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The *ex vivo* left and right atria, as particularly appropriate experimental systems for the general investigation of the receptor function

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It is a capital mistake to theorize before one has data. Insensibly one begins to twist facts to suit theories, instead of theories to suit facts.

Sir Arthur Conan Doyle

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1 Introduction and objectives

1.1 Epidemiological background

The proper function of the cardiovascular system is essential for the good quality of life. In the absence of this, the chance of developing high blood pressure, with all its consequences (e.g. stroke and heart attack), increases. In the low-income countries, infectious diseases are still responsible for the most deaths, while in middle- and high-income countries, cardiovascular diseases are the most common cause of death. The group of cardiovascular diseases, including ischemic heart disease, has been the biggest problem in Hungary.

The life expectancy and quality of life for the affected patients can be improved by a more health-conscious lifestyle, so a high standard of living does not necessarily mean a high cardiovascular risk. For patients with a more severe status of the disease, pharmacotherapy is also available. From this point of view, the synthesis of new compounds that target diseases affecting the cardiovascular system is particularly important, and it may also be useful to explore the potential cardiovascular effects of non-cardiovascular drugs that are already on the market. Adenosine is a molecule of particular importance in terms of cardiovascular diseases that can activate inherent protective mechanisms in the body. Adenosine is often used as an initial structure in the development of new pharmaceuticals, which may be suitable for the treatment of certain cardiovascular diseases.

1.2 Adenosine and the adenosinergic transmission

Adenosinergic transmission is one of the most ancient and general regulatory systems in the living organisms. Adenosine, the main endogenous agonist of the adenosine receptors, exerts its effects through several signaling pathways contributing to protective and reparative processes. However, adenosine itself is only suitable for the acute treatment of some supraventricular arrhythmias and acute myocardial infarction, as its therapeutic value is limited by its short half-life. Another disadvantage of using adenosine is the excessively broad spectrum of its effects, due to the ubiquitous occurrence of adenosine receptors.

Regarding the inherent protective mechanisms of the cardiomyocytes, the A_1 type of adenosine receptors (A_1 receptor) seems to be one of the most important actors. Thus, the A_1 receptor is a promising target for new drug candidates to improve the condition of the heart and to treat certain heart diseases. Several selective A_1 receptor agonists entered clinical trials, including adenosine analogues, e.g. tecadenoson (to convert paroxysmal supraventricular tachycardia to sinus rhythm), selodenoson (to slow heart rate in atrial fibrillation) and trabodenoson (to treat ocular hypertension and primary open-angle glaucoma), furthermore compounds only remotely resembling adenosine, such as capadenoson (to treat angina pectoris) and neladenoson (to treat chronic heart failure). Further diseases and pathological conditions thought to be improved by A_1 receptor activation are ischemic heart disease, acute myocardial infarction, cardiac fibrosis, myocardial hypertrophy, cardiac remodeling and certain other supraventricular arrhythmias.

In one of our investigations, we focused on hypoxanthine-tricyclano, which was synthesized at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Debrecen. Hypoxanthine-tricyclano is an adenosine analogue in which adenine is replaced by hypoxanthine, and ribose is replaced by a fused tricyclic molecule derived from morpholino. Hypoxanthine-tricyclano was originally developed as an antiviral agent, cardiovascular effects of which were previously unknown.

1.3 The atrial myocardium as an experimental system

From anatomical and functional points of view, the cardiac muscle can be divided into two parts: supraventricular (atrial) and ventricular myocardium. On the atrial myocardium, adenosine, like acetylcholine, can induce significantly stronger negative tropic effects than on the ventricular myocardium. This is due to the higher density and more extensive postreceptorial signaling of the A_1 adenosine receptor and M_2 muscarinic receptor in the atria. The negative tropic effects of the A_1 receptor and M_2 receptor are mediated by two main signaling pathways: the opening of the muscarinic-operated K^+ channel and the inhibition of the adenylyl-cyclase enzyme. The muscarinic-operated K^+ channel is a member of the so-called GIRK (G protein-coupled inwardly rectifying potassium channels), which is mainly found in the atrial myocardium, while the adenylyl-cyclase enzyme occurs in most tissues, including the cardiac muscle.

