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Properties and *in silico* application of the receptorial responsiveness method (RRM)

BY: Dr. Adrienn Monika Szabo

SUPERVISOR: Dr. Rudolf Gesztelyi

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By Adrienn Mónika Szabó, MD

Supervisor: Rudolf Gesztelyi, MD, PhD

Doctoral School of Nutrition and Food Sciences, University of Debrecen

Head of the Examination Committee:	Miklós Vecsernyés, PharmD, PhD
Members of the Examination Committee:	Bence Kozma, MD, PhD
	Péter Dér, PharmD, PhD

The Examination takes place at
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Head of the Defense Committee:	Miklós Vecsernyés, PharmD, PhD
Reviewers:	Zsuzsanna Helyes, MD, DSc
	Gábor Kardos, MD, PhD
Members of the Defense Committee:	Bence Kozma, MD, PhD
	Péter Dér, PharmD, PhD

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1 Introduction

1.1 Main objectives

My PhD work consists of two studies. First, I investigated the interaction between two adenosine analogues with different site of action, FSCPX (8-cyclopentyl-3-[3-[[4-(fluorosulfonyl)benzoyl]oxy]propyl]-1-propylxanthine; a selective, irreversible A_1 adenosine receptor antagonist) and NBTI (S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine; a selective nucleoside transport inhibitor), by means of *in silico* reproduction of previous *ex vivo* experimental data. Second, I examined the perfect way to perform the receptorial responsiveness method (RRM) *via* evaluating data of another previous *ex vivo* experiment. The link between these two topics stems from the facts that, on one hand, all *ex vivo* data were generated using the same *ex vivo* techniques on the same tissue (guinea pig atrial myocardium), and, on the other hand, our most important tool was RRM in both studies.

1.2 Adenosine and adenosine receptors

Adenosine is a nucleoside consisting of an adenine and ribose. Adenosine serves as a metabolite for the nucleic acid metabolism, just like guanosine, uridine, thymidine and cytidine. The pivotal role of adenosine comes from the fact that it is also the endogenous agonist of a receptor family, the adenosine receptors. Adenosine is a substrate for enzymes with high activity, therefore it is characterized with a short *ex vivo* and *in vivo* half-life (0.6 – 10 s).

The adenosine receptors, belonging to the phylogenetically ancient purinergic receptors, are G protein-coupled receptors with seven transmembrane domains that hold their (orthosteric) binding site at the extracellular surface. Three types of adenosine receptors are distinguished: A_1, A_2 and A_3. Within A_2, there are two subtypes: A_{2A} and A_{2B}.

The A_1 adenosine receptor (A_1 receptor) exerts extensive regulatory (mainly protective and regenerative) functions in almost all tissues, including the myocardium. As a protective action, the A_1 receptor mediates strong negative inotropic effect consisting of an indirect component (decreasing the stimulated contractile force, seen in

both the atrium and ventricle) and a direct one (reducing the resting contractile force, only characteristic of the atrium in most species).

1.3 Adenosine analogues with agonist nature

Most of synthetic A_1 receptor agonists possess longer half-life than adenosine, as they are worse substrate for the adenosine-handling enzymes. Accordingly, E/c curves of the stable synthetic A_1 receptor agonists are to the left of the adenosine E/c curve generated otherwise the same way. In addition, E/c curves of the stable A_1 receptor agonists are more regular and more reliably reflect the function of the A_1 receptor. Thus, in our investigation, we used three synthetic A_1 receptor agonists: NECA (5′-(N-ethylcarboxamido)adenosine), CPA (N^6-cyclopentyladenosine) and CHA (N^6-cyclohexyladenosine). In contrast to the affinity and agonist activity of adenosine to/on all adenosine receptors, NECA stimulates only the A_1 and A_2 receptors, while CPA and CHA are selective A_1 receptor agonists. Although maximal effect (regarding negative inotropy) elicited by CHA proved to be somewhat smaller in our investigations than that of adenosine, NECA and CPA, all the four agonists are considered as a full agonist for the A_1 receptor.

It was observed that A_1 receptors activated by NECA, CPA or CHA bind to the different G proteins with distinct preference, although, for the A_1 receptor, functional selectivity of the different agonists does not result in radically diverse effects. Leastways, the existence of functional selectivity (“biased agonism”) underlines the benefit to use various agonists in the same biological system when investigating an effect or phenomenon.

