



# Isobolographic analysis of interactions – a pre-clinical perspective

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## Abstract

**Introduction.** Isobolographic analysis is the preferred method of assessment of pharmacodynamic interactions occurring among drugs administered in mixture in both pre-clinical and clinical studies. Despite its mathematical complexity, rigorous preliminary conditions and various prerequisites to be met, it assesses the pharmacodynamic interactions, classifying them as additive, antagonistic, synergistic or indifferent in nature. These interactions are usually plotted in the Cartesian system of coordinates forming isobolograms. The strength (power) of interactions is calculated and presented as an interaction index.

**Conclusion.** This report provides basic information on the isobolographic analysis used experimentally in preclinical conditions indicating the underestimation of this valuable method in pharmacology and toxicology.

## Key words

antagonism, isobolographic analysis, synergy, additivity, pharmacodynamic interaction, indifference

## INTRODUCTION

Joint administration of two or more drugs is always associated with interactions among the taken drugs, which can positively or negatively affect the human organism. Positive effects evoked by drugs in mixture are linked with either potentiation of their therapeutic effects in the course of therapy, or reduction of their adverse effects. In the case of potentiation, low doses of the drugs can be as efficacious as high doses of one of the taken drugs applied in maximally-tolerated doses [1]. On the other hand, negative effects evoked by drugs in mixture are linked with either enhancement of their side-effects (adverse reactions), or changing metabolic parameters of the taken drugs that reduce their therapeutic properties [2]. At present, according to the European Medicines Agency (EMA) and US Food and Drug Administration (FDA), drugs administered in combinations must be safe and effective in their therapeutic action, which means that the drugs in combinations must exert benefits overcoming their unwanted and/or unexpected drug reactions [3].

## STATE OF KNOWLEDGE

There are three main groups of interactions occurring among drugs: 1) pharmaceutical interactions (also called pharmaceutical incompatibilities) occurring outside the organism, 2) pharmacokinetic interactions (*in vivo*) occurring at various levels inside the organism (according to the acronym: LADME – liberation, administration, distribution,

metabolism and elimination), and 3) pharmacodynamic interactions (*in vivo*) occurring at the sites of actions of the drugs and their targets. The aim of this study was to shed more light on methods of assessment of pharmacodynamic interactions occurring within the organism.

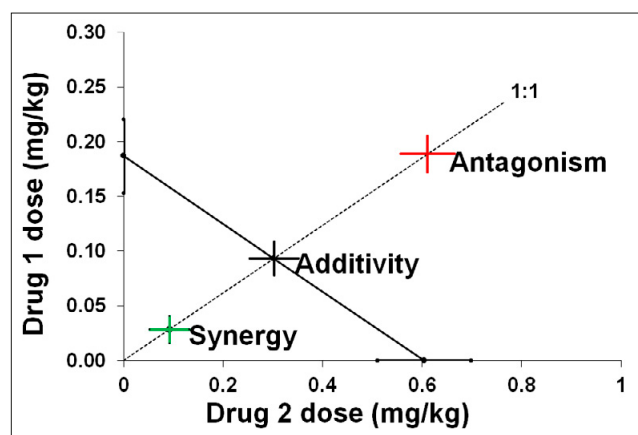
Isobolographic analysis (IA) is the preferred method in assessment of pharmacodynamic interactions between drugs administered together [9–14]. Theoretically, four main types of pharmacodynamic interactions can be distinguished with IA: synergy, antagonism, additivity and indifference [1, 15–17]. Synergy is defined as an effect exerted by a drug mixture which significantly exceeds the sum of effects produced by particular drugs used separately (Fig. 1). Antagonism is defined as an effect considerably lower than the sum of effects exerted by the particular drugs. Additivity is observed if drugs combined together produce an effect equals to the sum of effects produced by drugs present in the mixture (Fig. 1). Indifference is defined as the sum of effects produced by drugs, one of which produces no effect (zero-effect, i.e. a ‘pure’ placebo) [15] (Fig. 2).

The IA is a universal method in interaction analysis that can be applied in both clinical and preclinical studies, especially if the tested drugs exerted some measurable effects. At present, the IA is widely used for analysis of interactions between anticancer drugs [18–21], antiseizure medications [22–25], ototoxic drugs [26, 27], antibiotics, and antimicrobial drugs [28, 29], and when evaluating the antinociceptive activity of the drugs [30–35]. Quite recently, a large-scale and fully automatic IA has been incorporated into *in vitro* studies for assessing the interactions between anti-cancer drugs and various radiosensitizers to accelerate the discovery of anti-cancer drugs [36].