Stimulation of the A_1 receptor in the ventricular myocardium reduces the function of adenylyl-cyclase to the resting level, thus the contractile force also returns to the resting value (indirect negative inotropic effect). However, in the atrial myocardium, the activation of the A_1 receptor can reduce the inotropic function below the resting level (direct negative inotropic effect), which is usually attributed to the function of the muscarinic-operated K^+ channel. During our investigations, we never prestimulated the adenylyl-cyclase, so we always detected the direct negative inotropic action of the A_1 receptor.

This experimental arrangement is advantageous because we have the opportunity to collect well-reproducible data with (in general) exceptionally small scatter. Thus, the atrial preparations allow us to collect reliable data. The isolated, stimulated left atrium is particularly suitable for generating reliable data, since this preparation can (acutely) react to the stimuli (e.g. from the A_1 receptor) predominantly by changing its contractile force. This experimental arrangement is therefore also suitable for implementing investigations where quantitative analysis, sensitive to inaccuracy (such as regression), is performed.

Both of our studies underlying this PhD work were carried out in such an experimental system. During our *ex vivo* study, we measured both inotropic and chronotropic effects, so accuracy of data was important to ensure to differentiate even small effects. In turn, in our *in silico* study, we performed regression, which needed accurate and reliable data that can be provided by the isolated, paced left atrium.

1.4 The Hill equation and the receptorial responsiveness method (RRM)

The Hill equation describes the link between the concentration of an agonist and the biological effect evoked. The Hill equation, as the simplest model for the receptor function containing two variables (c or its logarithm logc, and E) and three variable parameters (E_{max}, EC_{50} or its logarithm $logEC_{50}$, and n), can be used to fit intact concentration-effect (E/c) curves.

The receptorial responsiveness method (RRM) is a simple (i.e. non-multiple) nonlinear regression-based procedure with a unique model containing two variables (c

or its logarithm logc, and E') plus at least one variable parameter (c_x or its logarithm $logc_x$). The c is the concentration of an agonist that is administered to generate an E/c curve, while E' is the effect (related to the E/c curve) that is partly (but not completely) evoked by c in a biological system. As for the obligate variable parameter (c_x or $logc_x$), its role is, in the broadest sense, to quantify something, which (before the generation of the E/c curve) has decreased the responsiveness of the given biological system. This quantification is made by RRM with the concentration of the agonist used for the E/c curve that is capable of producing the same reduction in the responsiveness as the original evoking factor. In a simple case, the "something" to be quantified is a single, constant concentration of an agonist (called "distorting agonist", the quantification of which is thus the goal of RRM). In turn, in the simplest case, the agonist used for the E/c curve and the distorting agonist are the same, a case when RRM directly estimates the concentration of the distorting agonist (as c_x or as $logc_x$).

Although RRM deals with an inherently linear issue, the relationship between a concentration (or dose) and a biological effect is typically nonlinear. Thus, we arranged the model of RRM (intended for curve fitting) to be nonlinear *via* combining the basic equation of RRM with a nonlinear receptor function model (being generally the Hill equation). This way, the model of RRM became suitable to evaluate raw (or minimally transformed) E/c data. This yields more reliable results because the more the data to be fitted are transformed, the greater the risk that the biological variability and measurement errors in the raw data will significantly bias the results.

1.5 Objectives

When examining hypoxanthine-tricyclano *ex vivo*, our goal was to reveal whether this molecule has an effect on the atrial contractility and chronotropic function, and whether it exerts its potential myocardial effects through binding to the A_1 receptor.

