SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

New method for determining receptor reserve of direct negative inotropic effect mediated by atrial A_1 adenosine receptor

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF CLINICAL MEDICINE
DEBRECEN, 2021.

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The PhD Defense takes place at
June 16, 2021.

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1. Introduction

1.1. Main objectives

My PhD work involves two studies built on each other. I validated a previously developed method by our group, which we could use to qualitatively determine the adenosine receptor reserve of the A_1 adenosine receptor (A_1 receptor) using an *in silico* computer simulation procedure, and then tested our new hypothesis based on the results of an *ex vivo* isolated guinea pig left atrium. I used a method suitable for determining the previously described receptor reserve, but I performed it with a modified method in order to confirm the newly emerged hypothesis.

1.2. The adenosinergic system

The receptors involved in purinergic signaling are divided into two parts: P1 and P2 receptors. Within P1 or adenosine receptors, the International Union of Basic and Clinical Pharmacology (IUPHAR) identifies four (sub)types: A_1, A_{2A}, A_{2B}, and A_3, all G-protein coupled receptors with seven transmembrane domains.

Adenosine is a natural purine nucleoside, the nucleobase is adenine, to which a ribose is attached by a glycosidic bond. Adenosine is continuously produced under physiological conditions, but not in very large amounts.

The physiological concentration of adenosine in human plasma is 0.1–1 μM. The half-life of adenosine in human plasma is very short, ranging from 0.6 to 1.5 seconds. The short half-life is attributed to the rapid elimination of adenosine from the extracellular space which depends to a large extent on nucleoside transporters. Adenosine can be produced in both the intracellular and extracellular spaces. In the cell, it is mainly formed by the enzymatic degradation of ATP, ADP, AMP, and cAMP by endo-5'-nucleotidases and other alkaline phosphatases. Intracellularly, adenosine can also be produced from S-adenosylhomocysteine (SAH) via SAH hydrolase. Extracellularly, adenosine is formed from ATP by the action of ecto-5'nucleotidases and ecto-apyrases. Ecto-5 'nucleotidases also include the enzymes CD39 and CD73. CD39 produces AMP via ADP, which is converted to adenosine by CD73. Adenosine is an important signaling molecule for changes in the body's energy turnover, as it is a metabolite of ATP and an endogenous agonist of adenosine receptors. Stimulation of the myocardial A_1 receptor results in negative tropic (inotropic, chronotropic, dromotropic and bathmotropic) effects. Atrial A_1 receptor-mediated direct negative inotropy results from multiple signaling pathways. A_1 receptor binds to Gi protein to increase the opening frequency of G protein-gated inward rectifying K^+ channels (GIRKs) and inhibits adenylate cyclase enzyme function.

1.3. The A_1 receptor ligands

The myocardial A_1 receptor is involved in extensive cardioprotective and regenerative functions by adenosine, thus, the development of A_1 receptor agonists for therapeutic purposes has great potential.

The elimination of the endogenous agonist adenosine makes it difficult to record accurate E/c curves, so more stable synthetic A_1 receptor agonists are more suitable for studying A_1 receptors. One such synthetic adenosine analogue is CPA (N^6-cyclopentyladenosine), which is the most commonly used in our studies. The CPA is a selective A_1 receptor full agonist, which, like adenosine, is a purine-based molecule. Its half-life is much longer than that of adenosine, about 6 minutes in a rat blood sample. The first selective A_1 receptor antagonists include reversible DPCPX (8-cyclopentyl-1,3-dipropylxanthine). From this, the FSCPX irreversible A_1 receptor antagonist (8-cyclopentyl-N^3-[3-(4-(fluorosulfonyl)benzoyloxy) propyl]-N^1-propylxanthine) was developed. It is the first to reversibly bind to the receptor but forms a covalent bond in a time-consuming process. In our studies, FSCPX was used to allow enough time for irreversible binding of FSCPX.

1.4. The A_1 adenosine receptor reserve

The term receptor reserve, first introduced and used in the traditional receptor theory, can be considered as an integrative measure of the response-inducing ability of the interaction between an agonist and a receptor system, the latter of which consists of a receptor specific for the given agonist and a postreceptorial signaling that can be activated by the particular agonist-receptor complex.

