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Evaluation of synergism in drug combinations and reference models for future orientations in oncology

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ABSTRACT

Current cancer therapy includes a variety of strategies that can comprise only one type of treatment or a combination of multiple treatments. Chemotherapy is still the gold standard for cancer therapy, though sometimes associated with undesired side effects and the development of drug resistance. For this reason, drug combination is an approach that has been proposed to overcome the problems related to monotherapy and several studies have already demonstrated the superiority of combined therapies compared to monotherapy. The main goal when designing and evaluating drug combinations is to achieve synergistic effects by demonstrating that the combined effects are greatly superior to the expected from the additive effects of the single drugs, allowing for dosage reduction and therefore decreasing toxicity. Nevertheless, synergism quantification is not a simple task due to the different definitions of additivity and over the years several reference models have been proposed based on different assumptions and with different mathematical frameworks. In this review, we begin to cover the available treatment options for cancer therapy, with emphasis on the importance of drug combinations in cancer therapy. We next describe the classical reference models that have been proposed for synergism evaluation, usually classified as effect-based and dose-effect based methods, with a brief analysis of the current limitations of these models. We also describe here the novel methods for the accurate quantification of drug interactions in combined treatments. At the end of this manuscript, we covered some of the most recent preclinical and clinical combination studies that reflect the importance of the appropriate, accurate and precise application of the concepts and methodologies here described for the evaluation of synergism.

1. Introduction

Biological organisms are composed of complex subsystems that interact dynamically at different levels, with functions that are complemented to avoid system malfunctions (Vakil and Trappe, 2019). When a disease develops, it means that these subsystems are not working in a proper way and rather than focusing on a single component of the condition, treatment should focus on the disease's multifaceted problems. (Vakil and Trappe, 2019). Current cancer therapy includes a variety of strategies that can comprise only one type of treatment or a combination of two or more types of treatment. The treatment plan is usually defined based on criteria related to the type of cancer, the stage of the tumor and patient characteristics (National Cancer Institute, 2015a). Next, according to the National Cancer Institute (NIH) guidelines, we will describe the currently available cancer treatment options (Fig. 1).

Chemotherapy includes all drugs used in cancer therapy and acts on the cell cycle by preventing or reducing the growth of tumoral cells, whose growth rate is higher than normal cells (National Cancer Institute, 2015b). This strategy can be used for the treatment of cancer or for ameliorating associated cancer symptoms, by shrinking tumors. This pharmacological approach is used in different cancers, alone or combined with other strategies, such as surgery, radiotherapy, etc. Chemotherapeutic drugs can also be administered before surgery or radiation therapy to decrease tumor size (neoadjuvant chemotherapy) or after surgery or radiotherapy to destroy the remaining cells (adjuvant chemotherapy) (National Cancer Institute, 2015b). Chemotherapy lacks

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Fig. 1. Available options for cancer therapy. The most common approaches are surgery (whenever possible), chemotherapy (mono or combination therapy) and radiotherapy or a combination of these approaches.

cell specificity affecting both tumoral and normal cells with elevated growth rates, such as mouth, hair, or intestine cells, resulting in several side effects such as mouth lesions, sickness, and hair loss. Chemotherapy can be administered orally, intravenously, by muscular injection, intrathecal, intraperitoneal, intraarterial or topical. Intravenous chemotherapy may also be administered using catheters or ports (National Cancer Institute, 2015b).

Hormone therapy is a strategy used to treat cancer by slowing or stopping cancer cell growth or to ease cancer symptoms. This strategy acts by decreasing the body's capacity to produce hormones that contribute to cancer development and growth or by changing hormones' normal behavior in the body (National Cancer Institute, 2015c). Hormone therapy is commonly used to treat hormone-dependent cancers, such as prostate and breast cancer, being co-administered with other cancer therapeutics. Hormone therapy can be administered as neoadjuvant or adjuvant therapy (National Cancer Institute, 2015c). This strategy can also cause several side effects by interfering with the hormonal balance and include hot flashes, weakened bones, diarrhea, fatigue, etc. (National Cancer Institute, 2015c). Hormone therapies can be given orally or by intramuscular injection. Ovaries or testicles' removal can also be done to stop hormone production (National Cancer Institute, 2015c).

Radiotherapy makes use of high doses of radiation to destroy or reduce cancer cell growth by damaging their DNA. Cancer cells with DNA damage that cannot be repaired cannot divide and trigger mechanisms of cell death (National Cancer Institute, 2010). This process is not immediate, and some time may be needed for the DNA to be damaged enough to kill cancer cells. This treatment can cause side effects as it can cause cell damage to healthy cells surrounding the tumor. Radiotherapy can be external or internal and the choice of administration depends on the type and size of the tumors, the intrinsic characteristics of the tumor cells as well as the characteristics of the patient (National Cancer Institute, 2010). Both external and internal radiation therapies are local treatments and aim at the specific location of the tumor. Internal radiation therapy can be solid (brachytherapy) or liquid (systemic therapy). Brachytherapy consists of the administration of seeds, ribbons, or capsules that contain a radiation source and is commonly applied for the treatment of head and neck, breast, cervix, and prostate cancers (National Cancer Institute, 2010). Systemic therapy usually refers to a treatment that extends all over the body, looking for and destroying tumoral cells. Radioactive iodine, for example, is commonly used for the treatment of thyroid cancer. Commonly, radiotherapy is combined with surgery or chemotherapy (National Cancer Institute, 2010).

The immune system is involved in the detection and destruction of abnormal cells. Several times, immune cells known as tumor-infiltrating lymphocytes (TILs) are found in and around tumors, suggesting that the immune system recognizes the tumor cells as strange bodies. In fact, patients with TILs-positive tumors have a better prognosis than patients whose tumors are absent of these cells (National Cancer Institute, 2019). In most cases, the cancer cells can avoid the destruction by the immune system and start growing and dividing. This response happens when cancer cells undergo genetic changes, leading to the production of surface proteins or changes in the tumor microenvironment, masking them to the immune system and interfering with the way the immune system responds to the cancer cells (National Cancer Institute, 2019). Immunotherapy modulates the immune system of the patient to destroy cancer cells and although it has been approved for many types of cancer, it is not as used as the previously mentioned types of treatment. It includes the use of inhibitors of immune checkpoints, T-cell transfer therapy, monoclonal antibodies, treatment vaccines or modulators of the immune system (National Cancer Institute, 2019). Immune checkpoint inhibitors are drugs that block immune checkpoints, decreasing the immunological responses (National Cancer Institute, 2019). T-cell transfer therapy involves the removal of immune cells from the tumor and their

modification to improve their activity against tumor cells. The most active T cells in the tumor are selected, modified in the lab, and put back into the patient's body (National Cancer Institute, 2019). Monoclonal antibodies are proteins that recognize and bind to specific targets on cancer cells, for better recognition by the immune system. Treatment vaccines act by enhancing the response of the patient immune system to cancer cells and immune system modulators modulate the patient's immune response against cancer cells. Immunotherapy can be given via oral, intravenous, topical or intravesical (National Cancer Institute, 2019).

Surgery is indicated for *in situ* solid tumors and is currently used in several types of cancer (National Cancer Institute, 2015d), except for leukemia or more advanced cancers. It consists of the surgical removal of the tumor from the body and usually involves cuts with scalpels, which results in side effects such as pain or elevated risk of infections. There are other alternatives such as cryosurgery, lasers, hyperthermia, and photodynamic therapy (National Cancer Institute, 2015d).

Laser treatment makes use of beams of light to cut through the tissue and can be applied for precise surgeries or to destroy tumor cells (National Cancer Institute, 2015e). Lasers are mostly used to treat basal cell carcinoma, cervical, vaginal, esophageal, and non-small cell lung cancer. Hyperthermia damages and destroys cancer cells by exposing them to high temperatures (National Cancer Institute, 2015e). This treatment can also increase the sensitivity of cancer cells to radiation or chemotherapy. Hyperthermia is not yet commonly used but is under investigation in clinical trials (National Cancer Institute, 2015e). Photodynamic therapy makes use of light-reacting drugs and when the tumor is irradiated, drugs are activating, destroying cancer cells (National Cancer Institute, 2015f). This type of treatment is used to treat or alleviate symptoms caused by skin cancer, mycosis fungoides, and non-small cell lung cancer (National Cancer Institute, 2015f).

Targeted therapy is an approach that involves the targeting of important proteins involved in carcinogenesis. Most targeted therapies rely on the use of small-molecule drugs and monoclonal antibodies (National Cancer Institute, 2014). Monoclonal antibodies, such as trastuzumab, pembrolizumab, and rituximab, are synthetic proteins that bind to specific sites in cancer cells, marking them for destruction by the immune system. They can also directly stop cancer cell growth and induce cell death (National Cancer Institute, 2014). This therapy only works for patients whose tumors express targets for these monoclonal antibodies, so biomarker testing (by biopsy) is usually performed prior to treatment choice. In general, targeted therapies can act in different ways: help the immune system to destroy tumor cells, act directly on cancer cell growth, inhibit angiogenesis, cause cancer cell death and can also affect hormone production (National Cancer Institute, 2014). They can also be combined with toxins, chemotherapeutic drugs or even radiation to perform selective delivery of cell-killing substances. Targeted therapy has some weaknesses such as the appearance of resistance and the difficulty of developing new drugs for some targets (National Cancer Institute, 2014).

Blood-forming stem cells are responsible for the formation and renewal of blood cells. A stem cell transplant originated from bone marrow, bloodstream, or umbilical cord allows restoring blood-forming stem cells in patients affected by chemotherapy or radiotherapy, helping them to a faster recovery after these treatments (National Cancer Institute, 2015g). In multiple myeloma and other types of leukemia, this therapy is the most frequent type of treatment and makes use of donor healthy leukocytes to defeat the cancer cells from the patient (National Cancer Institute, 2015g).

Despite the wide availability of treatments for cancer therapy, chemotherapy still plays a major key role in the treatment of this disease. Nevertheless, it is often accompanied by off-target effects that result in undesired effects and also by the development of drug resistance (Smalley et al., 2006). The discovery and approval of novel drugs for cancer therapy is a process that is time and cost-consuming, making the pharmaceutical industry reformulate existing drugs into combination products. Although treatment with single compounds may be beneficial, several recent studies have reported the improved results of combinations of two or more compounds compared to the use of single drugs (Dear et al., 2013; Kim et al., 2020; Klimaszewska-Wiśniewska et al., 2018; Lee et al., 2019; Sasaki et al., 2010; Vakil and Trappe, 2019). With the recent advances in Omics and Cell Biology, the understanding of cancer as a complex disease comprised of interconnected pathways has increased the interest in the use of drug combinations in oncotherapy (Keith et al., 2005; Podolsky and Greene, 2011; Zimmermann et al., 2007).

