ORIGINAL ARTICLE

Journal of Pre-Clinical and Clinical Research 2025, Vol 19, No 3, 91-95
www.jpccr.eu

Two isobolographic approaches in the evaluation of drug-drug interaction for non-parallel concentration-response curves in an *in vitro* study

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Łuszczki JJ, Błaszkiewicz-Tyniec A, Gałęziowska E, Kowalik A, Krajewska I. Two isobolographic approaches in evaluation of drug-drug interaction for non-parallel concentration-response curves in an *in vitro* study. J Pre-Clin Clin Res. 2025;19(3):91–95. doi:10.26444/jpccr/210907

Abstract

Introduction and Objective. In *in vitro* cell line studies, two isobolographic analysis approaches are commonly used when assessing interactions between drugs. Both – the log-probit accompanied with Tallarida method and Chou-Talalay-Martin method – can distinguish pharmacodynamic types of interactions occurring between drugs.

Materials and Method. The aim of the study is to assess the interaction between LY-2183240 and mitoxantrone in the malignant melanoma SK-MEL28 cell line in the MTT assay, by means of two methods – isobolographic (log-probit) accompanied by the Tallarida and Chou-Talalay-Martin method.

Results. Log-probit accompanied by the Tallarida method revealed that LY-2183240 and mitoxantrone had non-parallel concentration-response curves, and produced an additive interaction in the SK-MEL28 cell line in the MTT assay. In contrast, the Chou-Talalay-Martin method showed that the combination of LY-2183240 and mitoxantrone exerted a synergistic interaction with a combination index value of 0.354.

Conclusions. The observed discrepancy in the types of interactions between LY-2183240 and mitoxantrone in both methods resulted from the lack of assessment of mutual parallelism of the drugs concentrations-response curves in the Chou-Talalay-Martin method. The utmost caution is advised when utilizing the Chou-Talalay-Martin method in the detection of interactions in *in vitro* studies, especially for the combinations of drugs with non-parallel concentration-response curves.

Key words

isobolographic analysis, CompuSyn, log-probit, Chou-Talalay-Martin method, Tallarida method

INTRODUCTION

Overwhelming evidence indicates that cancer therapies usually need the combination of two or more chemotherapeutic drugs in order to efficiently cure cancer patients [1]. However, to combine the proper chemotherapeutic drugs together, providing the most effective cancer treatment, some preclinical studies involving cell lines and animal experimentations are very helpful by allowing the choice of the most efficacious drug combinations which offer not only suppression of the tumour growth, but also the inhibition of its propagation, limitation of neighbouring tissue infiltration, and metastasis [2–4].

Evaluation of interactions in preclinical conditions seems to be the optimal option allowing adjustment of specific fixed-ratio combinations of the drugs, whose anti-cancer (anti-proliferative) effects could be optimal in preclinical conditions [5]. Of note, the adjusted proportions of the anticancer drugs in mixtures (elaborated in *in vitro* studies) could be easily translated to clinical conditions [6].

In *in vitro* experiments, one can also test the nature of interactions between chemotherapeutic drugs using two models of the isobolographic analysis: the Chou-Talalay-Martin method based on mass-action law calculated by CompuSyn software [7], and the log-probit method accompanied by the Tallarida method [8]. Of note, the CompuSyn software can automatically perform analysis and calculate isobolographic parameters, including the combination index value [9]. The log-probit and Tallarida method is based on the evaluation of the parallelism of two drugs, concentration-response relationship curves, and further statistical evaluation of interactions. Confirmation of mutual parallelism is crucial because two variants of isobolography exist in the Tallarida method: for parallel and for non-parallel concentration-response relationship curves [10].

