Journal of Clinical and Basic Cardiology

An Independent International Scientific Journal

Journal of Clinical and Basic Cardiology 1999; 2 (1), 96-98

Dose-response curves of vasorelaxants: computerized calculation of combination effects using the example of nicorandil

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Indexed in Chemical Abstracts EMBASE/Excerpta Medica

Dose-response curves of vasorelaxants: computerized calculation of combination effects using the example of nicorandil

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Aim of this study was to develop a convenient calculation of dose-response curves (DRCs) including those of theoretical model effects of combinations, using a commercial computer program which enables a clear visualization of confidence interval statistics and high quality graphs. The usefulness of this approach is exemplified by reevaluation of data from interaction experiments with nicorandil.

Combination experiments were performed on bovine coronary artery strips in classical organ bath design. For the computerized calculation of DRCs, a "program package" was developed for SigmaPlot, consisting of user-defined worksheet/graph and program files (curve fits and transforms). Visualization of statistical deviations of experimental from dose-additive = competitive interaction was achieved by showing bars of 95 % confidence intervals (CIs) and DRCs with CIs of dose-additive combinations. Non-overlapping CI-ranges were considered significantly different. Dual relaxant effects of nicorandil could be split into single components of action by using combination experiments.

The comparison of experimental with theoretical model effects of combinations allows conclusions with respect to the molecular sites of drug action and gives an indication as to the mechanisms of vasorelaxant actions. *J Clin Basic Cardiol 1999; 2: 96–8.*

Key words: Dose-response curves, computerized calculation, combinations, dose-additivity, confidence intervals, nicorandil

Dose-response curves (DRCs) best characterize the quantitative effects of drugs, including vasorelaxant effects. It has been shown recently that combination effects can also be expressed by DRCs and be compared with calculated model effect DRCs, from which hints to the sites and mechanisms of drugs can be obtained. The calculation of experimental as well as theoretical DRCs together with publication-ready graphs is now possible using powerful commercial software, of which SigmaPlot was chosen recently [1] for the generation of user-defined worksheets with graphical properties and user-defined program files. We here report on an extension of such DRCs calculation for immediate statistical comparison of observed vs. "expected" effects using the 95 % confidence interval (CI). This report deals with application of this method for delineating the two components of the vasorelaxant action of nicorandil. For the latter purpose, previously published data of interaction experiments [2] were reevaluated.

Due to its nitrate component [3], nicorandil activates guanylyl cyclase and raises cGMP levels and due to its nicotinamide part of the molecule it hyperpolarizes the cell membrane by opening cromakalim-sensitive potassium channels [3]. Separation of these two components relies on the availability of specific blockers, such as methylene blue and glibenclamide. In order to identify the mechanism of action of the respective component, combination experiments proved very useful, in as much as the effects of nicorandil added to a nitrate compound on the one hand or to a cromakalim-like compound on the other hand could be compared with calculated effects of dose-additive combinations, representing competitive synergism. The latter is expected to occur whenever drugs share the same molecular site of action. In order to statistically investigated deviations from dose-additivity, we calculated 95 % confidence intervals (CIs) both of experimental and theoretical effects (DRCs).

Methods

Isolated coronary artery strips

We have described the preparation of bovine coronary artery strips and the *in vitro* procedure of interaction studies, ie, testing vasorelaxant agents in combination previously [2], and this procedure was used with some modifications. Briefly, arteries were cut into Z-formed strips which were suspended in Tyrode solution, gased with 95 % oxygen and 5 % CO_2 at 37.0 °C. Strips were precontracted before addition of relaxing agents by the stable thromboxane A2 analog U 46619 [4]. Changes in length were measured isotonically and calculated as % of maximum relaxation obtained at the end of each experiment by addition of 266 μmol/L papaverine as described earlier. Varying concentrations of one agent were tested alone and in the presence of fixed concentrations of nicorandil, allowing DRCs to be constructed.