2. Classical reference models for the analysis of drug combinations

Drug combination has been used in several areas such as cancer (Webster, 2016), asthma (Saleh, 2008), AIDS (Moreno et al., 2019), etc. This strategy exploits the susceptibility of different molecular pathways involved in the genesis of a certain disease to the different mechanism of actions of each individual drug, aiming to improve the efficiency of the treatment, decrease cytotoxicity to normal cells and reduce the development of drug resistance (Foucquier and Guedj, 2015). When combining two or more drugs, the main objective is to achieve positive interaction effects, by demonstrating superior evidence on the beneficial combination of two or more drugs compared to each drug individually (Tang et al., 2015). Basically, achieve more with less. To make the best of the combination of drugs, it is important to efficiently find a way to prove that the drug combination has more benefits than both drugs alone. Over the years, research in this area resulted in several theoretical and experimental manuscripts (Chou, 2010; Lederer et al., 2019; Roell et al., 2017), that described those interaction effects mostly as synergistic or antagonistic, that represent, respectively, more or fewer effects than the expected additive effect from each drug individually (Foucquier and Guedj, 2015).

Defining additivity is not as simple as it may seem and throughout the years several authors have proposed different formal definitions and approaches to apply this concept in clinical practice (Chou, 2006; Geary, 2013). In this review, we provide the most commonly used reference models for the evaluation of synergism in drug combinations, with a mathematical framework to perform the accurate evaluation of drug interactions in combination models. Next, we will describe the most

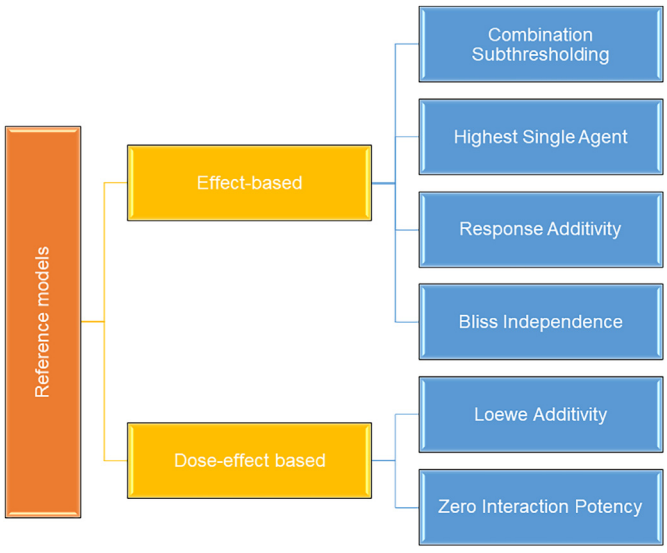

Fig. 2. Most well-known reference models for the analysis of drug combinations. Current approaches can be divided into effect-based or dose-effect based and are described by different mathematical frameworks, based on different definitions of additivity.

common reference models for drug combinations, which can be classified as effect-based and dose-effect based (Fig. 2).

2.1. Effect-based approaches

These methods are based on the effects of each individual drug within a combination to assess a positive interaction effect (Foucquier and Guedj, 2015). Effect-based strategies englobe four main strategies: Combination Subthresholding, Highest Single Agent, Response Additivity, and Bliss Independence model (Foucquier and Guedj, 2015). The term "effect", sometimes referred to as response, is usually evaluated by cell death, viability, growth rate, among others, being indicative of the measured or phenotype effect. Throughout the manuscript, the description of theorems assumes two drugs, A and B, given at doses a and b, with observed effects E_A and E_B. The effect of drug A combined with drug B is given by E_{AB} (Foucquier and Guedj, 2015).

2.1.1. Combination Subthresholding

The Combination Subthresholding is the most simple approach based on the concept that the combination of ineffective doses of drugs generates significant effects. Contrary to other reference models, this significant effect is defined based on P-values obtained from statistical tests by comparison with control (untreated groups) (Foucquier and Guedj, 2015). The observed effect is usually considered statistically significant when $p < 0.05$ (Fig. 3). Although this approach is sometimes still used, the observed effects may not be accurate and do not necessarily be representative of significant differences if the difference between what is significant or not, is not necessarily significant (Foucquier and Guedj, 2015; Nieuwenhuis et al., 2011).

2.1.2. Highest Single Agent (HSA)

This reference model is also known as the Gaddum's noninteraction or cooperative effect (Geary, 2013; Lehár et al., 2007) and assumes a positive combination interaction when the drug combination (E_{AB}) elicits a greater response than the highest single agent ($E_{AB} > \max(E_A, E_B)$) (Fig. 4). This represents an improvement of the Combination Subthresholding as it allows the calculation of a combination index (CI) by the following equation: $CI = \frac{\max(E_A, E_B)}{E_{AB}}$. If the value obtained for CI is positive, statistical significance is evaluated by comparing drug combination effects with the single drug more effective using the p value of the statistical test (Foucquier and Guedj, 2015). This reference model is more advantageous as it evaluates if differences are significant rather than the difference of significance. Nevertheless, this model only compares the drug combination effect to the most effective individual drug (highest single agent), not taking into account the expected additive effect of both drugs involved in the combination (Foucquier and Guedj, 2015). This makes this model suitable only for drug combinations where one of the drugs is inactive for all tested concentrations. Normally, this approach usually gives more optimistic results because it has the lowest threshold for considering synergism among all reference models (Vlot et al., 2019).

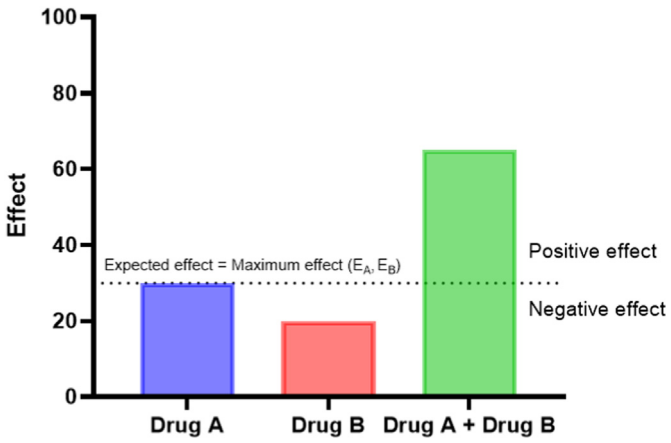

Fig. 4. Demonstration of the Highest Single Agent approach. This model assumes a positive interaction effect when the drug combination induces a greater response than the highest single agent. Based on EA=30, EB=20 and EAB=65. Adapted from (Foucquier and Guedj, 2015).

2.1.3. Response additivity

This reference model is also known as Linear Interaction Effect (Slinker, 1998) and assumes that a positive interaction occurs when the drug combination (E_{AB}) elicits a greater effect than the sum of the individual drugs' effects ($E_{AB} > E_A + E_B$) (Fig. 5). The CI can be calculated as: $CI = \frac{E_A + E_B}{E_{AB}}$, where the P-value means the significance of the interaction effect in factorial analysis of variance of the individual and combined effects (Slinker, 1998). Compared to the previously mentioned reference model, the Response additivity approach represents an improvement as it compares the effects from the combination with the expected effects from the single drugs, assuming their effect to be additive. Nevertheless, the determination of synergism using this model implies the drugs have linear dose-effect curves, which may not be true for many drugs used in pharmacological studies (Caudle and Williams, 1993).

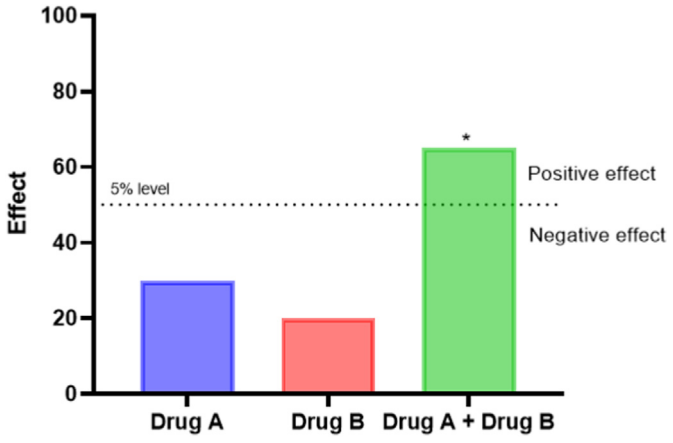

Fig. 3. Demonstration of the Response Additivity approach. This model assumes synergistic effects when the drug combination induces a greater response than the sum of the individual drugs' effects. Based on EA=30, EB=20 and EAB=65. Adapted from (Foucquier and Guedj, 2015).

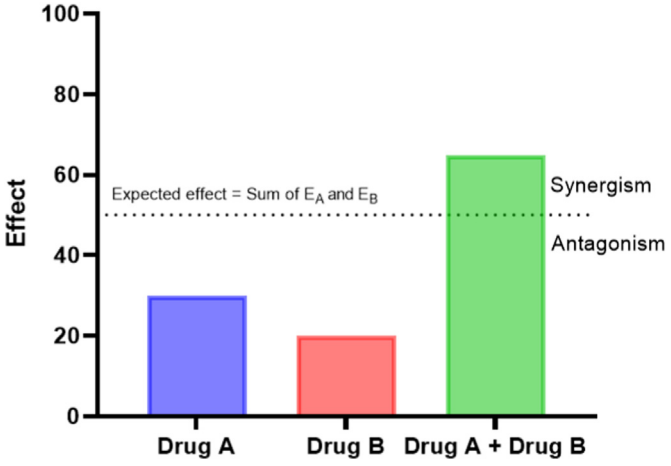

Fig. 5. Demonstration of the Response Additivity approach. This model assumes synergistic effects when the drug combination induces a greater response than the sum of the individual drugs' effects. Based on EA=30, EB=20 and EAB=65. Adapted from (Foucquier and Guedj, 2015).

2.1.4. Bliss independence

This approach is one of the most popular models and was first introduced in the 1930s (Berenbaum, 1989; BLISS, 1939; Geary, 2013; Greco et al., 1995). This model assumes that both drugs used in drug combinations act independently and do not interfere with the other, assuming that drugs act on different sites of action (Fig. 6) (Greco et al., 1995). Nevertheless, this model assumes both drugs contribute to the observed effect. In this approach, the drug effects from single and combination treatments are expressed in the form of a probability ($0 \leq E \leq 1$) and the expected combined effect can be defined as: $E_{AB} = E_A + E_B(1 - E_A)$, where E_A and E_B represent the observed effects of drug A and B, respectively, and E_{AB} the effect of drug A combined with drug B. The calculation index for following this approach can be calculated as $CI = \frac{E_A+E_B-E_AE_B}{E_{AB}}$, being indicative of synergy, antagonism or additivity when CI is under, above or equal to 1, respectively (Foucquier and Guedj, 2015). This model of non-interactivity is one of the effect-based approaches that has survived to the critics over time. Although this model is widely used in the pharmaceutical field and is more accurate than the previous ones, it still presents some limitations (Goldoni and Johansson, 2007): it depends on the knowledge of mechanisms of action and most of the times drugs have several, intricate and possibly undetermined mechanisms of action (Greco et al., 1995); then, this model presumes that drugs follow an exponential dose-effect response, which is not applicable for all drugs (Berenbaum, 1989); finally, this model only fits for effects that can be ranged in probabilities from 0 to 1 (Foucquier and Guedj, 2015).