During the *in silico* investigation of RRM, one of our goals was to investigate whether the results could be improved by simplifying the RRM model (involving a division by c_x). The next goal was to explore whether c_x or $logc_x$ is more appropriate in the RRM model. As an additional question, we raised whether individual, one-model global or two-model global ways of regression could give more accurate and precise

results, as well as which procedure was more convenient to use. We also aimed to clarify that, in the case of E/c curve families (one intact E/c curve plus more than one related distorted E/c curve), which could be the better choice: to fit all the curves at once, or fit the curves in pairs (one intact E/ c curve plus a distorted one). In addition, we examined how the results of RRM are affected if we also consider the distribution of the scatter of the E/c curve data.

2 Materials and methods

2.1 *Ex vivo* methods

2.1.1 Chemicals and solutions

The chemicals used in the study were hypoxanthine-tricyclano, produced by the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Debrecen (Debrecen, Hungary), furthermore adenosine and 8-cyclopentyl-1,3-dipropylxanthine (CPX), both purchased from Sigma (St. Louis, MO, USA).

Adenosine and hypoxanthine-tricyclano were dissolved in modified Krebs-Henseleit buffer (hereinafter referred to as Krebs solution) at 36 °C. CPX was dissolved in dimethyl sulfoxide (DMSO). The adenosine and hypoxanthine-tricyclano stock solutions were diluted with Krebs solution. The composition of Krebs solution was (in mM): NaCl (118), KCl (4.7), $CaCl_2$ (2.5), NaH_2PO_4 (1), $MgCl_2$ (1.2), $NaHCO_3$ (24.9), glucose (11.5) and ascorbic acid (0.1). Ascorbic acid, in the concentration used here as an antioxidant, did not affect the pH of the buffer (about 7.4, after a few minutes of carbogenization).

2.1.2 Animals and preparations

The left and right atria were isolated from male, Wistar rats weighing 400-500 g. The animal use protocols were approved by the Committee of Animal Research, University of Debrecen, Hungary (25/2013 DEMÁB and 5/2020/DEMÁB).

Each animal was quickly guillotined (without prior anesthesia to prevent subsequent drug interactions), and then the heart was rapidly removed and put in ice-cold Krebs solution. First, the left atrial appendage was cut off (hereinafter referred to as the left atrium). Next, the rest of the supraventricular myocardium was cut off (hereafter referred to as the right atrium), and then the stumps of the aorta and the main pulmonary artery were removed from the atrial tissue.

One thread was tied to the apex of each appendage and another one to the point on the appendage farthest from the apex (regardless of which side the atrium came from). Thus, atrial contractions were later measured between these two points on the

appendage.

Both the left and right atria were mounted at 10 mN resting tension in 10 mL vertical organ chambers, filled with Krebs solution, gassed ("carbogenized") with 95% O_2 and 5% CO_2 (36 °C; pH = 7.4). In the case of the right atria, the rest of the supraventricular myocardium (other than the tissue of the two appendages) floated in the bathing medium alongside the suspended right atrial appendage. The importance of this tissue arose from the fact that it contained the sinuatrial node responsible for the spontaneous beating.

The left atria were paced with platinum electrodes (3 Hz, 1 ms, twice the threshold voltage) using a programmable stimulator and power amplifier. The right atria were allowed to work spontaneously. The isometric twitches of atria were measured by a transducer and strain gauge, and recorded by a polygraph.

2.1.3 Protocols and groups

All atria were first allowed to equilibrate in Krebs solution for 45 min. Next, a cumulative concentration-effect (E/c) curve was constructed with adenosine (from 1 nM to 1 mM, to obtain an „Ado" E/c curve), which was followed by a 15-min wash-out with Krebs solution. Afterwards, the atria were randomized into two groups to perform two different protocols (P1, P2).

P1 (n=14): A cumulative E/c curve was generated with hypoxanthine-tricyclano (from 1 nM to 300 µM: „HT" E/c curve), followed by a cumulative adenosine E/c curve without wash-out (from 1 nM to 1 mM: „Ado+HT" E/c curve).

