A Pragmatic Definition of Therapeutic Synergy Suitable for Clinically Relevant In Vitro Multicompound Analyses

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Abstract

For decades, the standard procedure when screening for candidate anticancer drug combinations has been to search for synergy, defined as any positive deviation from trivial cases like when the drugs are regarded as diluted versions of each other (Loewe additivity), independent actions (Bliss independence), or no interaction terms in a response surface model (no interaction). Here, we show that this kind of conventional synergy analysis may be completely misleading when the goal is to detect if there is a promising in vitro therapeutic window. Motivated by this result, and the fact that a drug combination offering a promising therapeutic window seldom is interesting if one of its constituent drugs can provide the same window alone, the largely overlooked concept of therapeutic synergy (TS) is reintroduced. In vitro TS is said to occur when the largest therapeutic window obtained by the best drug combination cannot be achieved by any single drug within the concentration range studied. Using this definition of TS, we introduce a procedure that enables its use in modern massively parallel experiments supported by a statistical omnibus test for TS designed to avoid the multiple testing problem. Finally, we suggest how one may perform TS analysis, via computational predictions of the reference cell responses, when only the target cell responses are available. In conclusion, the conventional error-prone search for promising drug combinations may be improved by replacing conventional (toxicology-rooted) synergy analysis with an analysis focused on (clinically motivated) TS. Mol Cancer Ther; 13(7); 1964–76. ©2014 AACR.

Introduction

Multicompound treatment of cancer as well as other complex diseases is already a well-established practice in clinical settings. This area has nonetheless attracted mounted interest in recent years due to vast unexplored prospects for future pharmacotherapeutic gains (1–6). This development is also partly driven by a systems biology perspective acknowledging that many cellular processes are difficult to control using a single-drug compound, and partly by modern high-throughput laboratory equipment that make multicompound screening fast and cost effective.

One of the bottlenecks in this development is the lack of powerful methods for detection and analysis of useful drug combinations already at the early stages of the drug development process. Conceptually, the current practice in drug synergy analysis may be divided into two different strategies (7). The traditional one is to detect deviations from additivity as suggested by Loewe and Muischnek (8, 9) or from independence as outlined by Bliss (10). The second dominating strategy evaluates synergy in terms of the estimate of at least one interaction parameter in a response surface model, essentially according to that introduced by Box (11), estimated directly from data. For reviews of the broad range of already reported approaches to synergy analysis, see the influential article by Berenbaum (12), the critical survey by Greco and colleagues (7), and the mechanistic perspectives provided by Chou (13).

It is worth noting that the current practice in synergy analysis mainly has its intellectual roots in toxicology in which the main aim is to detect synergistic harmful effects of a multicompound provocation. In that case, it is natural to search for strong deviations from models of trivial interactions, suggesting that something potentially hazardous and mechanistically interesting occurs. In contrast, in synergy analysis used in pharmacology for drug discovery and development, one is usually only interested in drug combinations with large therapeutic windows characterized by relatively strong desirable (therapeutic) effects compared with the associated adverse effects (AE). Thus, in this pharmacologic context the detections made...
in a classical synergy analysis are only interesting in the quite uncommon situation when the AEs are negligible and/or approximately equal for all combinations studied. It, thus, seems natural and promising to develop an alternative synergy analysis, clinically and pharmacologically motivated, based on a therapeutic index (TI) that reflects an in vitro therapeutic window.

Although therapeutic synergy (TS) based on a TI has been explicitly ignored, for example, in the already mentioned review by Greco and colleagues (7), its importance has been acknowledged many times in the past (2, 4, 14, 15). The concept therapeutic synergism was probably introduced for the first time in 1956 by Venditti and colleagues (16) and is simply defined to occur whenever a combination treatment provides improved therapy compared with the best-component single-drug therapy (17–19). The importance of TS was strongly emphasized in a review by Goldin and Mantel (20) the following year in which they wrote (p.635):

“The increasing use of drug combinations in non-therapeutic situations such as in mixtures of insecticides, fungicides, or other poisons has tended to lead the evaluation of effectiveness of drug combinations in a direction different from that applicable in the therapeutic case. In place of evaluations in terms of improvement of therapy, drug combinations were evaluated on the basis of the extent of biological effect produced.”