1.4 Adenosine analogues with antagonist nature

CPX or DPCPX (8-cyclopentyl-1,3-dipropylxanthine) and FSCPX (8-cyclopentyl-3-[3-[[4-(fluorosulfonyl)benzoyl]oxy]propyl]-1-propylxanthine) are adenosine analogues with selective A_1 receptor antagonist property. CPX exerts a reversible effect. First, the structurally related FSCPX also binds to the A_1 receptor in a reversible manner. Later, however, this reversible binding is followed by a time-

consuming, probably covalent binding step that results in an irreversible receptor knock-out. A disadvantageous feature of FSCPX is that it is rapidly disintegrated in an aqueous solution. Interestingly, despite its sensitivity to water, FSCPX can elicit its irreversible antagonist effect *ex vivo* and *in vivo*. This may be due to that the lipid-soluble FSCPX can quickly enter the lipid compartment of the biological systems, becomes immune from water and can access its target, the membrane-bound A_1 receptor.

1.5 Adenosine analogues with inhibitory properties

Under physiological conditions, adenosine homeostasis of the myocardium is characterized by a net interstitial formation and net intracellular elimination, therefore, *via* adenosine transporters of the cardiomyocytes, adenosine flows into the cells. The most important adenosine transporter in the heart is ENT1, the equilibrative and nitrobenzylthioinosine-sensitive nucleoside transporter. Nitrobenzylthioinosine-type chemicals are S-(4-nitrobenzyl)-6-thioinosine and S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine, which latter was used in our investigations (abbreviated as NBTI). ENT1 blockade elicited by NBTI increased the interstitial level of the endogenous adenosine in our *ex vivo* guinea pig left atrium model as well, due to the inhibition of the inward adenosine flux and thereby prevention of the intracellular adenosine elimination (being much more intense than the interstitial one). For the investigation of the effect of NBTI on the endogenous adenosine, our work team previously developed its own method, the receptorial responsiveness method (RRM). During the use of RRM, E/c curves are generated in the absence and presence of NBTI with an A_1 receptor agonist, interstitial concentration of which is not affected by NBTI.

1.6 Background of the *in silico* study

In earlier *ex vivo* studies carried out in isolated, paced guinea pig left atria (a simple and reliable model to investigate the myocardial adenosinergic system), our work team observed a paradoxical phenomenon concerning FSCPX, a chemical widely known and used as a selective, irreversible A_1 receptor antagonist. Namely, in the presence of NBTI, a selective and potent inhibitor of the nucleoside transporter type ENT1 (the main carrier for the myocardial adenosine transport), FSCPX pretreatment

appeared to enhance the maximal response to adenosine, the physiological full agonist for the A_1 adenosine receptor. Back then, this phenomenon was considered as a misleading plotting peculiarity that was caused by neglecting the effect evoked by the surplus endogenous adenosine accumulated due to NBTI in the cardiac interstitium.

In a subsequent study, our work team *in silico* reconstructed some concentration-response (E/c) curves selected from a previous *ex vivo* investigation. Based on the behavior of the simulated E/c curves of different adenosine receptor agonists, it has been hypothesized that pretreatment with FSCPX alters the influence of NBTI on the E/c curves. As a mechanism, it has been assumed that FSCPX may modify ENT1 (the equilibrative and NBTI-sensitive nucleoside transporter) in a way that ENT1 preserves its ability to transport adenosine but NBTI can less inhibit this transport.

Next, we tested this putative effect of FSCPX in the isolated, paced guinea pig left atrium. Based on results of that study, we have propounded a new hypothesis, i.e. FSCPX pretreatment inhibits only one effect of NBTI on the E/c curves of adenosine receptor agonists, the one that is mediated via increasing the interstitial concentration of endogenous adenosine. The other effect of NBTI is mediated by elevating the interstitial level of exogenous adenosine (if any), and that action is proposed to remain intact after an FSCPX pretreatment. As a mechanism for this phenomenon, we have supposed that FSCPX may inhibit one (or some) enzyme(s) participating in the interstitial formation of adenosine, an action not acknowledged thus far.

Addressing the strict distinction between endogenous and exogenous adenosine cannot be overemphasized given that in our experimental conditions, elevation in the interstitial level of endogenous *versus* exogenous adenosine exerts the opposite effect on the E/c curve of adenosine. In general, NBTI, by blunting the normally inward transmembranous adenosine flux in the heart and thereby preventing adenosine from the intracellular elimination, increases the interstitial level of adenosine of both origins. However, endogenous adenosine is accumulated by NBTI before the generation of the E/c curve, thus it consumes (in part) the response capacity of the A_1 receptors and thereby decreases the observable effect evoked later by an exogenous agonist (used for the E/c curve). In contrast, exogenous adenosine is accumulated by NBTI during the construction of the E/c curve, so it can elicit a greater effect. A distinction between effects of endogenous and exogenous agonists (in experimental arrangements such as

the present one) forms the basis for the so-called receptorial responsiveness method (RRM), theoretical concept of which was used for the current work too. It is also important that CPA, a synthetic A_1 receptor full agonist, is relatively resistant to the adenosine-handling enzymes, so its level is minimally affected by NBTI.