Relatively recently it has been reported that LY-2183240 when combined with mitoxantrone (a chemotherapeutic

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Received: 27.08.2025; accepted: 15.09.2025; publikacja online: 25.09.2025

drug) produced the additive interaction in the malignant melanoma SK-MEL28 cell line in the MTT test [11]. As regards LY-2183240, the drug is a putative endocannabinoid transport blocker and potent inhibitor of the re-uptake of anandamide (an endogenous cannabinoid), also acting as an inhibitor of fatty acid amide hydrolase (FAAH) and several brain serine hydrolases [12]. At present, the role of cannabinoids and endocannabinoids in anti-cancer therapy is undergoing thorough and intensive investigations [4, 13].

Although the results for the combination of LY-2183240 with mitoxantrone in the malignant melanoma SK-MEL28 cell line has been published earlier [11], the current study attempts to re-analyze them again with two isobolographic approaches, with the aim of making a direct comparison between the two isobolographic methods, especially with respect to the lack of parallelism of the drugs' concentration-response curves.

OBJECTIVE

The aim of the study is to evaluate the anti-proliferative effects of LY-2183240 in combination with mitoxantrone in the malignant melanoma SK-MEL28 cell line in the MTT assay, using both the Chou-Talalay-Martin and log-probit accompanied with Tallarida methods. The focus of the study is on a direct comparison of both methods to check whether they can provide similar results and draw the same conclusions in *in vitro* studies. Any differences found in the results would be crucial for further recommendations on the use of these methods during the evaluation of interactions for novel anti-cancer therapies to extrapolate the obtained results to clinical conditions.

MATERIALS AND METHOD

Log-probit and the Tallarida method. Concentrations of LY-2183240, mitoxantrone and their mixture in combination at the fixed ratio of 1:1 (in μM) were transformed to logarithms to the base of 10, whereas the anti-viability effects in the malignant melanoma SK-MEL28 cell line in the MTT assay (in %) were transformed to probits, according to Litchfield and Wilcoxon [14]. From the log-probit method, the median inhibitory concentration (IC_{50}) values for the drugs used alone (LY-2183240, mitoxantrone) and for their combination at the fixed-ratio of 1:1, were automatically calculated by means of the Microsoft Excel spreadsheet with equations elaborated by Tallarida [8]. Of note, in *in vitro* studies, the drugs can be tested in various proportions of concentrations, but typically, the fixed-ratio combination of 1:1 is recommended. The notification of 1:1 indicates that proportions of two drugs is constant and equal. In other words, the concentrations of drugs increase proportionally along with their anti-proliferative effects observed in a specific cell line, but the starting concentrations correspond to a half of the IC_{50} values of the drugs, as determined for the drugs used alone in this specific cell line [8]. The parallelism of concentration-response curves for LY-2183240 and mitoxantrone was assessed with the log-probit method. To verify the nature of interaction between LY-2183240 with mitoxantrone, the experimentally derived $IC_{50\ mix}$ value was statistically compared with its corresponding theoretically calculated and predicted to be additive $IC_{50\ add}$ value with unpaired Student's t-test by means of the GraphPad Prism (version 8.0). The $p<0.05$ was accepted as statistical significance.

Chou-Talalay-Martin method. Concentrations of LY-2183240, mitoxantrone and their mixture at the fixed ratio of 1:1 (in μM) along with their anti-viability effects in the malignant melanoma SK-MEL28 cell line in the MTT assay (in %), were inserted into the CompuSyn software [9]. All calculations and graphical illustrations of the results were performed automatically by means of CompuSyn software ver. 1.0.

RESULTS

Log-probit accompanied by the Tallarida method. LY-2183240, in a concentration-dependent manner, produced anti-viability effects in the SK-MEL28 cell line in the MTT assay and the experimentally-derived median inhibitory concentration (IC_{50}) for this drug was 1.653 μM (Fig. 1). Similarly, mitoxantrone exerted a clear-cut anti-viability effect in the SK-MEL28 cell line in the MTT assay, and the experimentally-derived IC_{50} value for mitoxantrone amounted to 1.743 μM (Fig. 1). Since the concentration-response curves of LY-2183240 and mitoxantrone (when used alone) crossed each other, the test of parallelism revealed that both curves were not parallel (Fig. 1). The two-drug mixture of LY-2183240 and mitoxantrone, when administered at the fixed-ratio combination of 1:1, produced the definite anti-viability effects in the melanoma malignant SK-MEL28 cell line in the MTT assay; the experimentally-determined $IC_{50\ mix}$ value was 0.602 μM (Fig. 1).