Seemingly, with few exceptions only, their concerns have had little impact on the subsequent development in pharmacologic synergy analysis. Considering that the importance of TS in drug development was stressed already in the 1950s and that Carter and colleagues developed powerful tools for its analysis and optimization over the subsequent decades (19, 21–26), we find it quite remarkable that current practices in pharmaceutical synergy analysis are still chained to synergy according to Loewe, Bliss, and different response surface models. Presently there are very few reports addressing analysis and optimization of TS. One such example is a general approach suggested by Carter and colleagues in which optimal concentration mixtures are found to control the physiologic state of individual patients by searching for the maximum of a desirability function in which therapeutic effects as well as AEs may be embedded (27). Another example is a concentration mixture optimization method proposed by Zanderigo and colleagues in which optimal concentration mixtures are obtained from Horizon Discovery Ltd., (details about the cell lines are available from the company website). The cell lines were authenticated by short tandem repeat profiling (LGC Standards) by Horizon Discovery Ltd. in 2012. No additional authentication was done by the authors. All cell lines were cultured in McCoy 5A medium supplemented...
with 10% heat-inactivated FBS, 2 mM/L penicillin. These reagents were purchased from Sigma-Aldrich. The cells were cultured at 5% CO2 and 37°C.

**Drugs used**

A total of six drugs used in the presented examples. Of note, 5-fluorouracil (5-FU) and oxaliplatin purchased from Sigma-Aldrich, whereas sorafenib, erlotinib, and sunitinib were obtained from LC Laboratories. An experimental drug, EIC600, was also used in experiments.

**FMCA-based measurement of cytotoxicity**

Drug effects on the cells were measured by means of fluorometric microculture cytotoxicity assay (FMCA; ref. 30). Cell survival is measured by measuring the fluorescence, generated from the hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells that have their plasma membranes intact after 72 hours incubation with the compound. This means that the assay (implicitly) detects cell death irrespective whether the loss of membrane integrity (cell death) is preceded, for example, by apoptosis or necrosis. Compounds are added to the plates in a 2-fold dilution series by using an Echo 550 acoustic liquid handler (Labcyte Inc.). Every plate has wells with medium only that serve as blank controls, as well as wells with medium and cells but without drugs that serve as growth control wells. After incubation, wells are washed with PBS and then FDA is added to every well. These plates are incubated for 40 minutes with FDA and the emitted fluorescence from the blank wells and the fluorescence signal from the growth control wells. The fluorescence signal from the wells is proportional to the number of living cells in that well. The measured intensity of the fluorescence is used to calculate the survival index as

\[
S = \frac{f_{\text{sample}} - f_{\text{blank}}}{f_{\text{control}} - f_{\text{blank}}}
\]

where \(f_{\text{sample}}\) denotes the fluorescence signal from the well of the sample, \(f_{\text{blank}}\) denotes the average fluorescence signal from the blank wells and \(f_{\text{control}}\) denotes the average fluorescence signal from the growth control wells.

For an acceptable experiment the ratio of fluorescence signal between growth control wells and empty control wells must be >5. Moreover, an assay is only approved when the coefficient of variation, based on the control wells only, is less than 50%.

**Univariate statistical analysis**

The two-sample Mann–Whitney–Wilcoxon test was used to find significant differences in survival between reference cells and target cells or between predicted reference cell survival and real target cell survival. The significance level was set to 5% and in the resulting "heatmaps" produced, the concentration combinations corresponding to nonsignificant differences were displayed in green by simply resetting the observed nonsignificant difference by the value zero. All calculations were performed in the statistical programming environment R (31) using the function wilcox.test available in the package stats.

Standard nonlinear least squares fitting was used to estimate the two unknown parameters IC50 and \(H\) in the survival model

\[
S(d) = \frac{1}{1 + \left(\frac{d}{IC50}\right)^{H}}
\]

where \(S(d)\) denotes the survival at concentration \(d\). IC50 is the IC50 value for the drug considered and \(H\) denotes the Hill coefficient that mainly determines the slope in the resulting graph. The fitting was performed in the statistical programming environment R (31) using the function nls, available in the package stats, using default settings.

