals, and will help provide a comprehensive and solid foundation on which future chemical combination analyses could be performed. Such studies could have a significant impact on our understanding and applications of compound combinations. These include not only combination therapeutics but also pesticides, household chemicals and cosmetics that could otherwise have a potential health and environmental impact.

Acknowledgements

K.B., Y.K.M. and A.B. thank the European Research Council (ERC Starting Grant 2013 to AB) for funding. D.J.M. thanks Unilever and R.L. thanks the EPSRC for funding. E.M. thanks the financial support from NIH (GM 096967), EPA (RD 83499901) and UNC (2014 Junior Faculty Development Award). M.C. was supported by the Turkish Academy of Sciences GEBIP Programme.

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