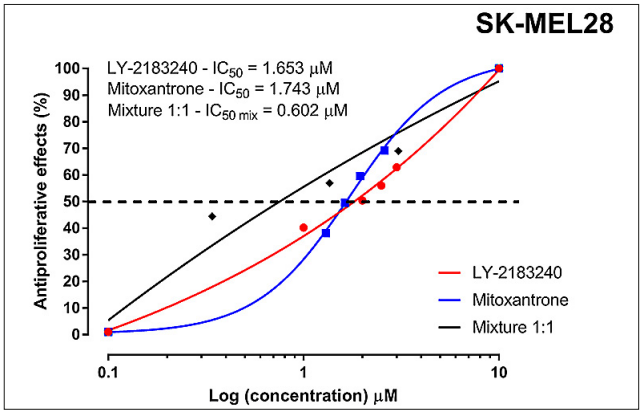

Figure 1. Sigmoidal concentration-response curves for LY-2183240 and mitoxantrone when used alone and their mixture in combination at the fixed-ratio of 1:1, with respect to their anti-proliferative effects in the malignant melanoma cell line SK-MEL28 in the MTT assay

The isobolographic analysis of interaction revealed that the experimentally-derived $IC_{50\ mix}$ value amounted to 0.602 ± 0.355 μM, and did not differ significantly (with unpaired Student's t-test) from the theoretically calculated and predicted to be additive $IC_{50\ add}$ value, which was 1.067 ± 0.569 μM (Fig. 2). Thus, the observed interaction between LY-2183240 and mitoxantrone was additive.

Chou-Talalay-Martin method. Concentrations of LY-2183240, mitoxantrone and their mixture in combination

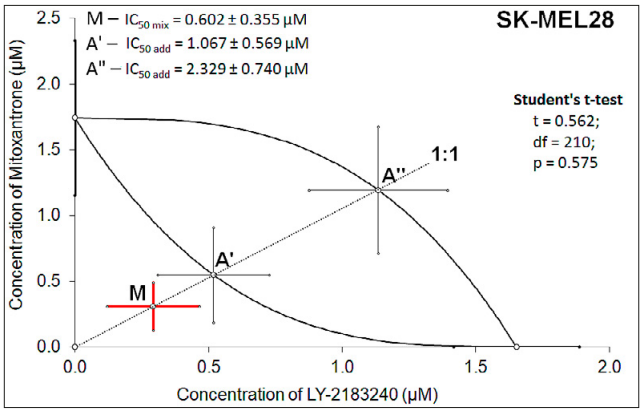

Figure 2. Isobologram illustrating additive interaction of LY-2183240 and mitoxantrone (at the fixed-ratio of 1:1) with respect to the anti-proliferative effects in the malignant melanoma SK-MEL28 cell line in the MTT assay.
The IC_{50} values for LY-2183240 and mitoxantrone, when used alone, are placed on the abscissa and ordinate, respectively. The lower and upper curves connecting these IC_{50} values of LY-2183240 and mitoxantrone are the lines of additivity limiting the area of additivity. The dotted line starting from the beginning of the Cartesian plot system and crossing the lower and upper lines of additivity, reflects the fixed drug concentration ratio combination (1:1). The points placed on the dotted line (in the shape of the crosses, as their SEM values) indicate either the experimentally-derived $IC_{50\ mix}$ value for the combination of LY-2183240 and mitoxantrone in the MTT assay (point M), or the theoretically calculated and predicted to be additive $IC_{50\ add}$ values (points A' and A''). Statistical analysis of data was performed with the Student's t-test

at the fixed ratio of 1:1, along with their anti-viability effects in the malignant melanoma SK-MEL28 cell line in the MTT assay, were inserted into the CompuSyn software (version 1.0). This software performed automatic computer-assisted calculations and graphical illustration of the concentration-response curves for LY-2183240, mitoxantrone and their mixture (Fig. 3).