**Test for TS**

Given our definition of TS (see below), for a single combination TS can be inferred by evaluating if the TI of that combination is larger than the TI of the best single compound. However, when multiple concentration pairs are considered in parallel, a large number of replicates are needed to provide enough statistical power for multiplicity correction. Therefore, we instead perform an omnibus (global) test for TS by considering the null hypothesis:

\[
H_0 : \max_{d_a, d_b} T(d_a, d_b) \leq \max_{d'_a, d'_b \in M_{\text{single}}} T(d'_a, d'_b).
\]

Here, \(T(d_a, d_b)\) denotes the TI (formally defined below) for a combination consisting of compound \(a\) at concentration \(d_a\) and of compound \(b\) at concentration \(d_b\). Moreover, \(M_{\text{single}}\) is the set of single concentrations tested, formally defined as the set \(M_{\text{single}} = \{(d_a, d_b) | d_a = 0 \lor d_b = 0\}\), where \(\lor\) denotes logical OR. Thus, rejecting \(H_0\) will suggest some TS between the compounds. The test statistic used is

\[
t = \max_{(d_a, d_b) \in M_{\text{single}}^\text{obs}} T(d_a, d_b) - \max_{(d'_a, d'_b) \in M_{\text{single}}} T(d'_a, d'_b),
\]

the observed TIs. The distribution of the test statistic under \(H_0\) is derived computationally using a bootstrap estimate of the replicate error distribution. For more details, see the Supplementary Material.

**Results**

As already mentioned, throughout this result section, either a KRAS or a BRAF knockout cell line is used as the reference to be left essentially unaffected, whereas the corresponding WT cell line should be eradicated. This is in analogy with a more conventional experiment in which a nonmalignant cell line would act as the reference cell and a malignant cell line, of the same or different tissue of origin, would be the target. The use of the isogenic pairs instead of a more conventional pair, consisting of a "normal" and a "cancer" cell line model, is intended to demonstrate the generality of the TS concept as well as to discover drug combinations being more active against the clinically challenging mutants than the corresponding WT cell
genotypes. All figures are labeled with concentrations of single drugs used in combination and the unit used is \( \mu \text{mol/L} \).

**A formal definition of a pragmatic TI**

A TI should, in the form of a single numerical value, quantify the balance between the desired effects in the target cell line and the costly AEs in the reference cell line.

Several formal definitions of the TI are conceivable; in this work the following one is used:

The TI is defined as, for a concentration combination \((d_a, d_b)\) used to treat reference and target cell models, respectively, let \(S_r(d_a, d_b)\) denote the survival of the reference cells and \(S_t(d_a, d_b)\) denote survival of the target cells. Then the resulting TI is defined as

\[
T(d_a, d_b) = \frac{S_r(d_a, d_b)^{\lambda} - S_t(d_a, d_b)}{\text{DB}},
\]

where \(\lambda\) is a user defined parameter for weighting the reference cell survival (AE) relative to the desired effect.

The TI, as defined here, has the following convenient properties:

- Regardless of the value of \(\lambda\), the TI is restricted to the interval \([-1, 1]\), where the left endpoint is reached for the worst case \((S_r = 0, S_t = 1)\) and the right endpoint is reached for the ideal case \((S_r = 1, S_t = 0)\).
- For \(\lambda = 1\), the "neutral" (middle) value \(T = 0\) is obtained whenever the concentration combination kills the same fraction of reference and target cells, that is, when \(S_r = S_t\). Thus, \(T = 0\) may correspond to different cases like \(S_r = S_t = 1\) as well as \(S_r = S_t = 0.01\).
- More generally, for any \(\lambda > 1\), the "neutral" value \(T = 0\) is achieved when the fraction of surviving reference cells is larger than the fraction of surviving target cells.

In all the results presented in this work, we used \(\lambda = 1\), which yields the simplified definition

\[
T(d_a, d_b) = S_r(d_a, d_b) - S_t(d_a, d_b).
\]

**Conventional synergy analysis may be completely misleading**

Figure 1 presents a first example of how the TI can be determined and visualized using data obtained directly from a wet laboratory experiment. Here, TIs are calculated for different concentration combinations of erlotinib and sorafenib. In Fig. 1A and B, survival surfaces (survival indices) are visualized as color coded "heatmaps" for the reference cells line (DLD-1KRAS\(^{wt}\)) and the target cell line (DLD-1), respectively, upon treatment with this combination. The Fig. 1C shows the difference between the "heatmaps" in Fig. 1A and B. The experiment was performed in triplicate and the nonparametric Mann–Whitney–Wilcoxon test was used for each combination. For all nonsignificant differences, at the 5% level, the value in the "heatmap" is set to zero yielding a green square. Significant positive TIs are colored gradually from yellow to red, according to the colorbar to the right. The most significant positive TIs are obtained around the concentrations 6.2 \(\mu \text{mol/L}\) of erlotinib and 0.8 \(\mu \text{mol/L}\) of sorafenib.