2.2. Dose-effect-based approaches

More recently, novel approaches have been proposed to surpass the limitations of the previously mentioned reference models. Especially for drugs that have nonlinear dose-effect curves, dose–effect-based approaches represent an improvement as they take into account the dose of each drug that results in the same quantitative effect (Berenbaum, 1977). This reference model considers the dose-effect curves of each drug and provides significant and unequivocal definitions of synergy, additivity, and antagonism (Foucquier and Guedj, 2015). Importantly, to evaluate if there is an interaction effect in a drug combination, it is essential to first define a non-interactive effect, which can be achieved by null reference models (Lederer et al., 2019).

2.2.1. Loewe Additivity

The Loewe additivity model is a null reference model and the most well-known dose–effect-based approach. It was first mentioned by Frei in 1913 and further described using a mathematical framework by Loewe in 1926 (Berenbaum, 1977; Loewe, 1927; LOEWE, 1953; Loewe and Muischnek, 1926). This model is based on the isobole representation to define the additive effect. This reference model assumes the dose equivalence principle, meaning the dose a from Drug A is equivalent to the dose b_a of drug B and vice-versa, for any given effect. It also assumes the sham combination principle, meaning that dose b_a can be added to any dose b of drug B to achieve an additive effect, which is not a characteristic of other null reference models (Lederer et al., 2019). The additive effects depend on the dose-curves of each drug and can be defined as *Effect* $(a + b) = E_A(a + a_b) = E_B(b + b_a) = E_{AB}$.

This model assumes that individual drugs have a constant potency ratio $(R = \frac{A}{B})$, meaning their doses have the same ratio at each level of effect. Graphically, this means the drugs have parallel dose-response curves and achieve the same maximum effects (Tallarida, 2012). This interaction can be defined by the following mathematical equations: $a + a_b = A \leftrightarrow a + b \times R = A \leftrightarrow a + b \times \frac{A}{B} = A$, which is behind the Loewe Additivity equation and many other dose-based approaches that derived from this reference model: $\frac{a}{A} + \frac{b}{B} = 1$.

The CI derived from the Loewe Additivity is calculated by: $CI = \frac{a}{A} + \frac{b}{B}$ (Berenbaum, 1977; Chou and Talalay, 1983, 1984). When CI is under 1, it means that the doses a and b that are necessary to produce a combined effect are lower than the ones predicted from additivity, which is indicative of synergistic interactions between the two drugs. When the CI value is above 1, the doses from each individual drug that produces a combined effect are greater than the ones predicted from additivity, meaning that drug interaction is antagonistic (Berenbaum, 1977; Chou and Talalay, 1983, 1984).

Isobologram analysis was first introduced in 1995 by Greco et al. (1995) and is a graphical approach to the Loewe Additivity model, allowing an easy interpretation of the interaction of two drugs in a given combination (Tallarida, 2012). It is a graphical representation that has represented the doses of drug A and drug B in the x and y-axis and indicates the collection of all dose combinations of the drugs that achieve a desired percentage of effect.

In this graphical representation, there is a line with a negative slope that represents the additive effect (also known as additive isobole), where deviations from additivity indicate synergism or antagonism (Fig. 7) (Chou, 2006; Grabovsky and Tallarida, 2004). It represents the expected combined effect of a drug combination using doses a and b from

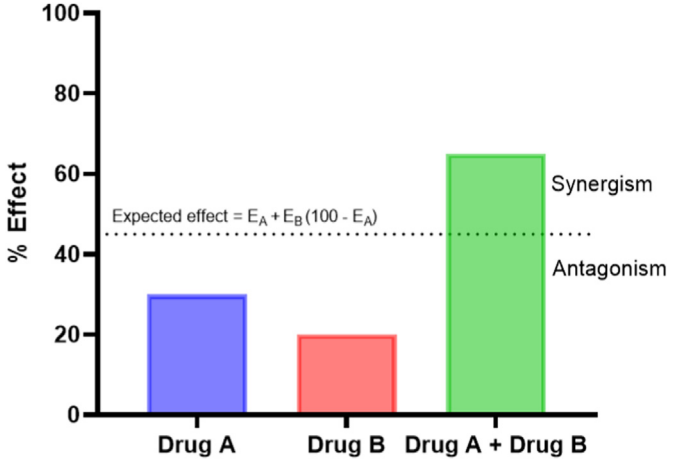

Fig. 6. Demonstration of the Bliss Independence approach. This model assumes that both drugs act independently and do not interfere with each other. Based on EA=30, EB=20 and EAB=65. Adapted from (Foucquier and Guedj, 2015).

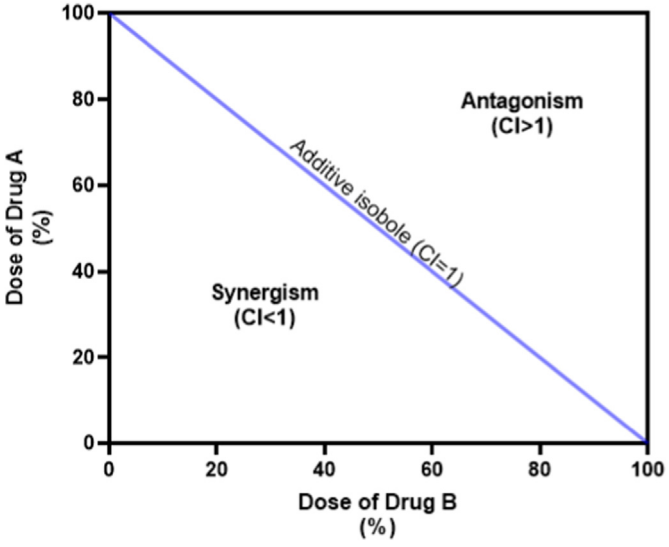

Fig. 7. Demonstration of the Loewe Additivity approach (isobologram). The diagonal line represents the additive effect (also known as additive isobole) and deviations from additivity are indicative of synergism or antagonism. Adapted from (Foucquier and Guedj, 2015).

two drugs A and B and can be calculated by the equation $b = B - \frac{B}{A} \times a$, where doses of A and B are represented on the x and y-axis. This means that when the doses from individual drugs necessary to achieve a combined effects are lower than the corresponding ones described from the additivity line (i.e., are located below the line), this can be translated as synergy (CI < 1). A drug pair located on the additive isobole means that CI = 1 and therefore indicates additivity, whereas a drug pair located above this isobole is indicative that higher doses of each individual drug are necessary to produce the expected combined effect and therefore are related with antagonism (CI > 1) (Foucquier and Guedj, 2015). This approach does not rely on the drugs' dose-response relationship neither their mechanism of interaction, being only affected by the concentration of the drugs used in the combination. This means that one drug pair can be synergic for one dosage and antagonist for another therapeutic regimen (Berenbaum, 1989).

Just like the previous reference models, the Loewe Additivity model also presents some limitations, mostly related to the dose-effect curves of each individual agent. It is only designed for drugs that have dose-response curves described by the Hill equation (also called sigmoid or logistic function) but sometimes the determination of these curves requires lots of data that can be time and money consuming (Sidorov et al., 2019). Also, some drugs have dose-response curves that are difficult to model, making the Loewe Additivity model useless (Zhao et al., 2014). In addition, many drugs do not fit in the isobole straight line: this can be a result of drugs that do not have a constant potent ratio, hence resulting in non-parallel dose-response curves and/or when the individual drugs do not achieve the same maximum effects (Grabovsky and Tallarida, 2004).

Many null reference models are based on the concept of isoboles. The Chou-Talalay approach, proposed by Chou and Talalay is still one of the most used in biological studies to quantify drug interactions, especially synergism. This reference model has been built based on the Loewe Additivity model (Chou and Talalay, 1983, 1984) and incorporates the median-effect equation, derived from the unified theory mass-action law principle, represented by the equation $\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^m$, where D represents the drug dosage or concentration, *fa* the inhibited fraction by the drug dose D, *fu* the unaffected fraction, Dm the dose that causes 50% inhibition and m the coefficient indicating the shape of the dose-effect curve. The model is also described by a (r) value, that indicates the fitting of the data to the mass-action law. The values of (m), (Dm), and (r) for each single drug are the dose-effect parameters required for the implementation of the Chou-Talalay theorem. In this way, the unified theory establishes the common link between single and multiple entities, and first order and higher order dynamics. The general equation that describes this model, previously mentioned, is derived from the Michaelis-Menten, Hill, Henderson-Hasselbalch, and Scatchard equations, the most important ones in biochemistry and biophysics, and also resulted in the development of the CI theorem, which offers a quantitative determination of synergism in drug combinations (Chou, 2010). It has been improved by including the principle of mass action to analyze the combined effects of a given combination and is now the subject of a huge number of publications. The combination index for a two-drug combination can be calculated as following: $CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}$, where $(Dx)_1$ represents the dose of the drug D_1 alone that inhibits the growth of cells by x% and $(Dx)_2$ is the dose of the drug D2 alone that inhibits the growth of cells by x%. It has its own graphical representation consisting of a plot of CI versus effect, where CI < 1, CI = 1 and CI > 1 indicate synergism, additivity and antagonism, respectively (Fig. 8).

2.2.2. Zero interaction potency (ZIP)

This is one of the most recent reference models proposed for the evaluation of expected responses in drug combinations, being a hybrid approach between the Bliss Independence and the Loewe Additivity models (Yadav et al., 2015). This approach evaluates the drugs' interactions by comparing changes in the potency of the dose-response curves between individual and combined drugs, not being affected by

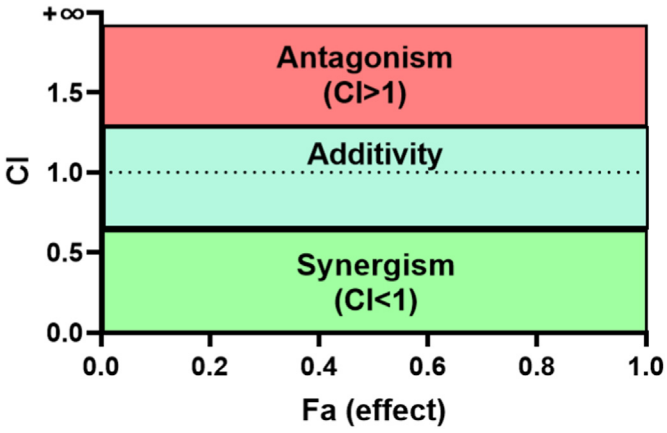

Fig. 8. Fa-CI plot proposed by Chou and Talalay, based on Loewe Additivity model. Fa indicates the observed effect and CI<1, CI=1 and CI > 1 indicate synergism, additivity and antagonism, respectively. Adapted from (Rodea-Palomares et al., 2015).

the pharmacodynamics of the compounds in combination. It assumes that drugs are independent and do not interact with each other when combined, resulting in minimal changes in their response curves when combined (Yadav et al., 2015). This means that the accurate fitting of the dose-response curves is crucial for the determination of parameters like the relative half-maximal effect concentration (EC_{50}) and the slope, which can be a difficult challenge if data is of poor quality, for example (Vlot et al., 2019).

Table 1 summarizes the advantages and disadvantages of the previously described references models used to assess the pharmacological interaction in drug combinations, together with the mathematical framework behind the calculation the expected additive effect and CI values for each model.

3. Current limitations of the classical combination models

As demonstrated in the previous sections, current reference models for the evaluation of synergism have been improved over the years but there are still some general limitations to the classical combination models (Chou, 2010; Foucquier and Guedj, 2015; Ma and Motsinger-Reif, 2019).