P2 (n=10): 10 µM CPX was added to the atria, and after a 10-min incubation period (without wash-out), a cumulative adenosine E/c curve was constructed (from 1 nM to 1 mM: „Ado+CPX" E/c curve), followed by a 10-min wash-out with Krebs solution. Next, another 10-min incubation period was carried out in the presence of 10 µM CPX, and (without wash-out) a cumulative hypoxanthine-tricyclano E/c curve was generated (from 1 nM to 300 µM: „HT+CPX" E/c curve).

2.1.4 Evaluation of the E/c curves

To assess inotropy, the distance between the lower and upper envelopes of the recorded consecutive isometric twitches of atria was considered as the contractile force. The inotropic effect was calculated from the change of the contractile force.

To assess chronotropy, the frequency of the mechanical activity of the right atria (beating rate) was computed. The chronotropic effect was then computed from the initial beating rate and the beating rate developed in the presence of the given agent concentration.

2.2 *In silico* methods

2.2.1 Data analyzed

The E/c curves evaluated in our *in silico* study were generated during two previous *ex vivo* studies of our work team on isolated, paced guinea pig left atria. The E/c curves were pairs (one intact E/c curve plus one distorted E/c curve) or families (one intact E/c curve plus three distorted ones). We reevaluated the E/c curves with the RRM in a way that we combined different regression options and searched for the most accurate, the most precise and the easiest to use.

The intact E/c curves were constructed with stable, synthetic A_1 receptor agonists (CPA, NECA or CHA), so that the generation of the E/c curve was not preceded by the use of any adenosine receptor agonist. In the case of the distorted E/c curves, we also used the former agonists, but shortly before the generation of the E/c curve, a known concentration of the agonist used later for the E/c curve was administered into the organ bath (the construction of the E/c curves started after the effect of the extra agonist concentration was completely developed). During the evaluation, the first administered concentration of the agonist (and its effect) was ignored, so it became a distorting concentration.

2.2.2 Regression models

To characterize the intact E/c curves, we used the form of the Hill equation

optimized for curve-fitting:

$$E = \frac{E_{max}}{1 + 10^{n \cdot (log\, EC_{50} - log\, c)}}$$

where: logc: the common logarithm of the agonist concentration used to generate the E/c curve; E: the effect; E_{max}: the maximal effect; $logEC_{50}$: logarithm of the agonist concentration causing half the maximum effect; n: the Hill coefficient (slope factor). This Hill model characterized the intact E/c curves with three parameters: E_{max}, $logEC_{50}$ and n.

Three, algebraically equivalent forms of the RRM model were fitted:

$$E' = 100 - \frac{100 \cdot \left(100 - E_{max} \cdot \frac{\left(c_x + 10^{logc}\right)^n}{\left(c_x + 10^{logc}\right)^n + 10^{n \cdot logEC_{50}}}\right)}{100 - E_{max} \cdot \frac{{c_x}^n}{{c_x}^n + 10^{n \cdot logEC_{50}}}}$$

The 1. RRM model, which is relatively complicated (no division by c_x) and contains c_x as a parameter.

$$E' = 100 - \frac{100 \cdot \left(100 - \frac{E_{max}}{1 + 10^{n \cdot \left(log\, EC_{50} - log\left(c_x + 10^{log\, c}\right)\right)}}\right)}{100 - \frac{E_{max}}{1 + 10^{n \cdot (log\, EC_{50} - log(c_x))}}}$$

The 2. RRM model, which is simplified (by dividing by c_x) and includes c_x as a parameter.

$$E' = 100 - \frac{100 \cdot \left(100 - \frac{E_{max}}{1 + 10^{n \cdot \left(log\, EC_{50} - log\left(10^{log\, c_x} + 10^{log\, c}\right)\right)}}\right)}{100 - \frac{E_{max}}{1 + 10^{n \cdot (log\, EC_{50} - log\, c_x)}}}$$

The 3. RRM model, which is simplified (by dividing by c_x) and includes $logc_x$ as a parameter. Variables and parameters of the RRM model: logc: the logarithm of the agonist concentration used to generate the distorted E/c curve; E': the distorted effect; E_{max}, $logEC_{50}$ and n: Hill parameters of the intact E/c curve related to the distorted E/c curve; c_x (or $logc_x$): the concentration (or its logarithm) of the agonist used to generate the distorted E/c curve, which is equivalent to the distorting concentration (administered first and then ignored). As here the distorting agonist and the agonist used for the distorting E/c curve were the same, c_x directly estimates the distorting concentration.