These results suggest that at each of the concentration combinations in which there is a significant difference, there is a positive TI (therapeutic window) of potential use. In Fig. 1D, the result of a Bliss synergy analysis is presented. For each concentration combination, a conventional Bliss analysis has been performed and using the four experimental values available, an associated P value has been calculated for the null hypothesis that there is no synergy. Then, in the same way as in panel Fig. 1C, the values for the combinations without significant Bliss synergy have been set to zero. By comparing Fig. 1C and D, it is possible to identify those combinations for which the conventional synergy analysis in Fig. 1D is completely misleading. In particular, it suggests no synergy around the most significant positive concentrations in Fig. 1C mentioned above. More generally, the Bliss analysis suggests very limited effects, whereas Fig. 1C suggests large therapeutic windows (indices) for combinations of low concentrations of sorafenib and intermediate concentrations of erlotinib. Figure 1E presents Loewe synergy results that also shows little synergy and, therefore, also fails to predict the promising TIs in Fig. 1C.

Note that, although we are presenting the result of 7 \(\times\) 9 different combinations in one image, each result and the associated Mann–Whitney–Wilcoxon test should be considered as an isolated independent experimental result. In other words, Fig. 1 displays in one single heatmap the results of a set of independent experiments in which a single-concentration combination has been tested in each of them. Many synergy analyses reported in the literature only test one or a few concentrations. Hence, the P values calculated and used here have not been adjusted for multiple testing. As discussed in the next section, when a massively parallel experiment is performed in which all concentration combinations are measured and evaluated simultaneously to detect if there is TS for at least one of the concentration combinations, one has do address the multiple testing problem.

Figure 2 presents a second example in which Bliss and Loewe synergy analyses are misleading. Here, the TIs in Fig. 2A are calculated on the basis of different concentration combinations of EIC600 and oxaliplatin exposed to the cell models DLD-1 and DLD-1KRAS\(^{wt}\). The results of a Bliss analysis in Fig. 2B show a strong isolated spot of synergy for intermediate concentrations (0.8\(\mu\text{mol/L}\)–6.2\(\mu\text{mol/L}\)) of EIC600 and high concentrations (32\(\mu\text{mol/L}\)–64\(\mu\text{mol/L}\)) of oxaliplatin. Similar results shown in Fig. 2C produced by a Loewe analysis suggest even stronger synergy, especially in the upper part (EIC600 0.8–3.1\(\mu\text{mol/L}\) and oxaliplatin 8\(\mu\text{mol/L}\)–64\(\mu\text{mol/L}\)). However, according to Fig. 2A there is actually no in vitro therapeutic window at all.

**Defining and using TS**

A promising large TI should be of clinical interest only if this large TI is not achievable by any of the individual
drugs alone. When this happens, the combination is said to provide a TS. In other words, TS is perceived to occur for a concentration combination when the desired drug effect relative to the AEs is stronger for that combination relative to any of the single-drug treatments alone within the concentration range studied. This is equivalent to the...
already discussed definition of TS, as proposed by Venditti and colleagues in 1956, considered by the Carter group, and more recently discussed by Laska and colleagues (32). This definition of TS may be expressed formally as follows (Note: In this definition the logical operator OR is denoted by $\lor$):

\[
\text{TS is defined as, let } T(d_a, d_b) \text{ denote the TI for concentration combination }(d_a, d_b). \text{ Also, let } M_{\text{single}} \text{ be the set of degenerated concentration combinations that contain only one of the drugs at a nonzero concentration: } M_{\text{single}} = \{(d_a, d_b) | d_a = 0 \lor d_b = 0\}. \text{ Then a concentration combination } (d_a, d_b) \text{ is said to cause TS if }
\]

\[
T(d_a, d_b) > \max_{(d'_a, d'_b) \in M_{\text{single}}} T(d'_a, d'_b),
\]

that is, when its TI has a greater value than the highest achievable TI using either component drug alone.