Thus, the first goal of my PhD work was to revisit the issue of the above-mentioned paradoxical phenomenon, and to *in silico* reevaluate the major conclusions of the relevant *ex vivo* and *in silico* investigations. For this purpose, eight (averaged) E/c curves, based on which these conclusions were drawn, were selected and reproduced *in silico*. Simulation was made using different assumptions, and then the different models were compared.

1.7 Background of the *ex vivo* study

Receptor theory is one of the most prominent concepts in pharmacology, with the great advantage of underlying a variety of methods suitable for quantitative analysis. A way to determine constants (or sometimes variables) characterizing receptors, ligands, or cellular (tissue/organ/whole body) functions that is governed by these substances is to develop exact models (equations) containing the relevant parameters of these substances or cellular (etc.) functions and then to fit them to properly prepared data that were obtained from biological measurements. The first quantitative model of receptor function is the Hill equation, originally developed to describe the kinetics of a simple ligand binding, which relates the concentration of an agonist to the response evoked. The Hill equation serves as the basis for all advanced quantitative (and semi-quantitative) receptor models, such as the widely used operational model of agonism and the most recent SABRE (Signal Amplification, Binding affinity, and Receptor activation Efficacy) model. Some models have been developed to address the special (rather than general) challenges, just like RRM, which combines the Hill equation with a simple relationship between simultaneous effects of two concentrations of one or two agonist(s), where one concentration is known and the other one is unknown (c_x). Under certain circumstances, RRM can be used to estimate an acute increase in the level of an agonist (as c_x) in the vicinity of its receptors in a biological system. Technically, it is of great impact to possess reliable input data and ensure the most appropriate implementation of the fitting of the model of RRM.

RRM is a procedure that is based on a simple nonlinear regression while using a model with two variables (X, Y) and (at least) one parameter to be determined (c_x). The fitting of this model requires two sets of concentration-effect (E/c) curves to be generated. E/c curves that are suitable for RRM are XY graphs where X is the (logarithm of the) concentration of a pharmacological agonist, while Y is a response of a biological system that is evoked by the given agonist concentration (indicated by the corresponding X value) alone (first set of curves) or together with a single extra concentration (c_x) of the same or another agonist, which was administered to the system before the generation of the E/c curve (second set of curves). Quantifying this extra agonist concentration as a c_x value is the goal of RRM.

Although an E/c curve in the second set relates the resultant effect of the two concentrations (consuming the same response capacity of a biological system) solely to the concentration that is administered for the E/c curve (that is depicted on the X-axis), the model of RRM attributes this resultant effect to two concentrations, namely to two concentrations of the agonist that was used for the E/c curve. One of these concentrations is indicated by the X-axis, while the other one, c_x, is a concentration of the agonist used for the E/c curve that is equieffective with the agonist added in a single extra dose. If the two agonists in question are the same, c_x is a real concentration, and, if not, c_x is a surrogate parameter of the single extra concentration of the other agonist. The application of RRM might be useful when the concentration of this extra agonist is unknown and difficult to determine in any other manners. As the single extra agonist concentration distorts (biases) the E/c curve in the second set as compared to the corresponding E/c curve in the first set (generated the same way except for the administration of the single extra agonist concentration), it will be referred to as “biasing” concentration.

This way, RRM can provide information regarding an acute increase in the concentration of an agonist in the microenvironment of its receptor. Importantly, the microenvironment of receptors is a tissue compartment that is difficult to access, especially in a moving organ. Theoretically, RRM can be applied for each receptor; however, the A_1 receptor is uniquely suitable for this method, due to its slow and incomplete desensitization in the presence of even a full agonist.