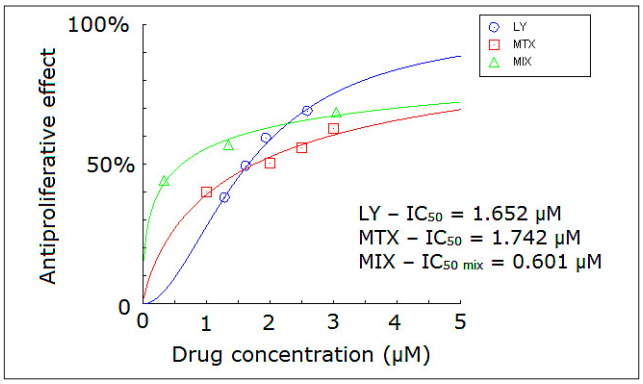

Figure 3. Sigmoidal concentration-response relationship curves for LY-2183240, mitoxantrone and their mixture.
LY – LY-2183240; MTX – mitoxantrone; MIX – mixture of both drugs at a fixed proportion
Source: automatically drawn by CompuSyn

Concentrations of both drugs (LY-2183240 and mitoxantrone) in the mixture along with their corresponding anti-viability effects in the malignant melanoma SK-MEL28 cell line in the MTT assay, allowed the automatic drawing of an isobologram illustrating the synergistic interaction between LY-2183240 and mitoxantrone with CompuSyn software (Fig. 4).

The point M reflects the experimentally derived $IC_{50\ mix}$ value for the combination of LY-2183240 and mitoxantrone in the MTT assay.

The synergistic interaction between LY-2183240 and

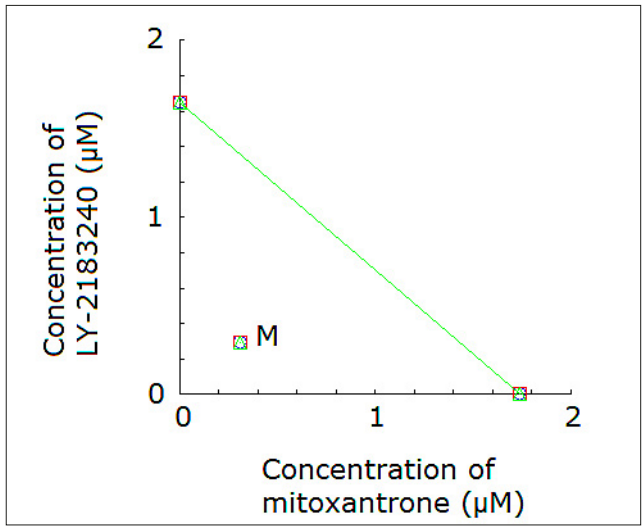

Figure 4. Isobologram illustrating synergistic interaction of LY-2183240 and mitoxantrone (at the fixed-ratio of 1:1) with respect to the anti-proliferative effects in the malignant melanoma SK-MEL28 cell line in the MTT assay
Source: automatically drawn by CompuSyn

mitoxantrone in the malignant melanoma SK-MEL28 cell line in the MTT assay was confirmed by calculating the combination index (CI), whose value amounted to 0.354 (Fig. 5).