The first limitation in the analysis of drug combinations is the misleading of the term "synergy" (Roell et al., 2017). In most clinical studies and throughout the literature, this term is often used to justify the combination of drugs in therapy, but many times this term is not clearly defined and commonly used without the proper knowledge underlying the concept and the methods necessary to evaluate it (Lederer et al., 2019; Ocana et al., 2012). Most of the time, synergy is not evaluated nor calculated using the appropriate reference models and results are interpreted as synergism only by comparison of results obtained from simple experimental assays, rather than included in a mathematical approach and without information about the dose-effect curves for single drugs. For example, sometimes, better-observed effects in drug combinations are often described as synergy when indeed it is only a potentiation of the effects of the two drugs combined if only one drug is active alone (Foucquier and Guedj, 2015).

Another limitation is that, to date, there is not a standard reference model to evaluate synergism. All available reference models have limitations and while some fail to provide clear definitions of additivity, others rely on data about the drug's mechanism of action, do not cover rare and specific cases like drugs that do not follow the ideal dose-response curves, or are not intuitive and user friendly (Shafer, 2012). The Loewe Additivity model is the most improved reference model, but it is also limited by the large amount of data required to make precise synergy analysis, which is particularly important when the data is

Table 1
Advantages, disadvantages and mathematical framework of the reference models described in this manuscript used to assess the pharmacological interaction in drug combinations.

Reference model	Pros	Cons	Mathematical Framework
Combination Subthresholding	• Most simple approach	• Effect-based approach • Significant effects are defined based on P-values • The observed effects may not be accurate and do not necessarily be representative of significant differences • Does not allow the calculation of a combination index (CI) • Least accurate reference model	The observed effect is considered statistically significant when $p < 0.05$
Highest Single Agent	• Allows the calculation of a combination index (CI) • Gives more optimistic results among all reference models	• Effect-based approach • Does not take into account the expected additive effect of both drugs involved in the combination • Suitable only for drug combinations where one of the drugs is inactive for all tested concentrations	• $E_{AB} = \max(E_A, E_B)$ • $CI = \frac{\max(E_A, E_B)}{E_{AB}}$
Response additivity	• Allows the calculation of a combination index (CI) • Takes into account the effects of both drugs in the combination, assuming their effect to be additive	• Effect-based approach • Implies the drugs to have linear dose-effect curves	• $E_{AB} = E_A + E_B$ • $CI = \frac{E_A + E_B}{E_{AB}}$
Bliss Independence	• One of the most popular • Allows the calculation of a combination index (CI) • Takes into account the effects of both drugs in the combination • Assumes that both drugs act independently and do not interfere with the other • Allows combinations of more than 2 drugs	• Effect-based approach • Depends on the knowledge of mechanisms of action of the drugs • Presumes that drugs follow an exponential dose-effect response • Only fits for effects that can be ranged in probabilities from 0 to 1	• $E_{AB} = E_A + E_B(1 - E_A)$ • $CI = \frac{E_A + E_B - E_A E_B}{E_{AB}}$
Loewe Additivity	• Most well-known dose–effect-based approach • Allows the calculation of a combination index (CI) • Take into account the dose of each drug • Assumes the dose equivalence and the sham combination principles • Graphical approach (isobologram) • Does not rely on the drugs' dose-response relationship nor their mechanism of interaction • Allows combinations of more than 2 drugs	• Only designed for drugs that have dose-response curves described by the Hill equation • Requires more data and sometimes raw data preprocessing • Only fit to drugs that have a constant potent ratio.	• $E_{AB} = E_A(a + a_b) = E_B(b + b_a)$ • $CI = \frac{a}{A} + \frac{b}{B}$
Zero Interaction Potency	• One of the most recent reference models • Dose-effect based approach • Assumes that drugs are independent and do not interact with each other when combined • It is not affected by the pharmacodynamics of the compounds in combination	• Requires accurate fitting of the dose-response curves • Requires data of good quality • Does not allow combinations of more than 2 drugs	• $E_{AB} = \frac{\left(\frac{[A]}{EC_{50,A}}\right)^{\lambda A}}{1 + \left(\frac{[A]}{EC_{50,A}}\right)^{\lambda A}} + \frac{\left(\frac{[B]}{EC_{50,B}}\right)^{\lambda B}}{1 + \left(\frac{[B]}{EC_{50,B}}\right)^{\lambda B}} - \frac{\left(\frac{[A]}{EC_{50,A}}\right)^{\lambda A}}{1 + \left(\frac{[A]}{EC_{50,A}}\right)^{\lambda A}} \frac{\left(\frac{[B]}{EC_{50,B}}\right)^{\lambda B}}{1 + \left(\frac{[B]}{EC_{50,B}}\right)^{\lambda B}}$

expensive or difficult to determine (Foucquier and Guedj, 2015). On the other hand, effect-based approaches are more limited but can still provide enough evidence of positive combination effects, with fewer data.

Another limitation is that most of the time, the analysis of drug interactions in the clinical trials involving drug combinations is impaired by intense practical and ethical constraints, which makes it hard to collect sufficient data to clearly and properly support synergy. Also, the choice of the reference model in each step of the investigational process must be tailored to the data available at each discovery step. For example, in *in vitro* studies, the Highest Single Agent, Bliss Independence, and Loewe Additivity may be the most appropriate reference models to find promising drug pairs for evaluation of their mechanisms of action or to proceed for further clinical research (Borisy et al., 2003; Cokol et al., 2011; Lehár et al., 2007, 2009; Wientjes, 2010; Zhao et al., 2004), whereas in further preclinical studies the Loewe Additivity with CI and Isobologram analysis may be more appropriate to determine more precisely the combination effects (Foucquier and Guedj, 2015). In clinical studies in humans, the recommendations from the Food and Drug Administration (FDA) (US FDA, 2013) together with the European Medicines Agency (EMA) (European Medicines Agency Committee for Human Medicinal Products, 2017) and the World Health Organization (WHO) (World Health Organization, 2005) determine that there must be a strong and rational basis for the use of combination therapies supported by previous preclinical studies that also justify the use of combined agents over individual drugs and their improved safety profile for the proposed disease. Nevertheless, extrapolating *in vitro* results to animals or even humans is not well established and raise several questions, being far from being consensual among the scientific community (Ram, 2019). Generally, drug combination clinical trials include four experimental groups that are treated with placebo, drug A, drug B or a combination of drug A + drug B and it must be clearly demonstrated that combination therapy has a great efficacy than both drugs alone, using the same or even

lower doses than the individual agents, while still maintaining a safety profile (Chou, 2010; Woodcock et al., 2011).

The fourth limitation is related to the optimization of dose ratios. Since cells do not differentiate single drugs or their combination, two drugs combined at a given ratio could be considered as a third that has its own dose-effect relation (Chou, 2010). So, rather than evaluating if a drug combination is synergistic, scientists must ask what dose ratio optimizes their synergy (Keith et al., 2005). To do so, the design of experimental analysis must evaluate a different set of fixed ratios and explore what doses fit well to reach the desired synergistic effects (Ma and Motsinger-Reif, 2019). These experiments should always be performed in preclinical studies before further clinical trials (Foucquier and Guedj, 2015).

When analyzing drug combination results, one must also take into account that biological experiments regularly have associated some type of experimental error (Foucquier and Guedj, 2015). Although the Highest Single Agent and Response Additivity reference models take into account the statistical significance, the Bliss Independence and Loewe Additivity models do not allow statistical significance interpretation, because they do not have the mathematical background necessary for statistical evaluation (Foucquier and Guedj, 2015). The software CompuSyn (http://www.combosyn.com), based on the Chou-Talalay theory, follows the Loewe Additivity model and assumes the Median-Effect approach of Chou and Talalay to assess the statistical significance of the experiments, representing an improvement over other approaches (Chou, 2010).

Another aspect when referring to the previously mentioned approaches is the use of drug combinations with more than two drugs, a common practice in cancer therapy, for example. Indeed, these approaches can be further extended for use of any drugs in combination. For example, for the Loewe Additivity model, the CI can be calculated by the following equation: $CI = \frac{a}{A} + \frac{b}{B} + \ldots + \frac{n}{N}$. Nevertheless, such generalization does not permit the quantification of each drugs' contribution to the combination, implying that a synergistic combination of three or more drugs could be the result of only two drugs, for example (Foucquier and Guedj, 2015). Therefore, it is really important to rationally design the experimental protocols to demonstrate that, for example, in a triple combination using drugs A, B and C, A and B are synergistic, and that the combination of A + B (considered as a new single agent) with the drug C is also synergistic (Foucquier and Guedj, 2015).

4. Recent methods for directly quantifying drug synergism

Loewe additivity and Bliss independence approaches have been dominant in the field of synergism, together with the work proposed by Chou and Talalay. Still, there is no consensus among the scientific community regarding the appropriate use of these reference models. Recently, there has been an increase in the number of publications related to the definition of synergism, such as the "lack-of-fit" model (Lederer et al., 2019) or the rediscovered Hand model (Hand, 2000; Sinzger et al., 2019). Novel ways to evaluate synergism in drug combination studies also appeared such as the ZIP model (Yadav et al., 2015), Combenefit (SANE) (Di Veroli et al., 2016), Bivariate Response to Additive Interacting Doses (BRAID) (Twarog et al., 2016), Schindler's Hill partial differential equation (Schindler, 2017), the SynergyFinder software (Zheng et al., 2022), the Multi-dimensional Synergy of Combinations (MuSyC) (Meyer et al., 2019), the effective dose model (Zimmer et al., 2016) and the copula model (Lambert and Dawson, 2019), for example. Next, we will describe some of the most common methods for directly quantifying and evaluating drug synergism (Ma and Motsinger-Reif, 2019).

Response surface modeling is an approach based on a 3D plot representation to describe the interaction effects in drug combinations. Basically, doses of drugs A and B are plotted on the x and y-axis and the expected combined effects (response) are plotted on the z-axis, creating a 3D surface. Synergism or antagonism is measured as deviations from this surface and depends on the value of z. It allows the use of both Bliss Independence and Loewe additivity models for the estimation of the expected effects (Ma and Motsinger-Reif, 2019).

Recently, more user-friendly approaches have gained attention for the quantification of drug interactions. CompuSyn, a computer software based on the Chou-Talalay theorem, has been developed to make synergy determination more user-friendly, enhancing the usage of this reference model for the evaluation of drug interactions in combination studies. To use this software, raw data must be analyzed and normalized to convert responses in value between 0 and 1 (Chou, 2010).

Another approach is the Mixlow (i.e. Mixed-effects Loewe) methodology, developed by Boik, Newman, and Boik in 2008 (Boik et al., 2008). This approach is based mainly on three elements: a nonlinear mixed-effects model for the estimation of the sigmoidal curve parameters from the concentration-response curves, the Loewe index, and a method for evaluation of statistical significance for the index. It is an improvement of the Chou-Talalay method as it is more accurate in the estimation of the parameters, includes the evaluation of confidence intervals and dismisses the need for previous data processing (Boik et al., 2008).