Construction of DRCs using SigmaPlot

User-defined worksheets were prepared first. Concentrations of drugs and mean values of relaxation, expressed as percent of maximum response, were listed in predetermined x-/y-columns of the user-defined worksheet. 95 % CI-values of percent-relaxation values, obtained from a t-distribution table at a given degree of freedom (by multiplying SEM with the listed table value) were also listed in the worksheet. Parameter values of DRCs were then calculated by fitting sigmoid DRCs to these column values, using the four parameter logistic function [5] $Y = ((a–d)/(1+(x/c)\ b))+d$ with the four parameters: E_{min} = a, slope = b, ED_{50} = c, E_{max} = d. These parameter values were obtained by applying user-defined curve fits and were kept in predetermined columns, from which plots of DRCs were generated by user-defined transforms. Besides the mean experimental DRCs of nitroprusside-Na and cromakalim, curves of upper and lower CIs were also calculated by user-defined curve fits, from which

Received December 19th, 1997; accepted July 14th, 1998.
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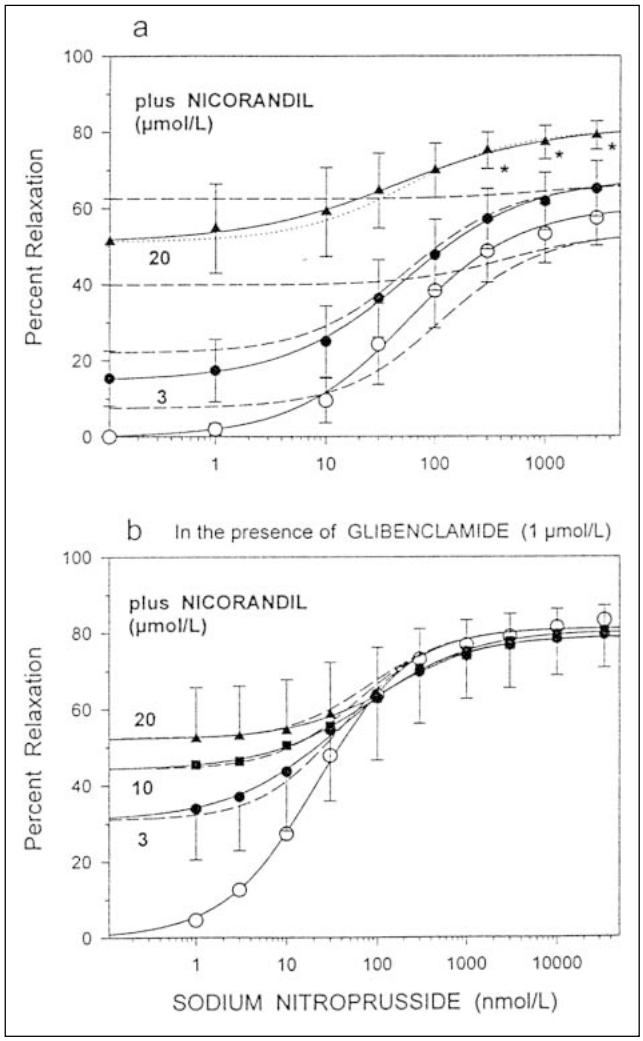

Figure 1. Cumulative dose-response curves (DRCs) of nitroprusside in the absence (open symbol) and presence of 3–20 µmol/L nicorandil (filled symbols), a) in the absence, b) in the presence of 1 µmol/L glibenclamide. The symbols and bars represent experimental mean values from 12 experiments and 95 % CI. Experimental DRCs are shown by solid lines, theoretical DRCs by dashed lines (dose-additivity) and dotted lines (independence). For graphical clarity, the following theoretical curves are shown in a) bands of 95 % CI with upper and lower boundary of dose-additive combinations of nitroprusside in the presence of 3 and 20 µmol/L nicorandil, curve of mean independent effect of nitroprusside in the presence of 20 µmol/L nicorandil, b) means of dose-additive combinations of nitroprusside with 3, 10, and 20 µmol/L nicorandil. The effects of nicorandil alone are shown on the y-axis. Significant deviation from dose-additivity is indicated by asterics.