Regression analysis (especially its nonlinear form) is one of the most common

ways to analyze E/c curves that might provide several useful pieces of information, e.g., about properties of receptors, receptor ligands and cell functions, which would be otherwise difficult (if possible) to gain. The goal of regression (curve fitting) is to find the best-fit values for the parameters of the model that is used for the regression, and thereby to create the best-fit regression function (curve), which is closest to the data points (XY data obtained usually from repeated measurements). Minimizing the sum of the squares of the vertical distances of data points (replicate Y values related to the same X value) from the curve (the Y value of the curve that relates to the corresponding X value) is the earliest and most common technique to find best-fit values. Accordingly, this procedure is called the least-squares method. The use of this method is based on two major assumptions:

1. normality, i.e., the scatter of the data points (related to the same X value) around the curve (the Y value on the curve related to the corresponding X value) follows a Gaussian distribution;

2. homoscedasticity, i.e., the extent of this scatter is the same for all values of X.

Ad 1. Assuming a Gaussian distribution for the scatter of data (and performing an ordinary regression) is useful for most cases. Nevertheless, assuming a Lorentzian distribution (and carrying out robust regression) makes the curve fitting more robust to outliers, although it hinders the calculation of data characterizing the reliability of the best-fit values and the curve (e.g., standard errors, confidence intervals, confidence, and prediction bands). In some cases, it is worth considering a Poisson distribution (and performing Poisson regression), but never for normalized Y data (used in the present study as well). While the Gaussian and Lorentzian distributions (being t distributions with infinity and 1 as degree(s) of freedom, respectively) allow for the use of the least-squares method, the Poisson distribution requires an alternative way for finding best-fit values (the so-called maximum likelihood-based parameter estimation).

Ad 2. To counteract heteroscedasticity, the standard equation of the least-squares method can be transformed ("weighted") by a factor, a procedure that is called weighting. The most common factors are $1/Y^2$ (relative weighting) and $1/|Y|$ (Poisson weighting). The relative weighting and, to a lesser extent, the Poisson weighting reduce the influence of the higher Y values on the best-fit values and the regression curve. In addition, the Poisson weighting (performed with ordinary regression) can serve as an

inferior alternative of the Poisson regression. A rarely used, although theoretically meaningful, choice is weighting by $1/SD^2$ (the inverse of the variance of Y values related to the same X value), which is expected to reduce the undue impact of Y replicates with bigger scatter.

The simplest and most common way of curve fitting is the individual regression, i.e., to find best-fit values for a single data set, e.g., a single E/c curve (or a set of E/c curves resulted from repeated measurements). An advanced way of curve fitting is when the model of regression defines a family of curves, i.e., some parameters (at least one) of the model to be fitted are (is) shared among several (at least two) data sets, called global regression. In the case of global regression, one sum of squares is computed for all Y replicates of all data sets.

The second goal of my PhD work was to explore the influence that different curve fitting ways (individual vs. global fitting) and curve fitting settings (assuming different distributions and scatter patterns for the Y values) might exert on the outcome of RRM. The experiments consisted of the construction of two E/c curves. For the first E/c curve, adenosine was administered to assess the naïve response of the atria to A_1 receptor stimulation. Adenosine is particularly suitable for this purpose, because it is quickly eliminated without yielding confounding byproducts. For the second E/c curve, one of three widespread, relatively stable, synthetic A_1 receptor agonists (CPA, NECA, and CHA) was used, in the absence or presence of a "biasing" concentration of the same agonist. The accuracy and precision of RRM was investigated via assessing this known "biasing" concentration in a well-established isolated and paced guinea pig left atrium model. Accordingly, c_x, estimate yielded by RRM, has been expected to directly provide the "biasing" concentration.

During this investigation, by measuring the left atrial contractile force, the negative inotropy that was elicited by the A_1 receptor agonists was determined as an effect. In the ventricular myocardium of most mammalian species, adenosine receptor agonists fail to directly evoke a negative inotropic effect, i.e., without a previous increase of the cellular cAMP level. In the mammalian atrium, however, stimulation of the A_1 receptor can considerably reduce even the resting contractile force, exerting a significant direct negative inotropic effect. As, for our *ex vivo* study, paced left atria were used, the negative tropic effects that were mediated by the A_1 receptor can manifest

themselves only in a decrease of the resting contractile force. This feature has made our results more reliable and easier to interpret, as the direct negative inotropy is sensitive to any change in the frequency of contractions.

2 Materials and methods

2.1 *In silico* methods

The effect of A_1 receptor agonists on the contractile force of the isolated, paced guinea pig left atrium were modelled with *in silico* E/c curves. To generate the simulated E/c curves, the operational model of agonism was applied, both for the action of one agonist and the co-action of two agonists. The operational model provides a general, (fully) quantitative description of the relationship between the concentration of bioactive agents and the effect mediated by a receptor specific for the given agents. Moreover, this model contains the appropriate parameters, by means of which the effects of FSCPX (concentration of the operable receptors) and NBTI (parameters for two different agonists) can be considered.