Using the definition of TS introduced above, one is able to go beyond the search for TIs illustrated in Figs. 1 and 2. One example of a resulting TS analysis is shown in Fig. 3. Notably, in contrast with Figs. 1 and 2, in Fig. 3 single-drug responses are also included. The spot of TIs at the upper left corner in Fig. 3E is clearly higher than the TI for any single-drug concentrations and the omnibus test used (see Materials and Methods) is indeed suggesting that a TS has been detected ($P = 0.0471$).

Also, in this figure the color coding is based on individual nonadjusted $P$ values determined independently for each concentration combination. However, in addition the omnibus test (see Materials and Methods) is also performed to test if there is at least one concentration combination that provides TS. The main advantage of this omnibus test is that one can avoid the classical approach of using adjusted $P$ values by the Bonferroni correction or similar, which often are too conservative to yield any statistical significance. Thus, in the particular example presented here the omnibus test rejects the null hypothesis ($P = 0.0471$), which suggests that there is synergy for at least one concentration combination.

Figure 2. A completely misleading synergy analysis of the combination EIC600 and oxaliplatin. All TI values for nonsignificant concentration combinations are set to zero (green) in all three panels. All concentrations are in \(\mu\)mol/L. A, therapeutic windows defined as the difference between the reference (DLD-1KRASwt) and the target (DLD-1). B, results of a classical Bliss synergy analysis showing a strong spot (yellow/orange) of synergy contradicting the results in A. C, results of a classical Loewe synergy analysis showing a strong and quite extended area of synergy contradicting the results in A.
Figure 4 shows a set of additional examples of selected TSs. In Fig. 4A, the combination of sorafenib and 5-FU is detected \((P = 0.0086)\) to yield TS with DLD-1 and DLD-1KRASwt/C0. The most significant individual TIs are obtained at around 0.08 \(\mu\)mol/L of sorafenib and 2 \(\mu\)mol/L of 5-FU. Figure 4B also shows a detected TS \((P = 0.02)\) by the same combination with RKO as target cells and RKOBRAFwt/C0 as reference cells. Here, the two most significant individual TIs are obtained for 0.02 \(\mu\)mol/L of sorafenib and 32 \(\mu\)mol/L of 5-FU as well as for 1.6 \(\mu\)mol/L of sorafenib and 16 \(\mu\)mol/L of 5-FU. In Fig. 4C, the cell line models RKO and RKOBRAFwt/C0 produce TS...
with the most significant individual TIs at 3.1 μmol/L of erlotinib and 12.5 μmol/L of sunitinib.

Modeling adverse drug effects using single-drug responses

As already shown above, the use of TI according to equation (E) used here requires access to the survival (effect) surface describing reference cell toxicities (AEs) for the drug concentration combinations studied. Because experimental modeling of reference tissue/cells in vitro may be expensive and time consuming, old studies often lack experimental information about AEs. Therefore, in this section, we investigate to what extent models for AEs derived directly from the single-drug concentration–response curves of the target cells studied are practically useful. To obtain such AE models, first we make a simplifying assumption on how AEs of reference cells are related to the target cell concentration–response curves. Because many malignant cells divide more rapidly than nonmalignant cells, they typically suffer a worse fare due to treatment. Thus, we propose a model for reference cell survival that can be derived from the single-drug survival function (the concentration–response curve) of target cells supposing the difference in effect is solely due to the drug being killing cells only while dividing, and that reference cells divide at a lower rate. This assumption can be expressed mathematically as

$$S_{r}(d) = [S_{t}(d)]^\alpha, \quad \alpha \in [0, 1],$$

where $S_{r}(d)$ denote survival of the reference and target cells, respectively, at concentration $d$. The parameter $\alpha$ is user defined and should not be confused with the other user defined parameter $\lambda$ introduced earlier. Thus, the generality of this model is restricted by the assumption that, for single-drug exposures, reference cells can be regarded as drug-resistant target cells with a constant degree of resistance across the entire concentration range considered, as specified by the parameter $\alpha \in [0, 1]$. At the two interval extremes, $\alpha = 0$ would correspond to total...
cytotoxic resistance in the reference cells, whereas \( \alpha = 1 \) would translate to reference cells and target cells being equally sensitive to cytotoxic action by the single-drug treatment. More details about the underlying probabilistic dynamical model of cytotoxic action in relation to cell growth that can motivate the above equation are provided in the Supplementary Material.