Determination of receptor reserve is based on this phenomenon, during the course of which a fraction of receptors is irreversibly inactivated in a way that the remaining fraction retains its functional integrity. The greater the receptor reserve, the greater the fraction of receptors need to be inactivated in order to achieve detectable diminution of maximal response is. Thus, the measure of receptor reserve is the “resistance” (or “inertia”) of the receptor system, activated with a given agonist, against an intervention that reduces the number of operable receptors. Traditionally, the receptor reserve is thought to be determined by the agonist, tissue and effect measured. Due to the multifactorial origin of receptor reserve, it is worthwhile assessing it for every agonist, receptor system (tissue) and effect having pathophysiological importance. Receptor reserve can only be determined in the case of a full agonist. The most common way to do this is to record E/c curves with the agonist in the absence and presence of the irreversible antagonist, which can then be analyzed in several ways.

Information about receptor reserve has high utility when predicting the behavior of an agonist in a tissue. If receptor reserve in a tissue is small, only high-efficacy agonists can evoke a significant effect (acting often as a full agonist), whereas low-efficacy agonists cannot elicit an effect (or at most they behave as a partial agonist). In turn, if receptor reserve is great, even low-efficacy agonists are able to generate a significant effect (moreover, sometimes they may act as a full agonist). Thus, application of low-efficacy agonists can ensure tissue selectivity in a sense that their effect will only be significant in tissues possessing great receptor reserve.

In an earlier study, we investigated the receptor reserve belonging to the direct negative inotropic effect several synthetic A_1 adenosine receptor agonists with long half-lives, in the guinea pig atrium, then also with degradable adenosine.

1.5. The applied mathematical models

1.5.1. The Hill equation

The Hill equation, as the oldest quantitative receptor function model, serves as the basis for many receptor theories. To date, the Hill equation is often used for regression analysis of E/c curves.

The regression analysis performed using the Hill equation is two variables (c, E), three-parameter (E_{max}, EC_{50}, n) and nonlinear (since it does not follow the formula $y = a \cdot x + b$). The Hill equation is flexible in terms of curve fitting, as it usually provides a good estimate even for E (effect value) data with significant measurement errors or high biological variability.

The Hill equation should be fitted by plotting the logarithm of the concentration on the x-axis. There are several benefits to this. The data set for concentration-type quantities (e.g., EC_{50}) is usually not normally distributed, but their logarithm is usually (which facilitates their statistical analysis). In addition, semilogarithmic E/c curves are more illustrative.

1.5.2. The operational model of agonism

The operative model of agonism is to quantify the affinity and effect-generating ability of agonists to the receptor.

The operative model of agonism is a two variable (c, E), four-parameter (E_m, K_A, τ, n_{op}), nonlinear regression analysis that must be fitted globally to an naive and an irreversible antagonist pretreatment E/c curve (for which only the presence or absence of antagonist treatment may differ). The irreversible antagonist should be used, where possible, at a concentration that significantly reduces the maximum of the E/c curve relative to the naive E/c curve. The antagonist should be removed from the system before recording the E/c curve so

that it is not in free form. Thus, naive and antagonist-treated E/c curves differ in the concentration of functional receptors.

1.5.3. The receptorial responsiveness method

RRM is based on a simplified mathematical modelling of the interaction between two agonists that consume the response capacity of the same (or at least greatly overlapping) signal-transduction. This way, RRM enables the quantification of an acute increase in the concentration of an agonist *via* generating concentration-effect (E/c) curves with the same or another agonist (which latter is more stable or preferred for any other reasons) in the given tissue. As a limitation, if the two agonists are different, the surplus concentration in question can be quantified only with a surrogate parameter, i.e. the equieffective concentration of the other agonist. A characteristic feature of this procedure is that these E/c curves should be constructed both in the absence and presence of an irreversible antagonist against the investigated receptor.

1.6. Antecedents of the research on which the dissertation is based

In contrast to previous methods developed for receptor reserve determination, our working group introduced the use of NBTI (S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine), a selective inhibitor of the equilibrative and NBTI-sensitive nucleoside transporter (ENT1; SLC29A1), in the experimental protocol. As the physiological adenosine transport is directed into the cells (e.g. cardiomyocytes), NBTI prevents adenosine, administered for the E/c curve, from the intracellular degradation and reutilization, allowing enough time for the exogenous adenosine to exert its effect.