To address the different impact of adenosine of endogenous and exogenous origins, a procedure, previously developed from RRM by our work team, was applied. By means of this procedure, the neglect of one agonist from two co-acting agonists was simulated. The overlooked agonist concentration modelled the extra interstitial concentration of endogenous adenosine accumulated by NBTI, which came into being before the construction of an E/c curve with (exogenous) adenosine or CPA (agonists for the same receptor).

Using different input data and assumptions, four models and two additional model variants of Model 4 were defined that resulted in six sets of E/c curves, each set containing eight curves, four ones belonging to agonists C and four ones belonging to agonists A. To characterize and illustrate the simulated E/c curves, the Hill equation was fitted to them.

Adenosine, the physiological agonist for the A_1 adenosine receptor, the major adenosine receptor type of the supraventricular myocardium, was modelled with an agonist A. Based on its location, two agonist A concentrations were considered, one “in the organ bath” (a “bathing medium concentration”), and another one “at the receptors” (a “near-receptor” concentration). Based on its origin, an “exogenous” (administered to generate an E/c curve) and an “endogenous” (produced in the atrial tissue) agonist A were distinguished.

The inward transport of adenosine was simulated differently for the exogenous and endogenous agonist A. In the absence of a transport inhibitor, the concentration "in the organ bath" designated for the exogenous agonist A was divided by 400, when computing its effect. This maneuver simulated the fact that, *in vivo* or *ex vivo*, the concentration of an intensively transported agonist is lower at its receptors (in the interstitial fluid) than in the blood plasma or bathing medium. In the presence of a transport inhibitor, the concentration of the exogenous agonist A in the organ bath was not divided, or it was but by a number much less than 400 (6 or 14.8952), during the calculation of its effect. In turn, the surplus concentration of the endogenous adenosine, accumulated by a transport inhibitor, was considered as a c_{bias} value of agonist A, using arbitrary values or values measured in the most recent *ex vivo* study, when calculating its effect. All E/c curves generated with the consideration of c_{bias} (i.e. all E/c curves reflecting the effect of a transport inhibitor) were regarded as "biased". This is because it was simulated that c_{bias} and its effect were neglected during the evaluation of the raw E/c data. (Indeed, this is the case during a conventional evaluation of E/c data measured in the presence of a transport inhibitor that accumulates an unknown amount of the endogenous agonist for the given receptor.)

CPA, a synthetic agonist of the A_1 adenosine receptor, was modelled with an agonist C. As CPA is eliminated by adenosine-handling enzymes to a much lesser extent than adenosine, the "bathing medium concentration" and "near-receptor concentration" of agonist C were considered to be equal. Accordingly, when calculated its effect, the "bathing medium concentration" of agonist C was never divided throughout the simulation.

FSCPX, an irreversible A_1 adenosine receptor antagonist, was modelled with an agent X. The effect of a pretreatment with agent X was considered with a division of the total receptor concentration by ≈5.556, simulating that 18% of the A_1 adenosine receptors remained intact. In Model 4 and its variants, if a transport inhibitor was present, the agent X pretreatment was considered with a second procedure as well, by introducing a further c_{bias} value (see: next paragraph). Moreover, in Model 4-v2 (one of the variants), if a transport inhibitor was present, the agent X pretreatment was considered with a third procedure as well, through an additional division by 14.8952, when computing the "near-receptor" concentration of agonist A.

NBTI, a nucleoside transport inhibitor, was modelled by an agent NB. Its effect was taken into account by omitting the division of the exogenous agonist A concentration "in the organ bath" by 400, or by dividing it using a smaller number (as anticipated above,), and, in addition, by considering a surplus endogenous agonist A concentration (c_{bias}), when computing an effect. The earlier models contained only one c_{bias} (Models 1-3), whereas the final one (Model 4) and its two variants possessed two c_{bias} values: one for a mere NB treatment, and the other one for an X + NB co-treatment. In Models 1 and 2, c_{bias} was an arbitrary value, while in Models 3 and 4, c_{bias} values equaled the surplus interstitial adenosine concentrations determined in the most recent *ex vivo* study. As mentioned at the end of the previous paragraph, in Model 4 and its variants, an interaction between agent X pretreatment and agent NB treatment was also taken into account.

When presenting the results, effect values of the simulated E/c curves were always plotted against the "bathing medium concentrations" of the given exogenous agonist, as usually these concentrations are only known during the *in vivo* and *ex vivo* experiments.