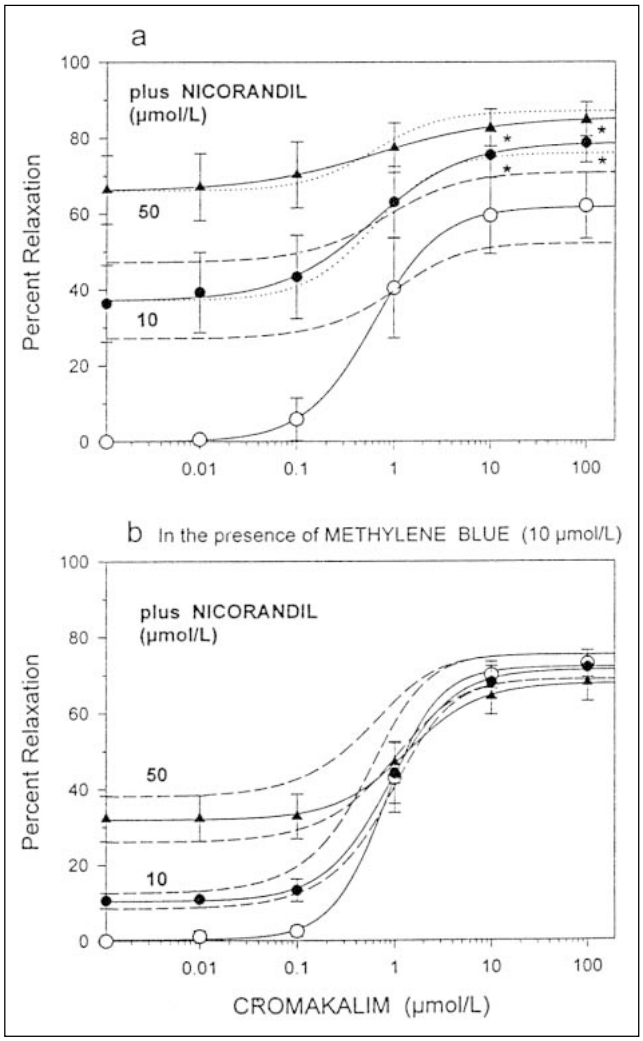

Figure 2. Cumulative dose-response curves (DRCs) of cromakalim in the absence (open symbol) and presence of 10 and 50 µmol/L nicorandil (filled symbols), a) in the absence, b) in the presence of 10 µmol/L methylene blue analogous to Figure 1. The symbols and bars represent experimental mean values from 6–12 experiments and 95 % CI. The following theoretical curves are shown in a) bands of 95 % CI of dose-additive effects of cromakalim in the presence of 10 µmol/L nicorandil, means of independent effects of cromakalim in the presence of 10 and 50 µmol/L nicorandil, b) 95 % CI bands of dose-additive combinations of cromakalim in the presence of 10 and 50 µmol/L nicorandil.

the means as well as bands of the 95 % confidence intervals of the theoretical curves were also calculated. Curves of dose-additive combinations were obtained by calculating dose x of A (nitroprusside or cromakalim) with which a fixed dose of B (nicorandil) applied was equieffective, exemplified for curve 2: x = {(((a-d)/(a2-d))-1)*(c ^ b)} ^ 1/b where a-d are the parameter values of the "control curve" of A, a2 = E_{min} of curve 2. According to the definition of dose-additivity, the effects of curve A alone are to be expected at the doses of A minus x. Hence, y-values of the simulated DRC of A correspond to simulated doses of A (xSIM)-x.

Curves of independent combinations were calculated on the basis of the effects E of A and B alone: $E_{A+B} = E_A + A_B-(E_A*E_B)$ where the effects E were expressed by the fraction of maximum response. Graphs show the DRCs of nitroprusside-Na (Fig. 1) or cromakalim (Fig. 2), alone and in the presence of fixed concentrations of nicorandil. CIs of experimental data points are shown by bars, those of dose-additive curves by curves of upper and lower CI. Alternatively, mean theoretical curves are shown, eg, for independent effects in Fig. 1a, and for dose additive combinations in Fig. 1b. The "program package" used, consisting of user-defined worksheet, curve fits and transforms to be used with SigmaPlot, may be obtained from the authors. It was used with SigmaPlot for Windows, version 2.0 (Jandel) but can also be used with more recent versions of SigmaPlot.

Statistical evaluation

Statistical comparison of observed vs. "theoretical" effects was done by using 95 % confidence intervals (CIs), constructed as described above. Non overlapping of experimental CI-bars with CI-ranges of theoretical CI-DRCs were considered as a significant deviation from the respective model effect.