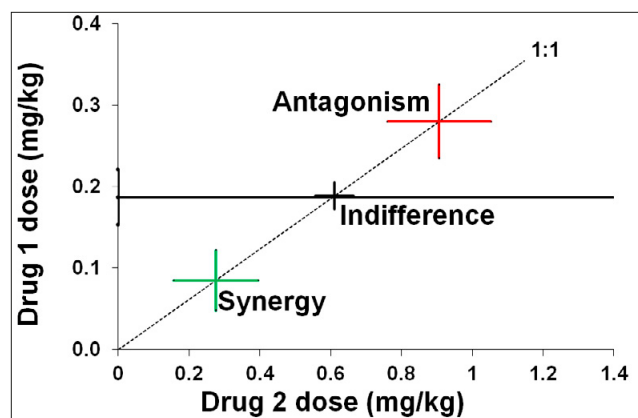
Despite a high index of universality of the IA in analytical studies on interactions, the application of IA is limited to only a few publications in experimental animal models and *in vitro*

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**Figure 1.** Isobologram illustrating various types of pharmacodynamic interactions (synergistic, additive and antagonistic) for two fully active drugs producing the clear-cut effects. The ED<sub>50</sub> values ( $\pm$  SEM as the errors bars) of the Drug 1 and Drug 2 are placed on the Y and X axes, respectively. The diagonal line connecting these ED<sub>50</sub> values on the X and Y axes reflects the line of additivity. The dashed line originating from the point (0;0) and crossing the line of additivity illustrates a fixed equi-effective proportion (1:1) of the tested drugs in mixture. Synergy (a green cross) is placed below the line of additivity, whereas antagonism (a red cross) is depicted above the line of additivity



**Figure 2.** Isobologram illustrating various types of pharmacodynamic interactions (synergistic, indifferent and antagonistic) for two drugs, one of which is ineffective. The ED<sub>50</sub> value ( $\pm$  SEM as the errors bars) of the active Drug 1 is placed on the Y axis. The parallel line to the X axis starting from the ED<sub>50</sub> value of the Drug 1 reflects the line of indifference. The dashed line originating from the point (0;0) and crossing the line of indifference illustrates a fixed equi-effective proportion (1:1) of the tested drugs in mixture. Synergy (a green cross) is placed below the line, whereas antagonism (a red cross) is depicted above the line of indifference

cell lines. Although several statistical softwares are available to characterize interactions between drugs, the IA has not been widely applied by researchers and scientists because of some methodological problems. One of the problems is the assessment of interactions between drugs, of which one is applied in low (sub-effective) doses and the other drugs are administered in fully effective doses. In such a case, application of the correct analytical methods is required. The drug administered in low doses usually produces 'no effect' or, more precisely, produces the effect which is undetectable by standard methods. During the combined therapy in humans, a drug producing 'no effect' is considered as 'ineffective', and is replaced with a more efficacious drug.

Previously in experimental studies, the therapeutic effects produced by a drug administered in sub-effective (ineffective) doses were neglected, and only the effects exerted by the other drugs in mixture were assessed. However, from a pharmacological viewpoint, any substance introduced to living organisms undergoes metabolic transformation

which affect the organs and tissues, changing absorption, distribution, metabolism and elimination of co-administered drugs. By neglecting the effect produced by a low-dose drug, scientists and researchers commit a common methodological error. In such cases, one ineffective drug can potentiate (even significantly statistically) the therapeutic effects of the other drugs used in mixture. Of note, this ineffective drug when introduced into the living organism is able to bind with specific target sites (receptors or other binding places on cellular and/or nuclear levels) in tissues and target organs. Generally, the ineffective drug can: 1) replace the other drugs from their binding sites in tissues or plasma proteins; 2) undergo metabolic transformation in the liver; 3) be eliminated via the digestive tract with faeces, or via the renal route in urine, affecting pharmacokinetic parameters of other drugs.

At present, to evaluate the interactions among drugs, even if one of the drugs in mixture is administered in 'ineffective' doses, the IA of interactions is used. Generally, two types of IA are designed to analyze interactions: type I – for drugs, which all are effective in the treatment (Fig. 1); type II – for drugs, of which one is ineffective (Fig. 2).