The three RRM models were used with individual, one-model global and two-model global fitting for both the intact and the distorted E/c curves.

During the global regression of an E/c curve family (i.e. when more than one distorted E/c curve belonged to an intact E/c curve), on one hand, we fitted all related E/c curves at once ("all-at-once fitting"), and on the other hand, we also fitted the intact E/c curve with only one related distorted E/c curve (“pairwise”). For the sake of simplicity, only the 3. RRM model was used to fit E/c curve families.

The fittings were performed in ordinary least-squares as well as robust manners.

2.3 Data analysis

The statistical analysis, curve fitting and plotting were performed using GraphPad Prism 8.4.2 (*ex vivo* investigations) and 9.5.1 (*in silico* investigations) software (GraphPad Software Inc., La Jolla, California, USA). We used Microsoft Excel 365 (Microsoft Co., Redmond, WA, USA) for some calculations.

During the *ex vivo* investigation, the normality of distribution of data were examined using D'Agostino-Pearson and Shapiro-Wilk tests. Two data sets with normal distribution were compared using unpaired t test (if the F test showed significantly different variances, with Welch's correction). The Mann-Whitney U test was used to compare two data sets with non-Gaussian distribution. More than two data sets with normal distribution were compared with one-way ANOVA and Tukey's post-test. For more than two data sets with non-Gaussian distribution, Kruskal-Wallis test and Dunn's post-test were used.

During the *in silico* investigation, accuracy of RRM was indicated by the difference between the determined distorting concentration values (c_x) and the related known distorting concentration values.

Precision of RRM was characterized by the width of the 95% confidence intervals (95% CIs) of the $\log c_x$ and c_x values provided directly by the regression. Precision of the curve fitting and E/c curve data was characterized by the width of the 95% confidence and prediction bands around the best-fit curves.

An additional information provided by the 95% CIs was the position of the

directly determined $\log c_x$ or c_x values within them. If the 95% CI was symmetrical (or close to it), then the parameterization of the fitted model could be considered adequate.

3 Results

3.1 *Ex vivo* results

3.1.1 Inotropic effects

As expected, adenosine, an orthosteric, reversible adenosine receptor agonist, exerted a strong, concentration-dependent, direct negative inotropic effect, which showed no statistically significant difference between the left and right atria.

CPX, a selective, orthosteric, reversible A_1 receptor antagonist, significantly antagonized the effect of adenosine at small and medium concentrations in both the left and right atria. However, adenosine at 1 mM concentration could surmount the inhibition.

In contrast, hypoxanthine-tricyclano evoked a moderate, concentration-dependent, direct positive inotropic effect that did not differed significantly between the left and right atria. The scatter around the mean effects of hypoxanthine-tricyclano was substantially greater than that of adenosine. CPX appeared to decrease the effect elicited by hypoxanthine-tricyclano, but this decrease did not reach the level of statistical significance.

The presence of hypoxanthine-tricyclano (in an about 411 μM concentration) slightly increased the response to adenosine that was statistically significant in the left atria.

3.1.2 Chronotropic effects

Our results for the chronotropic response were consistent with those obtained for the inotropic one. Adenosine elicited a strong, concentration-dependent, direct negative chronotropic effect on the spontaneously beating right atria that could significantly be inhibited with CPX.

In turn, hypoxanthine-tricyclano exerted a minor direct positive chronotropic effect. CPX seemed to reduce this positive chronotropy, but the extent of this (non-significant) effect did not reach that seen with inotropy. Furthermore, the presence of hypoxanthine-tricyclano (about 411 μM) slightly (and non-significantly) enhanced the

response to adenosine.