2.2 Materials and *ex vivo* methods

2.2.1 Chemicals and solutions

As a bathing medium for the preparations, Krebs–Henseleit buffer (referred to as Krebs solution) was used. As A_1 adenosine receptor agonists, adenosine, N^6-cyclopentyladenosine (CPA), 5′-(N-ethylcarboxamido)adenosine (NECA), and N^6-cyclohexyladenosine (CHA), all being purchased from Sigma (St. Louis, MO, USA), were used. Adenosine was dissolved in 36 °C Krebs solution. CPA, NECA, and CHA were dissolved in ethanol:water (1:4) solution (v/v). All stock solutions were adjusted to a concentration of 10 mM. Stock solutions were diluted with Krebs solution.

2.2.2 Animals and preparations

For the experiments, male Hartley guinea pigs, weighing 600–800 g, were used. The experiments were carried out by the approval of the Local Ethics Committee of

Animal Research, University of Debrecen, Hungary (code: 25/2013/DEMÁB; 12 December 2013), in the spirit of the XXVIII of 1998 Act on the Protection and Welfare of Animals.

The animals were guillotined and then the left atria were quickly removed and mounted at 10 mN resting tension in 10 mL vertical organ chambers filled with Krebs solution, aerated with 95% O_2 and 5% CO_2 (36 °C; pH = 7.4). Atria were electrically paced by platinum electrodes (3 Hz, 1 ms, twice the threshold voltage) by means of a programmable stimulator and power amplifier. The amplitude of the isometric twitches (contractile force) was measured by means of a transducer and strain gauge, and it was recorded by a polygraph.

2.2.3 Groups and protocols for the *ex vivo* study

The atria in the organ chambers were divided into six groups, according to the six experimental protocols that were carried out (Intact CPA group, Biased CPA group, Intact NECA group, Biased NECA group, Intact CHA group and Biased CHA group; n = 5-7). In the organ chambers, all atria were first incubated for 40 min (in Krebs solution). Next, a cumulative E/c curve was constructed using adenosine, followed by a washout period (Krebs solution for 15 min). Afterwards, in the "Intact" groups, a cumulative E/c curve was generated with CPA, NECA, or CHA. Meanwhile, a single CPA, NECA, or CHA dose was administered to the atria in the Biased CPA, NECA, or CHA group to reach 100 nM, 100 nM, or 300 nM concentration ("biasing" concentration) in the bathing medium, respectively. Next, a cumulative E/c curve was constructed with the same agonist as was previously administered in a single dose, i.e., with CPA, NECA, or CHA.

2.2.4 Evaluation of the *ex vivo* E/c curves

The effect was defined as the percentage decrease of the initial contractile force of atria. First, all of the E/c curves were fitted to the Hill equation. Then, the CPA, NECA, and CHA E/c curves (averaged within the groups) were fitted to the model of RRM. This latter equation was fitted two ways: individually and globally. In addition, the fitting of the equation of RRM was performed while using the following setting

options: ordinary vs. robust regression; furthermore, a lack of weighting vs. weighting by $1/Y^2$ vs. weighting by $1/SD^2$. The different ways and setting options were combined with one another in all possible manners. Replicate Y (effect) values were always considered as individual points, as recommended (except for the case of weighting by $1/SD^2$, where the mean of Y replicates could only be considered). For every other setting, the default option was used.

The outcome of regression has been characterized by the accuracy of $logc_x$, the best-fit value (indicated by the nearness of its antilog (c_x) to the corresponding "biasing" concentration) and by the precision of the curve fitting (as indicated by the 95% confidence interval (95% CI) of the best-fit value).

2.2.5 Data analysis

More than two data sets were compared with one-way ANOVA, followed by Tukey post-testing, after the verification of the Gaussian distribution of data with Shapiro-Wilk test.

Concentrations (c, EC_{50}, and c_x) in the equations used for curve fitting were expressed as common logarithms (logc, $logEC_{50}$, and $logc_x$). Statistical analysis and curve fitting were performed with GraphPad Prism 8.1.1 and 8.2.1 (GraphPad Software Inc., La Jolla, CA, USA) for the *in silico* and *ex vivo* study, respectively. Other calculations were made by means of Microsoft Excel 2016 (Microsoft Co., Redmond, WA, USA).

3 Results

3.1 *In silico* results

The *in silico* reconstruction of the eight E/c curves obtained from earlier *ex vivo* experiments have proved to be the best in the basal form of Model 4, which possesses the following properties and underlying assumptions:

1. The endogenous and exogenous agonist A (*in silico* adenosine) were treated totally separately, thus it was assumed that, beyond their different effect on the E/c curves, agent X (the *in silico* FSCPX) also influenced differently the effect of agent NB (the *in silico* NBTI) on them. This difference manifested in that, in the Model 4, agent X only inhibited the effect of agent NB on the interstitial concentration of endogenous agonist A.