More recently, Hennessey et al. (2010) (Hennessey et al., 2010) proposed a novel model for the evaluation of dose-response curves and synergy determination. The Bayesian model uses hierarchical nonlinear regression to describe the variability between and within experiments and in the observed responses of the controls. To do so, the authors used Markov chain Monte Carlo (MCMC) for data fitting and a modified version of Loewe additivity to infer about synergism in drug combinations while incorporating variables related to variability and uncertainty related intrinsically with the experiments. The authors found this approach to be more precise in drug synergism estimation than the Chou-Talalay methodology (Hennessey et al., 2010).

5. Oncological preclinical studies based on combination models

The evaluation of synergism in preclinical trials involving *in vitro* assays is a common practice and nowadays many *in vivo* drug combination studies using animal models are based on the results obtained in studies using cell lines. We searched the database PubMed using the terms '*in vivo*' and 'synergy' and 'cancer' and limited the results to a period from 2019 to 2022. The selected studies will next be analyzed to find if *in vivo* combination studies make correct use of the term synergy and to evaluate which reference models they employ to assess drug interactions.

One of the reference papers for the evaluation of drug synergism in *in vivo* studies is the one published by Fu et al., in 2016 (Fu et al., 2016), from the research group led by Ting-Chao Choug. The authors studied the combination of two anticancer drugs that target microtubule polymerization: Taxotere and T607 compound. They found these drugs are synergic against human colon carcinoma HCT-116 xenograft mice. The authors determine drug interactions quantitatively using the median-effect equation of the mass-action law and the CI theorem proposed by Chou-Talalay, demonstrating the basic concepts and experimental design aspects important for the quantitative analysis of drug interaction dynamics in complex biological systems (Fu et al., 2016).

A recent study from Skeberdyte (Skeberdytė et al., 2020)evaluated the effect of the combination of salinomycin and dichloroacetate in lung carcinoma. Salinomycin acts on cancer stem cells and dichloroacetate inhibits the pyruvate dehydrogenase kinase. The authors performed *in vitro* studies using two and three-dimensional cell cultures of Lewis lung carcinoma (LLC1) cells and also *in vivo* studies using an LLC1-C57BL/6 mouse model. The authors successfully proved that this drug combination has synergistic effects *in vitro* using the Chou-Talalay method by testing several doses below IC_{50} from both monotherapies using a non-constant ratio drug design. The authors also proved this combination increased the survival rate of mice, reduced metastasis occurrence and also decreased the population of cancer stem cells, proving the benefits of this combination both *in vitro* and *in vivo* (Skeberdytė et al., 2020).

Another study from Xu et al. (2019) attempted to evaluate the potential of the combination of ABT199 and irinotecan in RAS-mutant lung cancer cells. The authors selected this *in vitro* model as KRAS mutation is very frequent in non-small cancer lung cancer (NSCLC). Irinotecan is one of the most well-known chemotherapeutic drugs commonly used for metastatic colorectal cancer and ABT199 is an investigational drug with high efficacy of the BCL-2 inhibitor. Using different human NSCLC cell lines (H441, A549, H838 and H522), the authors have successfully demonstrated that this drug combination inhibits lung cancer cell growth and enhanced apoptosis, being synergistic with CIs under 0.7. Further *in vivo* studies suggests this combination induces tumor size and weight reduction, demonstrating the potent *in vivo* efficacy of the combination (Xu et al., 2019).

Ghosh et al. (2019) recently studied the anticancer effect of the combination of methylglyoxal, an agent with a well-established anticarcinogenic effect and 5-fluorouracil (5-FU), an antineoplastic agent commonly used for several types of cancer, for breast cancer treatment. This research group evaluated the effect of this drug combination both *in vitro* using MCF-7 breast cancer cells and *in vivo* and successfully found synergism using the Chou-Talalay theorem. *In vivo* studies revealed that EAC (Ehrlich Ascites Carcinoma) bearing mice and BALB/c mouse 4T1 breast tumor models exhibited tumor regression and fewer side effects when treated with this combination compared to single agents (Ghosh et al., 2019).

Buocikova and her colleagues (Buocikova et al., 2022) recently studied the combination of the DNA methyltransferase (DNMT) inhibitor decitabine with the anthracycline antibiotic doxorubicin for breast cancer. They first evaluated the synergism of sequential decitabine + doxorubicin treatment in three breast cancer cell lines (JIMT-1, MDA-MB-231 and T-47D) after assessing their influence on the cytotoxicity, genotoxicity, apoptosis, and migration capacity of these cells. The mathematical framework employed for drug synergism was based on the Chou-Talalay method using the CI to quantify drug interactions. They further confirmed these results using an orthotopic xenograft mouse model, demonstrating the potential of epigenetic drugs in the modulation of cancer cells' sensitivity to antineoplastic agents (Buocikova et al., 2022).

Another study combined Cisplatin-loaded poly(L-glutamic acid)-*graft*-methoxy poly(ethylene glycol) complex nanoparticles with different PD1/PD-L1 inhibitors and evaluated their cytotoxic effect in different types of cells (LLC, H1299, A549, B16F10, ID8, and U14) and female C57BL/6 mice. They found this combination to cause tumor PD-L1 overexpression time-dependent *in vitro* and increased tumor PD-L1 signals after 72 h of treatment *in vivo*. Different from the previously mentioned studies, this work employed the Q value method of Zhengjun Jin to analyze the synergistic interaction of the combination group for tumor therapy (*in vivo*). Taken together, these authors demonstrated the potential clinical treatment of Cisplatin-loaded poly(L-glutamic acid)-*graft*-methoxy poly(ethylene glycol) complex nanoparticles with different PD1/PD-L1 inhibitors for cancer therapy (Shen et al., 2021).

In another study, the authors attempted to combine gemcitabine, a deoxycytidine nucleoside analog commonly used for the treatment of advanced or metastatic pancreatic cancer, with Troxacitabine (Troxatyl™), an unnatural L-nucleoside analog that demonstrates potent antitumor activity. The authors performed *in vitro* studies using different pancreatic adenocarcinoma cell lines (AsPC-1, Capan-2, MIA PaCa-2 and Panc-1) and evaluated synergism using the isobologram and combination-index methods of Chou and Talalay. The authors then evaluated the effects of both drugs, alone or combined in nude mice transplanted with human pancreatic (AsPC-1) tumors and concluded that both drugs were more than additive at safer doses and schedules, being potential candidates for further evaluation in patients with advanced pancreatic cancer (Damaraju et al., 2007).

Sánchez et al. (2019) recently studied the combination of docetaxel, a chemotherapeutic agent, and capsaicin, an ingredient of hot chili peppers, for the treatment of prostate cancer. The authors used two prostate cancer cell lines (LNCaP and PC-3) for the *in vitro* studies and xenograft prostate cancer models to further evaluate this combination *in vivo*. Using the Chou-Talalay reference model, they found that docetaxel and capsaicin have synergistic effects in the inhibition of LNCaP and PC-3 cell growth, reflecting a CI under 1 for most of the combinations evaluated in the work. *In vivo* results reinforced the synergistic effects of this combination in the reduction of tumor growth in xenograft prostate cancer models (Sánchez et al., 2019).

Recently, another study aimed to study if the synergism between the drugs vincristine and irinotecan also extend to eribulin, another microtubule inhibitor for the treatment of childhood solid tumors. The authors combined vincristine or eribulin with irinotecan and studied their anticancer effect *in vitro* and xenograft models. CIs were calculated based on the Bliss model of independence and the Loewe additivity model. They found the eribulin combination to be very effective in these xenograft models, but not synergistic *in vitro* (Robles et al., 2020).

Despite the huge number of publications stating synergism in animal experiments, what we found is that most papers are not able to really prove drug synergism *in vivo*, assuming synergistic interactions based on the *in vitro* results and using only one reference model for assessing the drug interactions (usually the Chou-Talalay theorem). According to Chou (2010), the quantification of synergy *in vitro* and in animals is based on the same principles, requiring a minimum of 65 nude mice for accurate results (Chou, 2010). We believe that, for most of the combination studies, synergism is only determined *in vitro* because the determination *in vivo* is more expensive, time-consuming and introduces more variability. Most of the studies then extrapolate the results from *in vitro* to animals, "which is a general and separate biomedical problem which is not expected to be solved by the Chou-Talalay method" (Chou, 2010).

The recent studies from our research group explore the combination of repurposed drugs and antineoplastic agents for the treatment of breast and colon cancer, following the experimental design proposed for Chou-Talalay for the accurate prediction of drug synergism (Duarte et al., 2021, 2022; Duarte and Vale, 2020). Although our results were only performed *in vitro*, drug interaction was evaluated using more than one reference model (usually Loewe, Bliss, HSA, ZIP and Chou-Talalay methods), giving more support to the obtained results and providing a more robust basis for further research in animal models or clinical trials.

6. Oncological clinical trials based on preclinical synergism evaluation

Most clinical trials are often supported by preclinical studies that state to have found synergistic drug combinations. Although synergism is the gold standard desired when referring to drug combinations in cancer therapy, this term is commonly misused, as we previously stated. Another aspect is that, even if one achieves synergism in a certain combination of drugs, this does not necessarily mean that the combination will be indeed useful for cancer therapy. The potential for clinical use of combining drugs in clinical trials should also be measured by the therapeutic index, a concept that relates the relative toxicity of an anticancer treatment to its toxicity in normal tissues, rather than only by the quantification of synergism (Ocana et al., 2012).

A report from 2012 from Ocana et al. (2012) attempted to evaluate if clinical trials that frequently evaluate drug combinations were well designed and if they used correctly the term synergy, based on strong evidence from preclinical studies (Ocana et al., 2012). The authors have found, at the time, that only 13.6% of the preclinical studies included the evaluation of synergism based on isobologram analysis and 7.6% based on the Chou-Talalay method. Regarding preclinical studies involving animal models, only 39% evaluated the therapeutic index, concluding that most phase I and II studies did not perform the appropriate background methods for correctly assessing synergism (Ocana et al., 2012).

Also, a recent paper argues that synergism has not been proved in most clinical trials, especially those regarding studies that combine various checkpoint inhibitors with each other and with other

antineoplastic drugs (Palmer et al., 2022). This review analyzed thirteen Phase III clinical trials and found no synergistic interactions, just additive effects of each therapy, only synergism previously supported in animal models. Although this is not necessarily a bad thing, it is still not the good thing that all scientists have been looking at and reflects, once again, the misuse of the term synergism among clinical studies (Palmer et al., 2022).

Indeed, in a perspective published by Chou in 2010 (Chou, 2010), he states that it is generally not possible to determine synergism in clinical trials, based on scientific, practical and ethical reasons (Chou, 2010) that we already discussed in section 3. He claims that most clinical trials that employ the term "synergism" are not well supported by the available data, especially in studies where only a single dose was tested for a single drug, mentioning the importance of preclinical evaluation for accurate and robust results to support clinical trials in humans (Chou, 2010).

Here, we performed a Pubmed search using the same methodology as described by Ocana et al. (2012). We searched for the terms 'synergy' or 'synergistic' and 'cancer' and limited the search for studies in humans, clinical trials over the last year (2021–2022). Next, we will mention some of the most recent clinical trials in oncology that are based on preclinical studies claiming synergism.