Additionally, one of the most important advantages of IA is the fast graphical presentation of results which, due to the isobolar presentation, allows for unambiguous interpretation of pharmacodynamic interactions occurring between drugs. The isobolographic illustration of interactions is usually based on median effective doses (ED<sub>50</sub>) of the tested drugs in *in vivo*, or median inhibitory concentrations (IC<sub>50</sub>) of the tested drugs in *in vitro* studies [4, 5]. The isobole is the simplest illustration of interactions. Due to the placement of interaction into the Cartesian plot system of coordinates, it is possible to differentiate the additive interaction from synergy or antagonism (Fig. 1). Briefly, synergistic interaction is expected if its graphical presentation is placed below the line of additivity, which reflects the summation of effects exerted by particular drugs present in the mixture. Antagonism is observed if the interaction is placed above the line of additivity. By definition, the antagonism reflects an interaction for which doses of particular drugs are higher than those predicted to be additive. Of note, the isobole is based on equi-effective doses or drugs' concentrations [6–9]. In such a case, the effect is unchanged during the time of experiments and usually is equal to a 50% of the observed effects. Although other effects can be determined isobolographically (i.e., ED<sub>16</sub>, ED<sub>25</sub>, ED<sub>84</sub>, ED<sub>97</sub>), the most popular is the ED<sub>50</sub> or IC<sub>50</sub> based on a 50% effect – usually equal to the median effect.

When analyzing the interactions between drugs, the most important prerequisite is the definition of proportions of the drugs used in mixture [9]. There exist two main descriptions of proportions of drugs in mixture. The first mode of description of drugs is based on their mass quantity which corresponds to particular doses of the drugs [4, 5]. In such cases, the notification of proportions of 1:1 means that 1 mg of the first drug is added to 1 mg of the second drug in mixture. In other words, this way of describing the proportions of drugs in mixture is based on the equivalent drug doses. This type of IA, based on doses of the studied drugs is a special way of analysis, under a principal prerequisite conditioning that the drugs must produce the same effects in similar doses, i.e., the ED<sub>50</sub> values of both drugs in mixture should be identical in their dose-range [37]. In contrast, the

most universal description of proportions of drugs in the mixture is also based on natural numbers, but the notion of 1:1 proportion is different, based on the measurable effects produced by the drugs [11, 17]. When the drugs produce the same effects, but in various dose-ranges (i.e., with diverse  $ED_{50}$  values), it is possible to describe the proportion of drugs in mixture of 1:1, as their effects corresponding to their half  $ED_{50}$  values. In such a case, the 1:1 proportion means that half  $ED_{50}$  value of the first drug combined with half  $ED_{50}$  value of the second drug should provide one  $ED_{50}$  value of either the first drug or the second drug [12, 13, 38]. This kind of description of drugs' proportions in mixture is the most popular in experimental studies because it does not take into consideration the particular drug doses, but their effects, produced by the investigated two-drug mixture. Research studies assessing types of interactions among drugs are conceived to experimentally detect effects produced by drugs, not doses of the drugs.

Additionally, to determine the strength of the observed interactions, it is recommended to calculate the interaction index, the values of which can univocally indicate the power (strength) of interactions [39]. The simple assessment that the examined interaction is synergistic or antagonistic in nature is not enough in contemporary experimental studies. Researchers and scientists are forced to determine the power of such interactions by calculating the interaction index. On the other hand, the IA involves a statistical test based on comparison of the experimentally-derived values with theoretically calculated and predicted to be additive values [6]. This rigorous statistical test allows for precise and adequate assessment of drug interactions, from which the p-value for each the studied interaction can be calculated. In the opinion of the authors of this study, the combination of both methods, i.e., the calculation of interaction index along with statistical analysis of the experimental and additive values, is the recommended method for analysing the interactions of drugs in experimental studies.

In experimental studies, a principal question arises: whether the IA is really needed. Despite its complexity and rigorous presumptions, the IA is underdetermined and not fully appreciated by researchers and scientists when evaluating interactions between drugs, or when a novel therapeutic regimen is compared to a firmly established standard therapy. Considering the above-mentioned facts, the IA is obviously needed for assessing the drug interactions in both preclinical and clinical studies.

Experimental evidence indicates that due to the IA, some drugs cannot be combined together because of their negative therapeutic (unfavourable) effects (i.e., the drugs produce antagonistic interactions in preclinical studies). In the case of antagonistic interactions, more doses of drugs are required to obtain the same therapeutic effects measured and analyzed with IA. In contrast, in toxicological studies, the potentiation of toxic effects exerted by drugs in target organs and tissues can be either beneficial (anti-proliferative effects, antimicrobial activity) or unfavourable (i.e., if potentiation of the toxic effects, side-effects of drugs, occurs when the drugs are administered jointly).

## CONCLUSION

The main advantages in applying the IA during experimental studies are, among others, precise evaluation of the studied effects exerted by a drug mixture, modification of a drug content when used in combination, and determination of the strength of interaction by calculating the interaction index. Since the IA was successfully applied in experimental pharmacology and toxicology when assessing the interactions between antiepileptic medications and ototoxic drugs in various experimental models in mice, its applicability to other experimental studies has been confirmed.

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