As the surplus endogenous adenosine accumulated by NBTI consumes in part the response capacity of A_1 receptors (and their postreceptorial signaling) before constructing the adenosine E/c curve, the response to adenosine (detected by the given adenosine E/c curve) will show an apparent diminution. Thus, effect values of adenosine E/c curves generated in the presence of NBTI should be corrected for the distortion caused by the increased endogenous adenosine level in the interstitium.

For this correction, we apply a procedure based on the receptorial responsiveness method (RRM). Finally, the corrected FSCPX-naive and FSCPX pre-treated adenosine E/c curves (generated in the presence of NBTI) are compared. Information on the receptor reserve in question can be obtained from the distance of the final (saturated) parts of the corrected curves: small distance means great receptor reserve, while large distance indicates a small one.

However, during the process of determining receptor reserve with our qualitative method, we encountered an astonishing phenomenon that, as pharmacological paradox, in the

presence of NBTI, FSCPX pre-treatment apparently increased the response to adenosine (when the FSCPX-NBTI co-treated adenosine E/c curve was compared to the solely NBTI-treated one)

The first of goal of the current study was to provide *in silico* validation of our new method developed to assess receptor reserve. Some E/c curves presented in our previous *ex vivo* work were simulated and then evaluated by means of this new method. For the sake of clarity, adenosine and CPA were simulated as agonist A and B, respectively, while FSCPX and NBTI were simulated as irreversible antagonist (IA) and transport inhibitor (TI), respectively. However, in the *in silico* study, unexpectedly, an interaction between FSCPX and NBTI was suspected, which I tried to confirm with a revised *ex vivo* study.

2. Materials and methods

2.1. *In silico* methods

In our guinea pig model, the most important adenosine carrier is ENT1. The interstitially formed adenosine rapidly enters the cells via ENT1 possessing high transport capacity that renders the interstitial adenosine level hard to determine. In our guinea pig model, ENT1 blockade produced by NBTI increases the interstitial level of endogenous adenosine by minimizing the adenosine flux into the cardiomyocytes, thereby preventing the intracellular adenosine elimination. Thus, NBTI affects the adenosine E/c curve in two opposing ways.

The net effect of these two influences is that NBTI slightly reduces E_{max} but markedly decreases EC_{50} of the adenosine E/c curve, at least in terms of the direct negative inotropy mediated by the guinea pig atrial A_1 receptor. On the contrary, due to the stability of CPA, the influence of NBTI on the CPA E/c curve in the same model is less complex: NBTI decreases E_{max} and increases EC_{50}.

Irrespectively of the A_1 receptor agonist used to construct the E/c curve, the surplus interstitial endogenous adenosine produced by NBTI tends to cause a characteristic bias (i.e., depression of maximal effect and dextral displacement) on the E/c curve. However, this bias is unmasked only if this effect is the single effect of NBTI, i.e., level of the agonist used for the E/c curve is not affected by NBTI (as is the case for CPA). In general, the mechanism by which this bias develops is that the surplus endogenous agonist concentration and its effect is overlooked during the evaluation of effect values that are assigned to the agonist concentrations administered for the E/c curve. Nevertheless, the extent of this E/c curve modification provides an opportunity to quantify the neglected extra concentration of the endogenous agonist by the equieffective concentration of an agonist (optimally a stable one with a long half-life) used for the E/c curve.

In this sense, three types of E/c curves are distinguished in the *in silico* study: unbiased, biased and a corrected one. An E/c curve is unbiased if every agonist with its whole concentration is taken into account. In turn, an E/c curve is biased if a concentration of any agonist is neglected during the course of evaluating the effect values. Correction of a biased E/c curve aims to reproduce the corresponding unbiased E/c curve (at least in terms of the effect values).

Functions provided by the equation for one agonist's action represented simple unbiased E/c curves, while those, yielded by the equation for the co-action of two agonists, simulated unbiased E/c curves that could be easily biased by ignoring the agonist concentration used with a single value (see below). This ignored agonist concentration (defined arbitrarily for the simulation) served as a model for the surplus interstitial concentration of endogenous adenosine produced by NBTI, while increasing concentrations of the other agonist simulated the concentrations administered for the E/c curve.