2. E_m parameter of the operational model of agonism (showing the possibly maximal effect in the system) was set to 90%, a smaller value than the theoretical maximum characteristic to our system (100%). It is not easy to assign a mechanism (or mechanisms) to this finding (the advantage of a submaximal E_m). This theoretic maximum might be an unattainable condition like the absolute zero temperature (0 K) or the absolute zero pressure (perfect vacuum).

3. Agent NB only slowed (and did not stop) the transport of agonist A.

4. The effect of agent NB was considered using two c_{bias} values. These two c_{bias} values resulted from our most recent *ex vivo* investigation (eight E/c curves of which were simulated): one c_{bias}, measured under pure NBTI treatment, was assigned to the treatment with agent NB, and the other c_{bias}, measured upon FSCPX+NBTI co-treatment, was assigned to the X+NB co-treatment. Thus, in the Model 4, an interaction between effects of agents X and NB was assumed.

3.2 *Ex vivo* results

The c_x values proved to be acceptable estimates of the "biasing" concentrations in all cases when RRM was carried out without any weighting. In contrast, weighting by $1/SD^2$ and, especially, by $1/Y^2$, dramatically worsened the accuracy of estimates;

moreover, in some cases, it hindered the curve fitting. Narrow 95% confidence intervals could be obtained when using ordinary regression (capable of yielding 95% confidence limits for the best-fit values) without weighting, if the model of RRM was fitted in an individual manner. If not (namely, in case of global regression), the curve fitting provided wide 95% confidence intervals.

Surprisingly, the global regression did not improve the results of RRM. The global regression, although ensuring a quicker and more convenient way to obtain c_x values, yielded less accurate estimates than the conventional individual fitting. Consistent with this, the global regression increased (rather than decreased) the uncertainty of the curve fitting, which produced wide 95% confidence intervals.

The robust regression (assuming Lorentzian distribution) moderately ameliorated the accuracy of the estimates in comparison with the ordinary regression (while assuming Gaussian distribution). With the use of our curve fitting software, robust regression could not be combined with different weighting options.

In sum, weighting is of the greatest importance regarding the accuracy of RRM, whereas the assumption made on the type of distribution (whether Gaussian or Lorentzian) has the least impact among the investigated fitting options and ways. With the use of RRM, data of our recent *ex vivo* investigation were able to be analyzed the best if no weighting was implemented; furthermore, individual fitting was chosen rather than global one.

4 Discussion

4.1 Interpretation of the *in silico* results

Our goal was to perform an *in silico* investigation regarding the background of a paradoxical phenomenon, first described in 2013 by our work team, sc. the irreversible A_1 adenosine receptor antagonist FSCPX apparently increases the maximal response to adenosine in the presence of NBTI (adenosine transport inhibitor). Our present *in silico* investigation used an improved approach for computer simulation (modelling the adenosine homeostasis and A_1 adenosinergic control of contractility in the isolated, paced guinea pig left atrium) by addressing the issue of E_m parameter of the operational model of agonism. Taking outcomes of the current *in silico* investigation together with previous *in silico* and *ex vivo* results of our work team, we have concluded that the above-mentioned paradoxical phenomenon can be ascribed to two simultaneous, additive, but independent factors. One is the interesting phenomenon that forms the basis of RRM, i.e. interstitial accumulation of the endogenous and exogenous adenosine exerts the opposite effect on the E/c curve of adenosine in our experimental arrangement. The other factor underlying the paradoxical phenomenon investigated herein is an interference between effects of two adenosine analogues, FSCPX and NBTI, in our experimental setting. Herein, we have provided *in silico* evidence for this interference, proposing that FSCPX, in addition to antagonizing the A_1 adenosine receptor, blunts the interstitial accumulation of endogenous (but not exogenous) adenosine produced by NBTI.

A theoretically interesting result of our *in silico* investigation is that E_m parameter of the operational model of agonism can influence the behavior of our complex E/c curves (reflecting the net of actions of two agonists while not accounting for one of them) to an unexpectedly great extent.

An additional finding of the present *in silico* investigation is that ENT1 blockade elicited by NBTI appears not to completely stop the inward transmembranous adenosine transport in the guinea pig atrium. This finding can be well explained with the existence of adenosine carriers other than ENT1 in the heart.