Kang et al. (2022) published the results from a recent randomized, multicenter, double-blind, placebo-controlled, phase II-III clinical trial (NCT02746796) for the evaluation of the combination of immune checkpoint inhibitor nivolumab and oxaliplatin versus placebo plus oxaliplatin. This combination is intended to be used as first-line therapy for the treatment of patients with HER2-negative, unresectable advanced or recurrent gastric or gastro-esophageal junction cancer (Kang et al., 2022). This study was based on previous preclinical studies that supported the additive or synergistic antitumor effects of the combination of immune checkpoint inhibitors with oxaliplatin. The authors found that a combination of nivolumab and oxaliplatin enhanced progression-free survival but not overall survival and concluded this drug combination has the potential to be used as a first-line treatment for patients with this type of cancer (Kang et al., 2022).

Jain et al. (2021) recently conducted a single-center, phase II non-randomized trial (NCT02756897) to study the combination of ibrutinib and venetoclax in the treatment of chronic lymphocytic leukemia (Jain et al., 2021). The study is based on preclinical studies that demonstrate the synergism of Bruton tyrosine kinase inhibitors with the Bcl-2 inhibitor venetoclax. The authors found this combination can be advantageous for previously untreated patients with chronic lymphocytic leukemia (Jain et al., 2021).

Another study from Cousin et al. (2021) performed a single-arm, multicentric phase II trial (REGOMUNE) to assess regorafenib plus avelumab for the treatment of patients with microsatellite stable colorectal cancer, based on previous preclinical results that demonstrated that this combination is synergic. The authors concluded that regorafenib combined with avelumab successfully modulates antitumor immunity in some patients with this type of cancer (Cousin et al., 2021).

A phase Ib randomized, open-label, multicenter study studied the combination of alpelisib with everolimus ± exemestane in solid tumors. This study claims to be based on preclinical models that demonstrated a synergistic effect between and with alpelisib. Everolimus inhibits mTORC1, alpelisib acts on the phosphatidylinositol 3-kinase catalytic subunit p110α blockage and exemestane is an antineoplastic drug already used for cancer therapy. The authors conclude that this combination is safe, manageable and reversible and the pharmacokinetics of each drug did not change significantly when in combination (Curigliano et al., 2021).

Another phase I clinical trial (NCT01677559) aimed to combine an aurora kinase A inhibitor (alisertib) and nab-paclitaxel, a chemotherapeutic agent, for refractory high-grade neuroendocrine tumors. The authors refer that this trial is based on preclinical studies that have demonstrated that the combination of alisertib plus paclitaxel is synergic in rapidly proliferative cancers. Moreover, the authors successfully found that the proposed combination has is safe, manageable and reversible and demonstrated promising preliminary efficacy (Lim et al., 2021).

Hong et al. (2021) recently published the results from a phase Ib study (NCT02124148) where it was evaluated the combination of prexasertib and samotolisib. Prexasertib is a CHK1 Inhibitor and samotolisib is a dual PI3K/mTOR inhibitor. This clinical trial is based on previous studies that demonstrated that prexasertib alone has moderate anticancer activity and other preclinical data in triple-negative breast cancer cells, MDA-MB-231 orthotopic xenograft tumors, and TNBC patient-derived xenograft mouse models that also supported this combination. The authors found this combination to be effective in preclinical models and preliminary effective, with some degree of toxicity associated (Hong et al., 2021).

Another phase I, open-label, dose-escalation study was performed by Amin et al. (2021), for the determination of the maximal tolerated dose of docetaxel in combination with temsirolimus for the treatment of refractory solid tumors. Temsirolimus selectively inhibits mTOR and docetaxel is an antineoplastic drug belonging to the class of taxanes. This trial was based on preclinical results that demonstrated that combination of taxanes and mTOR inhibitors to be additive or synergic in different types of cancer cells. The authors found this combination to be very toxic and not suitable for cancer treatment (Amin et al., 2021).

Taken together, the mentioned clinical trials, which are usually phases I and II, demonstrate that synergism in preclinical studies is usually the justification for the clinical evaluation of drug combinations in cancer-related studies, clarifying the importance of the accurate and reliable drug interaction quantification in this type of studies.

7. Conclusions and prospects

Drug combination is a strategy that has been studied extensively over the last century and its advantages over monotherapy have been recognized by several scientists. Due to the recent advances in the understanding of the biological concepts behind several diseases, the interest in using drug combinations to improve the efficacy of the available therapies has dramatically increased. Over the years, research in this area has resulted in many theoretical and experimental papers, involving researchers from different disciplines. Despite the different approaches developed to evaluate the interaction of drugs in combination therapies, all reference models still present some limitations, and their choice must be adequate to the available data and type of study (*in vitro, in vivo* or clinical trial). Future studies must aim to rigorously apply the models and concepts herein described for the appropriate interpretation of combination effects, and, whenever possible, the analysis of drug combinations may benefit from the cooperative use of different approaches. The use of the term synergism in animal studies must be carefully employed and only when the determination of drug synergism was indeed validated using an appropriate reference model. Synergism in clinical trials should also be mentioned when there is robust information from previous preclinical studies. Also, in addition to evaluating synergism, further research should be always performed in order to elucidate the mechanisms of action by which drugs act both alone and combined, to help improve the combination experiment design. In silico approaches, such as PBPK models, can also represent a potential tool for the evaluation of drug synergism *in vivo* or in further clinical trials.

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CRediT authorship contribution statement

Diana Duarte: Writing – original draft. **Nuno Vale:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Amin, M., Gao, F., Terrero, G., Picus, J., Wang-Gillam, A., Suresh, R., Ma, C., Tan, B., Baggstrom, M., Naughton, M.J., Trull, L., Belanger, S., Fracasso, P.M., Lockhart, A.C., 2021. Phase I study of docetaxel and temsirolimus in refractory solid tumors. Am. J. Clin. Oncol. 44 (9), 443–448. https://doi.org/10.1097/COC.0000000000000852.

Berenbaum, M.C., 1989. What is synergy? Pharmacol. Rev. 41 (2), 93–141.

Berenbaum, M.C., 1977. Synergy, additivism and antagonism in immunosuppression. A critical review. Clin. Exp. Immunol. 28 (1), 1–18.

Bliss, C.I., 1939. The toxicity OF POISONS applied jointly. Ann. Appl. Biol. 26 (3), 585–615. https://doi.org/10.1111/j.1744-7348.1939.tb06990.x.

Boik, J.C., Newman, R.A., Boik, R.J., 2008. Quantifying synergism/antagonism using nonlinear mixed-effects modeling: a simulation study. Stat. Med. 27 (7), 1040–1061. https://doi.org/10.1002/SIM.3005.

Borisy, A.A., Elliott, P.J., Hurst, N.W., Lee, M.S., Lehár, J., Price, E.R., Serbedzija, G., Zimmermann, G.R., Foley, M.A., Stockwell, B.R., Keith, C.T., 2003. Systematic discovery of multicomponent therapeutics. Proc. Natl. Acad. Sci. Unit. States Am. 100 (13), 7977–7982. https://doi.org/10.1073/pnas.1337088100.

Buocikova, V., Longhin, E.M., Pilalis, E., Mastrokalou, C., Miklikova, S., Cihova, M., Poturnayova, A., Mackova, K., Babelova, A., Trnkova, L., El Yamani, N., Zheng, C., Rios-Mondragon, I., Labudova, M., Csaderova, L., Kuracinova, K.M., Makovicky, P., Kucerova, L., Matuskova, M., Cimpan, M.R., Dusinska, M., Babal, P., Chatziioannou, A., Gabelova, A., Rundén-Pran, E., Smolkova, B., 2022. Decitabine potentiates efficacy of doxorubicin in a preclinical trastuzumab-resistant HER2-positive breast cancer models. January Biomed. Pharmacother. 147, 112662. https://doi.org/10.1016/j.biopha.2022.112662.

Caudle, R.M., Williams, G.M., 1993. The misuse of analysis of variance to detect synergy in combination drug studies. Pain 55 (3), 313–317. https://doi.org/10.1016/0304-3959(93)90006-B.

Chou, T.-C., Talalay, P., 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzym. Regul. 22 (C), 27–55. https://doi.org/10.1016/0065-2571(84)90007-4.

Chou, T.-C.T.C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58 (3), 621–681. https://doi.org/10.1124/pr.58.3.10.

Chou, T.C., 2010. Drug combination studies and their synergy quantification using the chou-talalay method. Cancer Res. 70 (2), 440–446. https://doi.org/10.1158/0008-5472.CAN-09-1947.

Chou, T.C., Talalay, P., 1983. Analysis of combined drug effects: a new look at a very old problem. Trends Pharmacol. Sci. 4 (C), 450–454. https://doi.org/10.1016/0165-6147(83)90490-X.

Cokol, M., Chua, H.N., Tasan, M., Mutlu, B., Weinstein, Z.B., Suzuki, Y., Nergiz, M.E., Costanzo, M., Baryshnikova, A., Giaever, G., Nislow, C., Myers, C.L., Andrews, B.J., Boone, C., Roth, F.P., 2011. Systematic exploration of synergistic drug pairs. Mol. Syst. Biol. 7 (1), 544. https://doi.org/10.1038/msb.2011.71.

Cousin, S., Cantarel, C., Guegan, J.-P., Gomez-Roca, C., Metges, J.-P., Adenis, A., Pernot, S., Bellera, C., Kind, M., Auzanneau, C., Le Loarer, F., Soubeyran, I., Bessede, A., Italiano, A., 2021. Regorafenib-avelumab combination in patients with microsatellite stable colorectal cancer (REGOMUNE): a single-arm, open-label, phase II trial. Clin. Cancer Res. 27 (8), 2139–2147. https://doi.org/10.1158/1078-0432.CCR-20-3416.

Curigliano, G., Martin, M., Jhaveri, K., Beck, J.T., Tortora, G., Fazio, N., Maur, M., Hubner, R.A., Lahner, H., Donnet, V., Ajipa, O., Li, Z., Blumenstein, L., Andre, F., 2021. Alpelisib in combination with everolimus ± exemestane in solid tumours: phase Ib randomised, open-label, multicentre study. Eur. J. Cancer 151 49–62. https://doi.org/10.1016/j.ejca.2021.03.042.

Damaraju, V.L., Bouffard, D.Y., Wong, C.K.W., Clarke, M.L., Mackey, J.R., Leblond, L., Cass, C.E., Grey, M., Gourdeau, H., 2007. Synergistic activity of troxacitabine (Troxatyl™) and gemcitabine in pancreatic cancer. BMC Cancer 7, 1–11. https://doi.org/10.1186/1471-2407-7-121.

Dear, R.F., McGeechan, K., Jenkins, M.C., Barratt, A., Tattersall, M.H.N., Wilcken, N., 2013. Combination versus sequential single agent chemotherapy for metastatic breast cancer. Cochrane Database Syst. Rev. 2021 (3). https://doi.org/10.1002/14651858.CD008792.pub2.

Di Veroli, G.Y., Fornari, C., Wang, D., Mollard, S., Bramhall, J.L., Richards, F.M., Jodrell, D.I., 2016. Combenefit: an interactive platform for the analysis and visualization of drug combinations. Bioinformatics 32 (18), 2866–2868. https://doi.org/10.1093/bioinformatics/btw230.