Below, we show how one can use the classic Bliss and Loewe models to predict the entire concentration–response surface. Doing so also add clarification to the relationship between classical synergy analysis and TS. In particular, classical synergy analysis using either Bliss or Loewe can be regarded as an analysis of TS in which one assumes that the AEs follow either the Bliss or Loewe predictions. Using \( \alpha = 1 \), the predicted TI of single drug is zero for all concentrations. Therefore, in this case the difference between the predicted combination effect and the observed combination effect will correspond to our difference between the predicted combination effect and predictions. Using this approach, we can now predict the entire concentration–response surface. Doing so also add clarification to the Loewe models to predict the entire concentration–response curve for the two drugs, respectively. Using the corresponding inverse functions \( S_a^{-1}(s) \) and \( S_b^{-1}(s) \), one may write

\[
D_a(s) = S_a^{-1}(s) \quad \text{and} \quad D_b(s) = S_b^{-1}(s).
\]

Thus, the response surface value \( S(d_a, d_b) \) may now be written formally as

\[
S(d_a, d_b) = \arg \min_s \left( \frac{d_a}{S_a^{-1}(s)} + \frac{d_b}{S_b^{-1}(s)} - 1 \right),
\]

where the expression \( \arg \min_s f(s) \), for any function \( f(s) \), denotes the value \( s^* \) that minimizes the function \( f(s) \). Here, the use of this model is explained with an example, see Fig. 6 for details.

In Fig. 6A and B, the single-concentration responses of sunitinib and 5-FU in the target cell line RKO are shown (solid) in both graphs. These single-concentration–response curves are used with \( \alpha = 0.75 \) to obtain predicted single-concentration responses of sunitinib and 5-FU (dashed curves). After applying standard nonlinear curve fitting (see Materials and Methods) to obtain conventional Hill models of the form \( S(d) = \frac{d}{d_{50}\text{exp}} \), the corresponding inverse functions \( S^{-1}(d) \) were used in equation (I) to calculate the corresponding Loewe AE surface shown in Fig. 6C. Figure 6D shows the survival surface produced as a result of treatment of all concentration combinations of sunitinib and 5-FU with the target cell line model RKO. The predicted TIs are shown in Fig. 6E as the difference between the “heatmaps” in Fig. 6C and D. The most prominent spot of TIs in Fig. 6E (mainly at sunitinib 6.2 \( \mu\text{mol/L} + 5\text{-FU 4} \mu\text{mol/L} \)) is similar to the actual spot of most significant TIs in Fig. 6F.

### A generally applicable additive AE model

In situations, in which the drugs to be analyzed have similar and, therefore, competitive AEs, it is natural to model the resulting AEs using a Loewe additivity model. Thus, the idea is to create a response surface for the AEs that correspond to Loewe additivity, for each level of survival \( s \in [0, 1] \). To achieve this, for each value of \( s \), we simply create a linear segment of the surface between the endpoints \( (D_a(s), 0) \) and \( (0, D_b(s)) \) of the standard linear isobole defined as

\[
\frac{d_a}{D_a(s)} + \frac{d_b}{D_b(s)} = 1.
\]

Here, \( D_a(s) \) denotes the concentration of drug \( a \) at which it yields the survival level \( s \). This equation may also be expressed more compactly as follows. Let \( S_a(d_a) \) and \( S_b(d_b) \) denote the individual concentration–response curves for the two drugs, respectively. Using the corresponding inverse functions \( S_a^{-1}(s) \) and \( S_b^{-1}(s) \), one may write \( D_a(s) = S_a^{-1}(s) \) and \( D_b(s) = S_b^{-1}(s) \). Thus, the response surface value \( S(d_a, d_b) \) may now be written formally as

\[
S(d_a, d_b) = \arg \min_s \left[ \frac{d_a}{S_a^{-1}(s)} + \frac{d_b}{S_b^{-1}(s)} - 1 \right],
\]

where the expression \( \arg \min_s f(s) \), for any function \( f(s) \), denotes the value \( s^* \) that minimizes the function \( f(s) \). Here, the use of this model is explained with an example, see Fig. 6 for details.