3.2 *In silico* results

3.2.1 More complex vs. simpler RRM models

The 3. RRM model (being simplified and contains $\log c_x$) provided the best results, the 2. RRM model (simplified but contains c_x) proved to be worse, while the 1. RRM model (complicated and contains c_x) gave the worst outcome. The advantage of algebraic simplicity was indicated by comparing the 1. and 2. RRM models.

3.2.2 $\log c_x$ vs. c_x in the RRM model

The inclusion of $\log c_x$ in the RRM model provided better results than that of c_x. The advantage of the logarithmic form was denoted by comparing the 2. and 3. RRM models.

3.2.3 Individual vs. one-model global vs. two-model global fitting

The least convenient individual regression was the most accurate, closely followed by the moderately convenient two-model global regression, and then the easy-to-implement one-model global regression. In turn, the two-model global fitting was the most precise, closely followed by the individual regression and, only from afar, by the one-model global fitting.

3.2.4 All-at-once vs. pairwise fitting (for E/c curve families)

Fitting more than one distorted E/c curve (and, of course, the inevitable native E/c curve) at once led to the same (in the lucky case), or worse (even significantly worse) results than the pairwise regression (when only one distorted E/c curve was fitted simultaneously with the native E/c curve). In this way, although the all-at-once fitting was more convenient, it was often less accurate and less precise than the pairwise fitting.

3.2.5 Ordinary least-squares vs. robust fitting

The ordinary least-squares or robust way of regression had little effect on accuracy and no effect on the ease of use. The impact on precision could not be assessed, as 95% CIs, confidence and prediction bands were not provided by the curve fitting software for robust regression.

4 Discussion

4.1 *Ex vivo* conclusions

The purpose of our *ex vivo* study underlying the present thesis was to uncover the potential inotropic and chronotropic effects of hypoxanthine-tricyclano on the rat left and right atrium. We have found that hypoxanthine-tricyclano has a weak positive inotropic effect and an even weaker positive chronotropic effect. In the presence of CPX, a selective, orthosteric and reversible A_1 receptor antagonist, both the positive inotropic and the positive chronotropic effects could be inhibited, although to a moderate extent that did not reach the level of statistical significance. This might be due to the relatively small effects and the presence of considerable scatter in the data.

As the most important pharmacological evidence for the phenomenon that an effect is produced by an agonist that binds to the orthosteric binding site of a receptor is the inhibition of the given effect with a selective, orthosteric antagonist, we cannot state with absolute certainty that hypoxanthine-tricyclano elicits its positive tropic effects entirely by binding to the orthosteric binding site of the A_1 receptor. If this is the case, hypoxanthine-tricyclano is an orthosteric inverse agonist of the A_1 receptor (as the endogenous agonist adenosine mediates negative tropic effects).

Inverse agonism of the A_1 receptor is otherwise a rarely observed phenomenon. So far, when inverse agonist behavior has been observed for an A_1 receptor ligand, it has typically involved weak effects. In the case of other receptors, their inverse agonists were also identified relatively late, as it is difficult to separate them from antagonists in many experimental arrangements.

The adenosine E/c curves constructed in the presence of hypoxanthine-tricyclano showed a similar course as the adenosine E/c curves generated alone. Assuming that the observed effects of hypoxanthine-tricyclano are mediated by the A_1 receptor, we can conclude that it can bind to the A_1 receptor with a significantly lower affinity than adenosine, and therefore it can be easily removed from the receptor. It should also be noted that hypoxanthine-tricyclano slightly enhanced the negative inotropic effect of adenosine (which was even statistically significant in the left atrium). Considering the positive inotropic action of hypoxanthine-tricyclano, it can be concluded that, owing to the increased contractile force in the presence of hypoxanthine-tricyclano, adenosine

could exert a greater negative inotropic effect.