Duarte, D., Cardoso, A., Vale, N., 2021. Synergistic growth inhibition of HT-29 colon and MCF-7 breast cancer cells with simultaneous and sequential combinations of antineoplastics and CNS drugs. Int. J. Mol. Sci. 22 (14), 7408. https://doi.org/10.3390/ijms22147408.

Duarte, D., Rêma, A., Amorim, I., Vale, N., 2022. Drug combinations: a new strategy to extend drug repurposing and epithelial-mesenchymal transition in breast and colon cancer cells. Biomolecules 12 (2), 190. https://doi.org/10.3390/biom12020190.

Duarte, D., Vale, N., 2020. New trends for antimalarial drugs: synergism between antineoplastics and antimalarials on breast cancer cells. Biomolecules 10 (12), 1–20. https://doi.org/10.3390/biom10121623.

European Medicines Agency Committee for Human Medicinal Products, 2017. Committee for Human Medicinal Products (CHMP) Guideline on Clinical Development of Fixed Combination Medicinal Products.

Foucquier, J., Guedj, M., 2015. Analysis of drug combinations: current methodological landscape. Pharmacol. Res. Perspect. 3 (3), e00149. https://doi.org/10.1002/prp2.149.

Fu, J., Zhang, N., Chou, J.H., Dong, H.J., Lin, S.F., Ulrich-Merzenich, G.S., Chou, T.C., 2016. Drug combination in vivo using combination index method: Taxotere and T607 against colon carcinoma HCT-116 xenograft tumor in nude mice. Synergy 3 (3), 15–30. https://doi.org/10.1016/j.synres.2016.06.001.

Geary, N., 2013. Understanding synergy. Am. J. Physiol. Metab. 304 (3), E237–E253. https://doi.org/10.1152/ajpendo.00308.2012.

Ghosh, S., Pal, A., Ray, M., 2019. Methylglyoxal in combination with 5-Fluorouracil elicits improved chemosensitivity in breast cancer through apoptosis and cell cycle inhibition. October 2018 Biomed. Pharmacother. 114, 108855. https://doi.org/10.1016/j.biopha.2019.108855.

Goldoni, M., Johansson, C., 2007. A mathematical approach to study combined effects of toxicants in vitro: evaluation of the Bliss independence criterion and the Loewe additivity model. Toxicol. Vitro 21 (5), 759–769. https://doi.org/10.1016/j.tiv.2007.03.003.

Grabovsky, Y., Tallarida, R.J., 2004. Isobolographic analysis for combinations of a full and partial agonist: curved isoboles. J. Pharmacol. Exp. Therapeut. 310 (3), 981–986. https://doi.org/10.1124/jpet.104.067264.

Greco, W.R., Bravo, G., Parsons, J.C., 1995. The search for synergy: a critical review from a response surface perspective. Pharmacol. Rev. 47 (2), 331–385.

Hand, D.J., 2000. Synergy in Drug Combinations. Springer, Berlin, Heidelberg, pp. 471–475. https://doi.org/10.1007/978-3-642-58250-9_38.

Hennessey, V.G., Rosner, G.L., Bast, R.C., Chen, M.Y., 2010. A Bayesian approach to dose-response assessment and synergy and its application to in vitro dose-response studies. Biometrics 66 (4), 1275–1283. https://doi.org/10.1111/J.1541-0420.2010.01403.X.

Hong, D.S., Moore, K.N., Bendell, J.C., Karp, D.D., Wang, J.S., Ulahannan, S.V., Jones, S., Wu, W., Donoho, G.P., Ding, Y., Capen, A., Wang, X., Bence Lin, A., Patel, M.R., 2021. Preclinical evaluation and phase Ib study of prexasertib, a CHK1 inhibitor, and samotolisib (LY3023414), a dual PI3K/mTOR inhibitor. Clin. Cancer Res. 27 (7), 1864–1874. https://doi.org/10.1158/1078-0432.CCR-20-3242.

Jain, N., Keating, M., Thompson, P., Ferrajoli, A., Burger, J.A., Borthakur, G., Takahashi, K., Estrov, Z., Sasaki, K., Fowler, N., Kadia, T., Konopleva, M., Alvarado, Y., Yilmaz, M., DiNardo, C., Bose, P., Ohanian, M., Pemmaraju, N., Jabbour, E., Kanagal-Shamanna, R., Patel, K., Wang, W., Jorgensen, J., Wang, S.A., Garg, N., Wang, X., Wei, C., Cruz, N., Ayala, A., Plunkett, W., Kantarjian, H., Gandhi, V., Wierda, W.G., 2021. Ibrutinib plus venetoclax for first-line treatment of chronic lymphocytic leukemia. JAMA Oncol. 7 (8), 1213. https://doi.org/10.1001/jamaoncol.2021.1649.

Kang, Y.-K., Chen, L.-T., Ryu, M.-H., Oh, D.-Y., Oh, S.C., Chung, H.C., Lee, K.-W., Omori, T., Shitara, K., Sakuramoto, S., Chung, I.-J., Yamaguchi, K., Kato, K., Sym, S.J., Kadowaki, S., Tsuji, K., Chen, J.-S., Bai, L.-Y., Oh, S.-Y., Choda, Y., Yasui, H., Takeuchi, K., Hirashima, Y., Hagihara, S., Boku, N., 2022. Nivolumab plus chemotherapy versus placebo plus chemotherapy in patients with HER2-negative, untreated, unresectable advanced or recurrent gastric or gastro-oesophageal junction cancer (ATTRACTION-4): a randomised, multicentre, double-blind, placebo-contr. Lancet Oncol. 23 (2), 234–247. https://doi.org/10.1016/S1470-2045(21)00692-6.

Keith, C.T., Borisy, A.A., Stockwell, B.R., 2005. Multicomponent therapeutics for networked systems. Nat. Rev. Drug Discov. 4 (1), 71–78. https://doi.org/10.1038/NRD1609.

Kim, H., Lee, S.J., Lee, I.K., Min, S.C., Sung, H.H., Jeong, B.C., Lee, J., Park, S.H., 2020. Synergistic effects of combination therapy with akt and mtor inhibitors on bladder cancer cells. Int. J. Mol. Sci. 21 (8), 1–12. https://doi.org/10.3390/ijms21082825.

Klimaszewska-Wiśniewska, A., Hałas-Wiśniewska, M., Grzanka, A., Grzanka, D., 2018. Evaluation of anti-metastatic potential of the combination of fisetin with paclitaxel on a549 non-small cell lung cancer cells. Int. J. Mol. Sci. 19 (3), 1–18. https://doi.org/10.3390/ijms19030661.

Lambert, R.J.W., Dawson, D.A., 2019. New models for the time dependent toxicity of individual and combined toxicants. Toxicol. Res. (Camb). 8 (4), 509. https://doi.org/10.1039/C9TX00005D.

Lederer, S., Dijkstra, T.M.H., Heskes, T., 2019. Additive dose response models: defining synergy. November Front. Pharmacol. 10, 1–15. https://doi.org/10.3389/fphar.2019.01384.

Lee, J.O., Kang, M.J., Byun, W.S., Kim, S.J.S.A., Seo, I.H., Han, J.A., Moon, J.W., Kim, J.H., Kim, S.J.S.A., Lee, E.J., In Park, S., Park, S.H., Kim, H.S., 2019. Metformin overcomes resistance to cisplatin in triple-negative breast cancer (TNBC) cells by targeting RAD51. Breast Cancer Res. 21 (1), 1–18. https://doi.org/10.1186/s13058-019-1204-2.

Lehár, J., Krueger, A.S., Avery, W., Heilbut, A.M., Johansen, L.M., Price, E.R., Rickles, R.J., Short, G.F., Staunton, J.E., Jin, X., Lee, M.S., Zimmermann, G.R., Borisy, A.A., 2009. Synergistic drug combinations tend to improve therapeutically relevant selectivity. Nat. Biotechnol. 27 (7), 659–666. https://doi.org/10.1038/NBT.1549.

Lehár, J., Zimmermann, G.R., Krueger, A.S., Molnar, R.A., Ledell, J.T., Heilbut, A.M., Short, G.F., Giusti, L.C., Nolan, G.P., Magid, O.A., Lee, M.S., Borisy, A.A., Stockwell, B.R., Keith, C.T., 2007. Chemical combination effects predict connectivity in biological systems. Mol. Syst. Biol. 3 (1), 80. https://doi.org/10.1038/msb4100116.

Lim, K.-H., Opyrchal, M., Acharya, A., Boice, N., Wu, N., Gao, F., Webster, J., Lockhart, A.C., Waqar, S.N., Govindan, R., Morgensztern, D., Picus, J., Tan, B.R., Baggstrom, M.Q., Maher, C.A., Wang-Gillam, A., 2021. Phase 1 study combining alisertib with nab-paclitaxel in patients with advanced solid malignancies. Eur. J. Cancer 154, 102–110. https://doi.org/10.1016/j.ejca.2021.06.012.

Loewe, S., 1927. Die Mischarznei - versuch einer allgemeinen Pharmakologie der Arzneikombinationen. Klin. Wochenschr. 6 (23), 1077–1085. https://doi.org/10.1007/BF01890305.

Loewe, S., 1953. The problem of synergism and antagonism of combined drugs. Arzneimittelforschung 3 (6), 285–290.

Loewe, S., Muischnek, H., 1926. Über kombinationswirkungen - mitteilung: Hilfsmittel der Fragestellung. Arch. für Exp. Pathol. und Pharmakologie 114 (5–6), 313–326. https://doi.org/10.1007/BF01952257.

Ma, J., Motsinger-Reif, A., 2019. Current methods for quantifying drug synergism. Proteomics bioinforma. Curr. Res. 1 (2), 43–48.

Meyer, C.T., Wooten, D.J., Paudel, B.B., Bauer, J., Hardeman, K.N., Westover, D., Lovly, C.M., Harris, L.A., Tyson, D.R., Quaranta, V., 2019. Quantifying drug combination synergy along potency and efficacy axes. Cell Syst 8 (2), 97–108. https://doi.org/10.1016/j.cels.2019.01.003 e16.

Moreno, S., Perno, C.F., Mallon, P.W., Behrens, G., Corbeau, P., Routy, J.P., Darcis, G., 2019. Two-drug vs. three-drug combinations for HIV-1: do we have enough data to make the switch? HIV Med. 20 2–2012. https://doi.org/10.1111/HIV.12716.

National Cancer Institute, 2019. Immunotherapy for cancer - National cancer Institute [WWW Document] Natl. Cancer Inst. URL https://www.cancer.gov/about-cancer/treatment/types/immunotherapy accessed 3.18.22.

National Cancer Institute, 2015a. Types of cancer treatment - National cancer Institute [WWW Document]. Natl. Cancer Inst. URL. https://www.cancer.gov/about-cancer/treatment/types. accessed 3.18.22.

National Cancer Institute, 2015b. Chemotherapy to treat cancer - National cancer Institute [WWW Document]. Natl. Cancer Inst. URL. https://www.cancer.gov/about-cancer/treatment/types/chemotherapy. accessed 3.18.22.

National Cancer Institute, 2015c. Hormone therapy for cancer - National cancer Institute [WWW Document]. Natl. Cancer Inst. URL. https://www.cancer.gov/about-cancer/treatment/types/hormone-therapy. accessed 3.18.22.