In Fig. 6A and B, the single-concentration responses of sunitinib and 5-FU in the target cell line RKO are shown (solid) in both graphs. These single-concentration–response curves are used with \( \alpha = 0.75 \) to obtain predicted single-concentration responses of sunitinib and 5-FU (dashed curves). After applying standard nonlinear curve fitting (see Materials and Methods) to obtain conventional Hill models of the form \( S(d) = \frac{d}{d_{50}\text{exp}} \), the corresponding inverse functions \( S^{-1}(d) \) were used in equation (I) to calculate the corresponding Loewe AE surface shown in Fig. 6C. Figure 6D shows the survival surface produced as a result of treatment of all concentration combinations of sunitinib and 5-FU with the target cell line model RKO. The predicted TIs are shown in Fig. 6E as the difference between the “heatmaps” in Fig. 6C and D. The most prominent spot of TIs in Fig. 6E (mainly at sunitinib 6.2 \( \mu\text{mol/L} + 5\text{-FU 4} \mu\text{mol/L} \)) is similar to the actual spot of most significant TIs in Fig. 6F.

### Some theoretical issues

#### Selection of the parameter \( \alpha \)

In the subsections above, we show that in the common situation when there are no results from a reference cell model available for the TS...
analysis, it can be performed using concentration-response data for the target cell combined with theoretical models of AE assuming either independence (Bliss) and competition (Loewe). This theoretical modeling requires the user to specify the value of the parameter $\alpha$ that transforms the two concentration–response curves available for the target cell line RKO.
cell model into two predicted concentration–response curves that should describe the corresponding responses in the reference cell model. For values of $\alpha$ on the interval $[0, 1]$, the resulting concentration–response curves are reflecting a cell line that is more drug resistant than the corresponding target cells. In this work, the parameter

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{An example of predicted TIs based on an additive AE model for the drug pair sunitinib + 5-FU. A, real single-compound concentration–response curve produced by the target cell line RKO (solid line) and predicted response curve to model the reference cell line RKOBRAFwt/C0/C0/C0 (dashed line) when treated with sunitinib. The responses of reference cells are modeled using RKO responses and the parameter value $\alpha = 0.75$. B, real concentration–response curves of 5-FU in the target cell model (solid) and predicted curve (dashed) modeling the response in the reference cells ($\alpha = 0.75$). C, predicted survival surface of reference cells obtained using the additive AE model for all concentration combinations of the drugs sunitinib and 5-FU. D, survival surface of the target cells RKO for all concentration combinations studied. E, surface generated by subtracting the surface of the real reference cell response (RKOBRAFwt/C0/C0/C0) from the response surface for the target cell line model (RKO). The most prominent spot of TIs in E (mainly at sunitinib $6.2 \mu\text{mol/L}$ + 5-FU at $4-32 \mu\text{mol/L}$) is similar to the actual spot of TIs in F.}
\end{figure}
values were selected manually to demonstrate that an appropriate selection may yield quite useful results. How to select an appropriate value for \( \alpha \) automatically based on the dataset available is a nontrivial problem beyond the scope of this article. One simple pragmatic procedure would be to build AE models using a set of different values of the parameter \( \alpha \) and then study how consistent the resulting TS analysis becomes across the different values tested. However, with access to modern laboratory instruments that enable massively parallel measurements, it is relatively fast and cheap to evaluate the combination responses to a reference cell model jointly together with the same analysis on the target cell model. Therefore, the need for the kind of predicted reference responses introduced here is expected to continue decreasing.