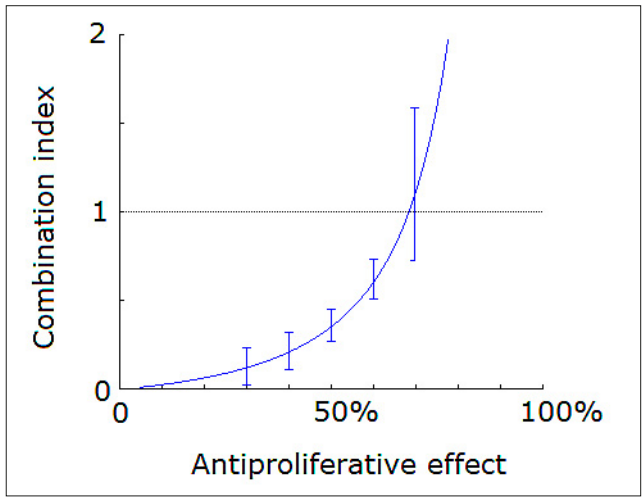

Figure 5. Combination index diagram illustrating the interactions between LY-2183240 and mitoxantrone depending on the anti-viability effects in the MTT assay.
Source: automatically drawn by CompuSyn

The straight line parallel to the abscissa depicts the line of additivity. Since the combination index value for 50% was 0.354, the observed interaction between LY-2183240 and mitoxantrone was synergistic.

To graphically visualize the observed interaction between LY-2183240 and mitoxantrone, a polygonogram was automatically created by CompuSyn (Fig. 6). On the graph, the strong synergy is marked as a thick green line connecting LY-2183240 and mitoxantrone (Fig. 6).

DISCUSSION

The presented results indicate that the additive interaction reported in the log-probit accompanied with the Tallarida

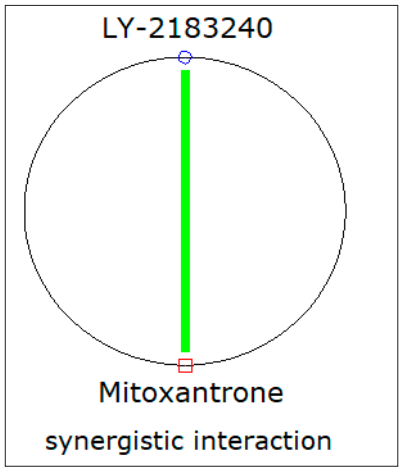

Figure 6. Polygonogram reporting synergistic interaction between LY-2183240 and mitoxantrone. *Source:* automatically drawn by CompuSyn

method is classified as a synergistic by CompuSyn software, according to the Chou-Talalay-Martin method. The observed discrepancy results from the lack of assessment of parallelism by the CompuSyn. In contrast, the log-probit methods confirmed that both, LY-2183240 and mitoxantrone have their concentration-response curves non-parallel to each other; therefore, the interaction was analyzed with the isobolographic analysis for non-parallel concentration-response curves. As illustrated in Figure 1, the concentrations-response curves for LY-2183240 and mitoxantrone crossed one another, confirming that both curves are not collateral. In such a situation, instead of one theoretically calculated and predicted to be additive ($IC_{50\,add}$) value, two lower and upper $IC_{50\,add}$ values were calculated following the Tallarida equations [15], bearing in mind the fact that the Chou-Talalay-Martin method does not assume parallelism, and the CompuSyn automatically preformed analysis without correcting the additivity lines. This is the main reason that the interaction between LY-2183240 and mitoxantrone was classified as synergistic according to the Chou-Talalay-Martin method.

Relatively recently the Chou-Talaly-Martin method has been applied in five various pancreatic tumour cell lines (MIA Paca-2, BxPC-3, Capan-1, AsPC-1, and Beta-TC-3) to detect synergistic, additive and antagonistic interactions among six focal adhesion kinase inhibitors (defactinib, CEP-37440, VS-4718, VS-6062, ifebemtinib and GSK2256098) in combination with the oncolytic coxsackievirus B3 strain PD-H [16]. Similarly, temozolomide and dacarbazine (two chemotherapeutic drugs) when combined with WT161 (a selective histone deacetylase 6 inhibitor) produced synergistic interactions in three various malignant melanoma CHL-1, SK-MEL-147 and WM1366 cell lines [2].