4.2 Interpretation of the *ex vivo* results

It is important – and somewhat self-critical – to state that, in all of the previous works, RRM was carried out with assumptions of Gaussian distribution for the scatter of data points around the curve, and of the same extent of this scatter along the curve. However, the long-established observation that these assumptions are usually true for non-transformed (at most normalized, in certain cases logarithmic) data obtained from biological systems was the only reason to do this. Thus, one goal of our *ex vivo* study was to explore whether other curve fitting options based on other assumptions ameliorate the accuracy and/or precision (and thereby the usefulness and/or reliability) of RRM. In addition, our other goal was to compare two ways of curve fitting, the individual and the global one. The global fitting is thought to be a powerful procedure that can reduce the uncertainty experienced when the corresponding E/c curves are individually fitted.

NECA, CPA, and CHA were decided as agonists to construct the E/c curves to be fitted in the *ex vivo* investigation. NECA stimulates both the A_1 and A_2 adenosine receptors, while CPA and CHA are highly selective for the A_1 adenosine receptor, the predominant adenosine receptor type of the myocardium. These synthetic compounds are less sensitive to adenosine-handling enzymes than adenosine, the endogenous agonist of the adenosine receptor family, so E/c curves of these synthetic agonists are more suitable for quantitative evaluation than those of adenosine. In addition, NECA, CPA, and CHA affect G proteins located in the cell membrane with somewhat different preference, so we considered it appropriate to carry out our investigation with all the three synthetic agonists.

A finding of our *ex vivo* investigation is that the well-established observation, i.e., assumption of both normality and homoscedasticity is a useful (and first choice) approach when analyzing biological data, is valid for the assessment with RRM as well. Consequently, ordinary regression without any weighting is the appropriate decision, when performing RRM. Regarding only the accuracy of the fitting, robust regression can also be chosen (moreover, somewhat more accurate estimates may be obtained), but the lack of 95% confidence intervals deprives the possibility of considering the precision (and thus reliability) of the curve fitting.

A further, unexpected finding of this *ex vivo* investigation is that, based on the

present results, the conventional individual regression is a more accurate and precise (although less comfortable) way to carry out RRM than the global regression. Thus, the individual fitting is the appropriate choice for RRM.

5 Summary

On one hand, during the *in silico* investigations underlying this thesis, we have found that E_m parameter of the operational model of agonism can influence the behavior of our E/c curves to an unexpectedly great extent regarding our previous ideas. Furthermore, we have gained *in silico* evidence for an interference between effects of FSCPX and NBTI in our *ex vivo* experimental setting used in several earlier studies. This finding extends beyond the well-established A_1 adenosine receptor antagonist property of FSCPX, indicating an inhibitory action exerted by FSCPX on the interstitial adenosine accumulation produced by NBTI, a selective and potent blocker of the nucleoside transporter type ENT1. Regarding the mechanism of this interference, *in silico* evidence has been obtained supporting that FSCPX only inhibits the interstitial accumulation of endogenous (but not exogenous) adenosine. As an additional result, we have found that NBTI seems not to completely inhibit the inward adenosine flux in the guinea pig atrium.

On the other hand, the major finding of the *ex vivo* investigations underlying the present thesis is that the best estimates of RRM can be obtained *via* individual fitting without any weighting, almost irrespectively of the fact of whether ordinary (assuming Gaussian distribution) or robust (optimized for Lorentzian distribution) regression is chosen. However, regarding the reliability, it is worthwhile to perform an ordinary regression, as only this method provides 95% confidence intervals for the best-fit values informing us about precision of the curve fitting. Accordingly, RRM is a relatively (by comparison to the possibilities it offers) easy-to-use procedure that requires neither a heavy-duty curve fitting software nor a high level of knowledge concerning regression analysis.

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List of publications related to the dissertation

1. **Szabó, A. M.**, Viczján, G., Erdei, T. D., Simon, I., Kiss, R., Szentmiklósi, J. A., Juhász, B., Papp, C., Zsuga, J., Pintér, Á., Szilvássy, Z., Gesztelyi, R.: Accuracy and Precision of the Receptorial Responsiveness Method (RRM) in the Quantification of A1 Adenosine Receptor Agonists.
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IF: 3.06 (2018)

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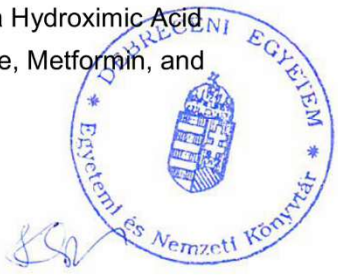

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