The parameter value \( \alpha = 1 \) yields Bliss and Loewe. As already mentioned, for \( \alpha = 1 \) the reference and target cell models have the same concentration–response curves and the resulting synergy analysis will in fact be equivalent to a conventional Bliss or Loewe analysis. Because in this special case the difference between the reference cell surface and the target cell surface is zero for single-compound treatment, synergy is identified for any concentration combination \((d_a, d_b)\) satisfying \( T(d_a, d_b) > 0 \). For example, Bliss synergy occurs whenever the effect exceeds that predicted from the Bliss model; \( T(d_a, d_b) = \text{S}_{\text{Bliss}}(d_a, d_b) - \text{S}(d_a, d_b) > 0 \). Consequently, conventional Bliss synergy is equivalent to TS assuming independent adverse side effects with \( \alpha = 1 \). Similarly, synergy analysis using the competitive AE model and \( \alpha = 1 \) identifies the same combinations as a classical Loewe isobologram analysis. Loewe synergy occurs when for a given combination of drugs \( a \) and \( b \) in concentrations \( d_a \) and \( d_b \) the survival (effect) measured is \( s \) and the following inequality is satisfied:

\[
\frac{d_a}{D_a(s)} + \frac{d_b}{D_b(s)} < 1. \tag{J}
\]

As above, \( D_a(s) \) is a function that returns the lowest concentration of drug \( a \) needed alone to attain the survival \( s \) (and similarly for \( D_b \)). Now, suppose a spot \((d_a, d_b)\) shows TS using the competitive AE model. This means that the observed survival \( s \) in the target cells is less than the predicted survival \( s' \) in the reference cells (as predicted by the model): \( s < s' \). By definition, the predicted survival \( s' \) for \((d_a, d_b)\) is such that the following equality holds:

\[
\frac{d_a}{D_a(s')} + \frac{d_b}{D_b(s')} = 1. \tag{K}
\]

As \( s < s' \) and \( D(s) \) is a decreasing (monotonic) function of \( s \), it follows that \( \frac{d_a}{D_a(s')} < \frac{d_a}{D_a(s)} \) (and similarly for drug \( b \)). Thus, \( \frac{d_a}{D_a(s)} + \frac{d_b}{D_b(s')} < \frac{d_a}{D_a(s)} + \frac{d_b}{D_b(s)} = 1 \) whenever the observed survival in the reference cells is less than the survival predicted for the reference cells. In other words, \( \frac{d_a}{D_a(s)} + \frac{d_b}{D_b(s)} < 1 \) whenever there is (predicted) TS. In conclusion, using \( \alpha = 1 \) and theoretical AE models will identify the same combinations as classical Loewe or Bliss analysis.

No omnibus test is performed when the AE are predicted. For the results presented in the two subsections above using a predicted reference model, no omnibus test is performed. Thus, only \( P \) values are calculated for each concentration combination to test for a positive therapeutic window. The omnibus test is not applied because the assumptions behind it are invalid in this case due to an inherent nontrivial statistical dependency between the values in the predicted reference values and the target values. The dependency is created simply because the predicted reference model is based on the single-drug responses for the target cell model.

Conclusion

In this work, we have introduced a pragmatic definition of TS that provides more informative analyses of candidate multicompound therapies than the current practice, which is focused on synergy in the target cells only. Because any chemotherapy that kills more target cells than reference cells per time unit has a promising clinical potential, we think one should search actively for multicompound therapies that achieve exactly this. More specifically, we think that main contributions of this work are:

- Demonstrating that conventional Bliss and Loewe synergy analysis can be completely misleading when searching for promising therapeutic windows (TIs).
- Reviewing and reintroducing the concept of TS for synergy analysis in drug development, including an omnibus test for TS, which is designed to circumvent the multiplicity problem when studying many combination concentrations simultaneously.
- Introducing and demonstrating the applicability of pragmatic definitions of TI and TS using real experimental data.
- Showing that conventional Bliss and Loewe synergy analysis may be interpreted as searching for TS under the often inappropriate assumption that the single drugs do not offer any TIs alone.
- Demonstrating the potential of replacing the true reference cell response surface by a prediction of according to an AE model.

These contributions make it possible for the first time to perform a proper search for TS in conventional in vitro drug combination screening projects. In addition, the following two important theoretical and historical perspectives about the problem of synergy analysis are provided in part C of the supplement: “Conventional synergy is simply a detected deviation from a noninteresting molecular interaction” (Supplementary Section C.1), and “Independent historical branches of non-TS analysis” (Supplementary Section C.2).
Disclosure of Potential Conflicts of Interest

T. Sjöblom has ownership interest (including patents) in ExScale Biospecimen Solutions AB. U. Hammerling has provided expert testimony for the National Food Administration, Uppsala, Sweden. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Kashif, C. Andersson, P. Nygren, R. Larsson, M.G. Gustafsson
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