National Cancer Institute, 2015d. Surgery for cancer - National cancer Institute [WWW Document]. Natl. Cancer Inst. URL. https://www.cancer.gov/about-cancer/treatment/types/surgery. accessed 3.18.22.

National Cancer Institute, 2015e. Hyperthermia to treat cancer - National cancer Institute [WWW Document]. Natl. Cancer Inst. URL. https://www.cancer.gov/about-cancer/treatment/types/hyperthermia. accessed 3.18.22.

National Cancer Institute, 2015f. Photodynamic therapy to treat cancer - National cancer Institute [WWW Document]. Natl. Cancer Inst. URL. https://www.cancer.gov/about-cancer/treatment/types/photodynamic-therapy. accessed 3.18.22.

National Cancer Institute, 2015g. Stem cell transplants in cancer treatment - National cancer Institute [WWW Document]. Natl. Cancer Inst. URL. https://www.cancer.gov/about-cancer/treatment/types/stem-cell-transplant. accessed 3.18.22.

National Cancer Institute, 2014. Targeted therapy for cancer - National cancer Institute [WWW Document]. Natl. Cancer Inst. URL. https://www.cancer.gov/about-cancer/treatment/types/targeted-therapies. accessed 3.18.22.

National Cancer Institute, 2010. Radiation therapy for cancer - National cancer Institute [WWW Document]. Natl. Cancer Inst. URL. https://www.cancer.gov/about-cancer/treatment/types/radiation-therapy. accessed 3.18.22.

Nieuwenhuis, S., Forstmann, B.U., Wagenmakers, E.J., 2011. Erroneous analyses of interactions in neuroscience: a problem of significance. Nat. Neurosci. 14 (9), 1105–1107. https://doi.org/10.1038/NN.2886.

Ocana, A., Amir, E., Yeung, C., Seruga, B., Tannock, I.F., 2012. How valid are claims for synergy in published clinical studies? Ann. Oncol. 23 (8), 2161–2166. https://doi.org/10.1093/annonc/mdr608.

Palmer, A.C., Izar, B., Hwangbo, H., Sorger, P.K., 2022. Predictable clinical benefits without evidence of synergy in trials of combination therapies with immune-checkpoint inhibitors. Clin. Cancer Res. 28 (2), 368–377. https://doi.org/10.1158/1078-0432.CCR-21-2275.

Podolsky, S.H., Greene, J.A., 2011. Combination drugs — Hype, Harm, and Hope. N. Engl. J. Med. 365 (6), 488–491. https://doi.org/10.1056/NEJMp1106161.

Ram, R., 2019. Extrapolation of animal research data to humans: an analysis of the evidence. In: Animal Experimentation: Working towards a Paradigm Change. BRILL, pp. 341–375. https://doi.org/10.1163/9789004391192_016.

Robles, A.J., Kurmasheva, R.T., Bandyopadhyay, A., Phelps, D.A., Erickson, S.W., Lai, Z., Kurmashev, D., Chen, Y., Smith, M.A., Houghton, P.J., 2020. Evaluation of eribulin combined with irinotecan for treatment of pediatric cancer xenografts. Clin. Cancer Res. 26 (12), 3012–3023. https://doi.org/10.1158/1078-0432.CCR-19-1822.

Rodea-Palomares, I., González-Pleiter, M., Martín-Betancor, K., Rosal, R., Fernández-Piñas, F., 2015. Additivity and interactions in ecotoxicity of pollutant mixtures: Some Patterns, Conclusions, and open questions. Toxics 3 (4), 342–369. https://doi.org/10.3390/TOXICS3040342.

Roell, K.R., Reif, D.M., Motsinger-Reif, A.A., 2017. An introduction to terminology and methodology of chemical synergy—perspectives from across disciplines. Front. Pharmacol. 8. https://doi.org/10.3389/fphar.2017.00158.

Saleh, J.A., 2008. Combination therapy in asthma: a review. Niger. J. Med. 17 (3), 238–243. https://doi.org/10.4314/NJM.V17I3.37377.

Sánchez, B.G., Bort, A., Mateos-Gómez, P.A., Rodríguez-Henche, N., Díaz-Laviada, I., 2019. Combination of the natural product capsaicin and docetaxel synergistically kills human prostate cancer cells through the metabolic regulator AMP-activated kinases. Cancer Cell Int. 19 (1), 1–14. https://doi.org/10.1186/s12935-019-0769-2.

Sasaki, K., Tsuno, N.H., Sunami, E., Tsurita, G., Kawai, K., Okaji, Y., Nishikawa, T., Shuno, Y., Hongo, K., Hiyoshi, M., Kaneko, M., Kitayama, J., Takahashi, K., Nagawa, H., 2010. Chloroquine potentiates the anti-cancer effect of 5-fluorouracil on colon cancer cells. July 2010 BMC Cancer 10, 370. https://doi.org/10.1186/1471-2407-10-370.

Schindler, M., 2017. Theory of synergistic effects: Hill-type response surfaces as "null-interaction" models for mixtures. Theor. Biol. Med. Model. 14 (1), 1–16. https://doi.org/10.1186/s12976-017-0060-y.

Shafer, S.L., 2012. All models are wrong. Anesthesiology 116 (2), 240–241. https://doi.org/10.1097/ALN.0B013E318242A4A7.

Shen, N., Yang, C., Zhang, X., Tang, Z., Chen, X., 2021. Cisplatin nanoparticles possess stronger anti-tumor synergy with PD1/PD-L1 inhibitors than the parental drug. Acta Biomater. 135, 543–555. https://doi.org/10.1016/j.actbio.2021.08.013.

Sidorov, P., Naulaerts, S., Ariey-Bonnet, J., Pasquier, E., Ballester, P.J., 2019. Predicting synergism of cancer drug combinations using NCI-ALMANAC data. Front. Chem. 7 (July), 1–13. https://doi.org/10.3389/fchem.2019.00509.

Sinzger, M., Vanhoefer, J., Loos, C., Hasenauer, J., 2019. Comparison of null models for combination drug therapy reveals Hand model as biochemically most plausible. Sci. Rep. 9 (1), 1–15. https://doi.org/10.1038/s41598-019-38907-x.

Skeberdytė, A., Sarapinienė, I., Krasko, J.A., Barakauskienė, A., Žilionytė, K., Prokarenkaitė, R., Sužiedėlis, K., Bukelskienė, V., Jarmalaitė, S., 2020. Salinomycin and dichloroacetate synergistically inhibit Lewis lung carcinoma cell proliferation, tumor growth and metastasis. Biochem. Biophys. Res. Commun. 523 (4), 874–879. https://doi.org/10.1016/j.bbrc.2019.12.107.

Slinker, B.K., 1998. The statistics of synergism. J. Mol. Cell. Cardiol. 30 (4), 723–731. https://doi.org/10.1006/JMCC.1998.0655.

Smalley, K.S.M., Haass, N.K., Brafford, P.A., Lioni, M., Flaherty, K.T., Herlyn, M., 2006. Multiple signaling pathways must be targeted to overcome drug resistance in cell lines derived from melanoma metastases. Mol. Cancer Therapeut. 5 (5), 1136–1144. https://doi.org/10.1158/1535-7163.MCT-06-0084.

Tallarida, R.J., 2012. Revisiting the isobole and related quantitative methods for assessing drug synergism. J. Pharmacol. Exp. Therapeut. 342 (1), 2–8. https://doi.org/10.1124/jpet.112.193474.

Tang, J., Wennerberg, K., Aittokallio, T., 2015. What is synergy? The Saariselkï¿½ agreement revisited. Front. Pharmacol. 6 (SEP), 1–5. https://doi.org/10.3389/fphar.2015.00181.

Twarog, N.R., Stewart, E., Hammill, C.V., Shelat, A.A., 2016. BRAID: a unifying paradigm for the analysis of combined drug action. Sci. Rep. 6 (1), 25523. https://doi.org/10.1038/srep25523.

US FDA, 2013. Guidance for industry: codevelopment of two or more new investigational drugs for use in combination. Fda (1), 1–5.

Vakil, V., Trappe, W., 2019. Drug combinations: mathematical modeling and networking methods. Pharmaceutics 11 (5), 1–31. https://doi.org/10.3390/pharmaceutics11050208.

Vlot, A.H.C., Aniceto, N., Menden, M.P., Ulrich-Merzenich, G., Bender, A., 2019. Applying synergy metrics to combination screening data: agreements, disagreements and pitfalls. Drug Discov. Today 24 (12), 2286–2298. https://doi.org/10.1016/j.drudis.2019.09.002.

Webster, R.M., 2016. Combination therapies in oncology. Nat. Rev. Drug Discov. 15 (2), 81–82. https://doi.org/10.1038/nrd.2016.3.

Wientjes, M.G., 2010. Comparison of methods for evaluating drug-drug interaction. Front. Biosci. E2 (1), 86. https://doi.org/10.2741/e86.

Woodcock, J., Griffin, J.P., Behrman, R.E., 2011. Development of novel combination therapies. N. Engl. J. Med. 364 (11), 985–987. https://doi.org/10.1056/NEJMp1101548.

World Health Organization, 2005. Annex 5 Guidelines for registration of fixed-dose. WHO Tech. Rep. Ser. (929), 94–142.

Xu, Y., Zong, S., Gao, X., Zhang, H., Wang, B., Li, P., Liu, T., Li, S., 2019. Combined treatment of ABT199 and irinotecan suppresses KRAS-mutant lung cancer cells. October 2018 Gene 688, 1–6. https://doi.org/10.1016/j.gene.2018.11.018.

Yadav, B., Wennerberg, K., Aittokallio, T., Tang, J., 2015. Searching for drug synergy in complex dose–response landscapes using an interaction potency model. Comput. Struct. Biotechnol. J. 13, 504–513. https://doi.org/10.1016/j.csbj.2015.09.001.

Zhao, L., Wientjes, M.G., Au, J.L.S., 2004. Evaluation of combination chemotherapy. Clin. Cancer Res. 10 (23), 7994–8004. https://doi.org/10.1158/1078-0432.CCR-04-1087.

Zhao, W., Sachsenmeier, K., Zhang, L., Sult, E., Hollingsworth, R.E., Yang, H., 2014. A new Bliss independence model to analyze drug combination data. J. Biomol. Screen 19 (5), 817–821. https://doi.org/10.1177/1087057114521867.

Zheng, S., Wang, W., Aldahdooh, J., Malyutina, A., Shadbahr, T., Tanoli, Z., Pessia, A., Tang, J., 2022. SynergyFinder plus: toward better interpretation and annotation of drug combination screening datasets. Genomics. Proteomics Bioinformatics. https://doi.org/10.1016/j.gpb.2022.01.004.

Zimmer, A., Katzir, I., Dekel, E., Mayo, A.E., Alon, U., 2016. Prediction of multidimensional drug dose responses based on measurements of drug pairs. Proc. Natl. Acad. Sci. U. S. A 113 (37), 10442–10447. https://doi.org/10.1073/PNAS.1606301113.

Zimmermann, G.R., Lehár, J., Keith, C.T., 2007. Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug Discov. Today 12 (1–2), 34–42. https://doi.org/10.1016/j.drudis.2006.11.008.