Alternatively, it can be supposed that hypoxanthine-tricyclano does not exert its positive tropic effects exclusively through the A_1 receptor, but also influences another signaling (or metabolic) pathway. This alternative pathway would explain why CPX could only partially inhibit the observed effects of hypoxanthine-tricyclano in the supraventricular myocardium.

Although the inotropic effects on the two types of atria were very similar, the measurement data obtained from the right atrium showed larger scatter than those from the left atrium. This indicates that, from the point of view of inotropy, the left atrium paced at a constant frequency offers a more reliable model than the spontaneously beating right atrium.

Taking all together, we can conclude that hypoxanthine-tricyclano elicits weak positive ino- and chronotropic effects in rat left and right atria. Based on our results, hypoxanthine-tricyclano is believed to be a low-affinity, orthosteric, reversible, weak inverse agonist for the A_1 receptor, but the existence of other mechanisms behind its effects cannot be excluded.

4.2 *In silico* conclusions

In the present study, accuracy of RRM has generally been found to be acceptable for all kinds of regression used in our *in silico* investigation. However, there were three noteworthy exceptions:

1) the one-model global fitting using the 1. RRM model (non-simplified, contains c_x);

2) the one-model global fitting to the intact E/c curves using the 2. RRM model (simplified, contains c_x);

3) the global fitting performed in all-at-once manner to determine the smallest distorting concentration of the E/c curve families.

From these observations, three major conclusions can be drawn:

1) the algebraically simplified model is better for RRM (even despite the theoretical concern of dividing by c_x, a parameter that should be allowed to be zero);

2) in the model of RRM, the use of c_x is rather a disadvantage than an advantage;

3) a small distorting concentration is a challenge for RRM, especially if it is determined by fitting an E/c curve family in all-at-once manner.

These conclusions are detailed as follows:

Ad 1) For RRM, a model as simple as possible should be used. The greater the complexity in a regression model, the greater the degree of correlation that can occur between the parameters of the model.

Ad 2) Rewriting the RRM's model to replace $\log c_x$ with c_x did not improve either accuracy or precision of the estimation. On the contrary, in some cases, the use of c_x worsened precision by increasing the correlation between some parameters, moreover, the one-model global fitting of the 1. RRM model (the non-simplified model containing c_x that entirely allows c_x to be zero) made the determination impossible. This finding has refuted our previous assumption about the poor performance of the one-model global regression implemented with a model containing $\log c_x$. So, foibles of RRM in any case do not stem from the fit of $\log c_x$ that would hinder the correct determination of the zero value of c_x for the intact E/c curves. This conclusion has been confirmed by another finding of this *in silico* study. Namely, the two-model global regression (with a model containing either $\log c_x$ or c_x) did not improve the accuracy of RRM in comparison to either the one-model global fitting or the individual regression, although it has proven to be the most precise.

Ad 3) This conclusion is consistent with our earlier *in silico* finding that too small and too large concentrations might be estimated with substantial inaccuracy by means of RRM. A distorting concentration can be regarded small or large if its effect is small or large relative to the maximal effect of the agonist used for the E/c curves. However, for E/c curve families, accuracy (when a small concentration was to be determined) and precision (in every case) of the global regression could be substantially improved by fitting the appropriate E/c curves in a pairwise manner. Thus, despite the acceptable estimation for most cases and the good usability, we do not recommend fitting globally to more than two E/c curves at once (for RRM). In line with this, we discuss below those results of global regression that were obtained with pairwise fitting technique (unless otherwise indicated).

As for the further fitting options, in terms of accuracy, the individual fitting

proved to be the best, followed closely by the global regression (when fitting the model with $\log c_x$, irrespective of the number of models). In turn, regarding precision (i.e. the reliability of the estimates), the two-model global fitting was the best, followed closely by the individual regression and, from afar, by the one-model global fitting. As regards manageability (ease of use), the one-model global regression was the best, followed by the moderately complicated two-model global fitting, and then by the most complicated individual regression.