Results

Dual action of nicorandil

As mentioned above, nicorandil's relaxant action consists of a nitroprusside- and a cromakalim-like component. Therefore, it is not surprising that the effect of nitroprusside in the presence of nicorandil significantly deviated from a simple dose-additive behavior (Fig. 1a). Likewise, the effects of cromakalim plus nicorandil were also significantly different from a calculated dose-additive combination (Fig. 2a). Significance was only seen at higher concentrations of nitroprusside and cromakalim, respectively, although differences between experimental combined effects and dose-additivity were noticed also at lower concentrations of these agents (Fig. 1a and Fig. 2a). Note that the effect of 50 μmol/L nicorandil alone exceeded the maximum of the cromakalim DRC in Fig. 2a. Hence, a dose-additive effect could not be calculated. However, a competitive interaction DRC would be a falling curve, starting at 67 % and finally reaching the maximum of the cromakalim curve (60 %). It is therefore clear that at least the highest concentrations of cromakalim in the presence of 50 μmol/L nicorandil must have exceeded the response expected for a competitive interaction. In contrast to dose-additivity and competitive interaction, respectively, the combined experimental effects rather closely follow the DRCs for independent effects which require different molecular sites of action.

Nitroprusside-like action of nicorandil

Under conditions in which the cromakalim-like action of nicorandil was effectively blocked by glibenclamide (1 μmol/L), the combination experiments revealed a single, nitroprusside-like action of nicorandil. This finding is evident from the DRCs in Fig. 1b, in which nicorandil behaved like a concentration of nitroprusside. The combined effects of nitroprusside and nicorandil closely followed the calculated effects for dose-additive combinations with no significant deviations. This fact is already evident from experimental effect bars crossing the mean DRCs for dose-additivity.

Cromakalim-like action of nicorandil

When the nitroprusside-like component of nicorandil was inhibited by methylene blue (10 μmol/L), the combined effects of cromakalim and nicorandil appeared dose-additive (Fig. 2b), although not as closely as observed with nitroprusside plus nicorandil in the presence of glibenclamide (Fig. 1b). Here, agreement with dose-additivity is in line with the reduction from a dual to a single action of nicorandil, which can be characterized as a cromakalim-like action.

Discussion

In this paper, we went one step further by including bars of experimental CI and theoretical DRCs of upper and lower boundaries of CI. As is evident from Fig. 1 and 2, this extension allows a kind of immediate statistical evaluation by looking where these bars are outside the CI-boundaries of "expected" effects.

The use of model combination effects for the delineation of sites and mechanisms of vasorelaxant agents requires some understanding of the underlying bases of these models. The dose-additivity model describes the combination effects of agents which share the same molecular site of action. The latter may be a classical receptor or a binding site on another macromolecule, eg, an enzyme or ion channel. Agents which compete for the same binding site are then expected to exhibit a dose-additive combination, which can be seen as a special case of a competitive interaction, termed competitive synergism by Ariens et al. [6]. Applying this model for the evaluation of vasorelaxants means that two agents acting at the same molecular site of action should behave according to the model of dose-additivity, as was evident in Fig. 1b and Fig. 2b.

On the other hand, any significant deviation from this model suggests either different sites of action of the compounds tested in combination or a dual action of one component or both agents. This phenomenon is evident from Fig. 1a and 2a. An interaction of a dual action compound with another agent possessing one of the two components is likely to result in a combination effect which is different from a pure competitive action. If nicorandil's relaxing effect is due to two different actions, one could expect that blocking of one of its two components would result in simple dose-additive combination effects. Indeed this was the case (Fig. 1b and Fig. 2b).

Similar results as shown in Fig. 1 were also obtained with SIN-1 instead of nitroprusside. Likewise, substituting pinacidil for cromakalim results in similar effects to those shown in Fig. 2. It is especially interesting that the combination effects of these compounds with nicorandil also appeared independent in the absence of glibenclamide and methylene blue, respectively.

The meaning of the independence model is quite different. This model is based on the assumption that two chemical agents do not share a common molecular binding site and further assumes that each of the compounds independently contributes to the total effect. Hence, the model assumes independence in action with respect to the molecular site and the subsequent reactions [3, 7]. There are, of course, other ways by which agents with different sites of action may interact, eg, by a special type of interaction resulting in a combination effect larger than the calculated independent effect. A typical example for this type of combination effect is the relaxant action of isoprenaline in the presence of phosphodiesterase (PDE)-inhibitors like theophylline or papaverine [8].

The results of Fig. 1a and Fig. 2a practically exclude such a "special" type of interaction with nicorandil. The effect of nitroprusside plus nicorandil as well as the effect of cromakalim plus nicorandil lend weight to the assumption underlying an independent combination, independent binding sites and independent mechanisms resulting in smooth muscle relaxation. In other words, the two components of nicorandil's action appear to result in an independent overall relaxing effect.

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