The classification of interactions according to the Tallarida method has also been performed recently for bromelain and N-acetylcysteine when combined with gemcitabine, 5-fluorouracyl and doxorubicin (three chemotherapeutic drugs) in various pancreatic and hepatic cancer cell lines [17]. Moreover, cisplatin when combined with AM1172 (a hydrolysis-resistant endocannabinoid analog inhibiting anandamide cellular uptake) exerted either a synergistic interaction in the neuroblastoma CHP-134 cell line, or an antagonistic interaction in the glioblastoma C6 cell line [18]. Similarly, cisplatin when combined with cannabidiol (a negative allosteric modulator of the cannabinoid CB1 receptor) produced either an antagonistic interaction in the glioblastoma T98G cell line, or additive interaction in the neuroblastoma (CHP-134 and KELLY) and glioblastoma (U-87MG and C6) cell lines [18].

Of note, in the Chou-Talalay-Martin method, classification of interactions is based on the calculated combination index values. If the combination index values are lower than one, the observed interactions are synergistic. In contrast, the combination index values higher than one indicate the antagonistic interactions. When the combination index value is equal to one, the observed interaction is classified as additive [7]. Since the combination index value in the Chou-Talalay-Martin method amounted to 0.354, the interaction between LY-2183240 and mitoxantrone was classified as synergistic. However, statistical analysis of data performed according to the Tallarida method for the combination of LY-2183240 with mitoxantrone, revealed that the observed interaction is additive.

Nevertheless, the observed differences in the same results analyzed independently by two isobolographic approaches, speak against application of the Chou-Talalay-Martin method only, especially when evaluating the real nature of the interactions in *in vitro* studies. The Chou-Talalay-Martin method may classify some combinations as those producing synergistic interactions, whereas the exact nature of interaction is additive, depending on the non-parallelism of two concentration-response curves. As reported herein, the parallelism should evidently be evaluated in *in vitro* study to correctly perform the isobolographic analysis of interaction.

On the other hand, the Chou-Talalay-Martin method can be readily used as a first screen test to choose and select the best (synergistic) interactions for the further testing of drug-drug combinations. Although both isobolographic approaches are used in experimental conditions, the superiority of the log-probit accompanied with Tallarida method over the Chou-Talalay-Martin method has been confirmed in this study. Undoubtedly, the main recommendation for future studies is to confirm parallelism of concentration-response curves. Besides, a comparative study reporting evaluation of interaction between lamotrigine and clonazepam in the mouse model of tonic-clonic seizures by means of both isobolographic methods (the Chou-Talalay-Martin and log-probit accompanied with Tallarida) has recently been published [19]. Confirmation of the parallelism of dose-response relationship curves for the drugs tested separately is crucial from a methodological viewpoint.

Limitations of the study. The main limitations are related to the experimental *in vitro* conditions and application of the drugs in a fixed drug concentration ratio of 1:1, in which both drugs exerted their equi-effective anti-proliferative activities in the MTT assay. Additionally, all the drugs were administered in the same time and no pre-treatments were available. It is highly likely that any pre-treatment could substantially modify the final anti-proliferative effects in the MTT assay. Besides, the combination of LY-2183240 and mitoxantrone was selected to treat the malignant melanoma SK-MEL28 cell line. Obviously, the same two-drug combination (i.e., LY-2183240 and mitoxantrone) would produce different types of interactions in various malignant

melanoma cell lines, especially if the concentration-response curves might (or might not) be parallel to one another.

CONCLUSIONS

Although the CompuSyn software makes the automatic calculations and is easy-going in inserting research data, a prior verification of the parallelism of concentration-response curves of the tested drugs should become a standard procedure before undertaking the isobolographic analysis and the isobologram drawn.

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