The most differences among the various implementations of RRM presented here have been found in terms of precision and ease of use. Accordingly, the choice between ordinary or robust regression had little impact in this study, as the robust regression prevented calculating confidence intervals for the best-fit values as well as confidence and prediction bands for the best-fit curves. Besides, the robust regression did not affect the manageability of curve fitting at all.

In summary, we recommend the implementation of the RRM as follows:

- with the simplified model containing $\log c_x$ (3. RRM model);
- with two-model global regression in a pairwise manner (if appropriate), or perhaps with individual regression (or, as a check, with both of them)
- with ordinary least-squares regression, or perhaps – in case of scattered data – with robust regression (or, as a check, with both).

5 Summary of the new findings

In our *ex vivo* study, we investigated the inotropic and chronotropic effect of hypoxanthine-tricyclano on the rat atrial myocardium. We found that hypoxanthine-tricyclano exerted a weak positive inotropic effect on both the left and right atrium, and, in line with this, it elicited a weak positive chronotropic effect on the right atrium. CPX (a selective A_1 adenosine receptor antagonist) slightly inhibited the inotropic and chronotropic effects of hypoxanthine-tricyclano (which action did not reach the level of statistical significance). The effects of hypoxanthine-tricyclano were easily surmountable with adenosine. Thus, we concluded that hypoxanthine-tricyclano may act as a weak (low-efficacy), reversible, orthosteric, low-affinity and inverse agonist of the A_1 receptor, although alternative mechanisms of action cannot be excluded.

In our *in silico* study, we compared the different implementation options of the receptorial responsiveness method (RRM) in terms of accuracy of the estimation, precision (reliability) of the procedure and convenience of use. The following regression options were combined: **1)** more complicated *vs.* simpler (although algebraically equivalent) RRM models; **2)** logarithmic ($\log c_x$) *vs.* non-logarithmic (c_x) main parameter in the RRM model; **3)** individual (local) *vs.* one-model global *vs.* two-model global fitting; **4)** in the case of global regression for curve families (more than two related data sets), all-at-once *vs.* pairwise fitting; **5)** ordinary (least-squares) *vs.* robust regression. We have found that: **1)** the simpler model is better than the more complicated one (even if the simplification is theoretically objectionable); **2)** better to use the main parameter as a logarithm ($\log c_x$); **3)** the individual fitting is the most accurate (and quite precise), the two-model global regression is the most precise (and quite accurate), while the two-model global regression is more convenient than the individual one; **4)** to fit curve families, the two-model global regression is only accurate and precise when performed in a pairwise manner; **5)** the ordinary and robust ways of regression are both suitable, but, for reliable data, it is worth choosing ordinary regression (so that the precision can be judged), while for scattered data, it is worth also performing robust fitting as a check. Thus, to implement RRM, two-model global regression performed with a pairwise technique is recommended (perhaps together with individual regression as a check), using the simplified model containing $\log c_x$, with ordinary (least-squares) fitting (together with robust fitting in case of scattered data).

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List of publications related to the dissertation

1. **Óvári, I.**, Viczján, G., Bege, M., Borbás, A., Herczegh, P., Zsuga, J., Papp, Z., Szilvássy, Z., Juhász, B., Gesztelyi, R., Erdei, T. D.: The ex vivo effects of hypoxanthine-tricyclano, a synthetic adenosine analogue, on rat left and right atria.
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2. **Óvári, I.**, Viczján, G., Erdei, T. D., Takács, B., Tarjányi, V., Zsuga, J., Szűcs, M., Szilvássy, Z., Juhász, B., Gesztelyi, R.: The influence of the way of regression on the results obtained by the receptorial responsiveness method (RRM), a procedure to estimate a change in the concentration of a pharmacological agonist near the receptor.
Front. Pharmacol. 15, 1-18, 2024.
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List of other publications

3. Viczján, G., **Óvári, I.**, Erdei, T. D.: A kannabidiol és az adenozinerg rendszer kapcsolata.
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