Adenosine and CPA were modelled with an agonist A and B, respectively. Therefore, a continuous extracellular production, intracellular elimination and transmembrane transport of agonist A were considered. NBTI and FSCPX were represented by a so-called transport inhibitor (TI) and irreversible antagonist (IA), respectively. When constructing E/c curves, effect values were always plotted against the bathing medium concentrations.

First step, we constructed the simple unbiased E/c curves with the operational model of agonist. We used the first of two equations in this model to determine the effect of a single agonist. Thus, two systems (without and with IA) and two agonists (A, B) were determined.

Second step, effect values belonging to the surplus endogenous agonist A concentration acting together with increasing concentrations of exogenous agonist A or agonist B were computed by means of second equation of the operational model of agonist. The unbiased E/c curves of agonist A served as a "built-in control", when comparing them with the (biased and then) corrected E/c curves of agonist A, in order to validate our recently published method to assess receptor reserve for degradable agonists.

The equations were used with the following parameters. Concentrations of agonist A and agonist B in the bathing medium ranged from 10^{-10} to $3.1623 \cdot 10^{-3}$ (being logarithm of which −10 and −2.5, respectively. The bathing medium concentration was designated as a concentration at the receptors for agonist B (in all circumstances) and for agonist A under TI treatment and without IA treatment (simulating complete ENT1 inhibition). Furthermore, the bathing medium concentration divided by 400 was designated as near-receptor concentration

for agonist A without IA and TI treatment (simulating the presence of intact ENT1) and also by taking a surplus near-receptor concentration of endogenous agonist A into account.

The effect of IA treatment was simulated with a division of the total receptor concentration by 5. Thus, according to our previous results with FSCPX, it was assumed that 20% of the A1 receptors remained intact after IA treatment. In addition, in the case of IA and TI co-treatment (possible interaction observed in the *in silico* study), bathing medium concentrations of exogenous agonist A were divided by 3 to compute its near-receptor levels. Furthermore, a third of the value of surplus near-receptor concentration of endogenous agonist A, which was designated to simulate the treatment with TI alone, was taken into account.

Third step, transformation of unbiased effect values into biased ones by means of RRM equation simulated the neglect of the surplus near-receptor concentration of endogenous agonist A and its effect by the previous obtained unbiased effect.

Finally, using another equation of RRM and knowing the biased effects, we were able to express the B-agonist concentration equivalent to the surplus endogenous agonist, which generated to their effect values using the Hill equation. When the effect of surplus endogenous agonist was computed for the system with a naive receptor population, Hill parameters of the simple unbiased E/c curve of agonist B generated in the system with normal receptor number were used. In turn, when the effect of surplus endogenous agonist was calculated for the system with reduced receptor number, Hill parameters of the simple unbiased E/c curve of agonist B constructed in the system with reduced receptor population were used. From these effects, RRM can be used to generate corrected effects in both the naive and IA-pretreated simulated systems.

2.2. Materials and *ex vivo* methods

2.2.1. Chemicals and solutions

The following chemicals were used: Adenosine, N^6-cyclopentyladenosine (CPA), 8-cyclopentyl-N^3-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N^1-propylxanthine (FSCPX) and *S*-(2-hydroxy-5-nitrobenzyl)-6-thioinosine (NBTI), purchased from Sigma (St. Louis, MO, USA).

Adenosine was dissolved in 36 °C modified Krebs-Henseleit buffer (Krebs solution). CPA was dissolved in 36 °C ethanol:water (1:4) solution (*v/v*). Dimethyl-sulfoxide (DMSO) was used as a solvent for FSCPX and NBTI. All stock solutions were adjusted to a concentration of 10 mM, except for the adenosine stock solution used to achieve 3 mM concentration in the bathing medium (it was 20 mM and was prepared always freshly before each use). Adenosine and CPA stock solutions were diluted with Krebs solution.

2.2.2. Animals and preparations

Male Hartley guinea pigs weighing 600-800 g were used for our experiments. The animal use protocols were approved by the Committee of Animal Research, University of Debrecen, Hungary (25/2013/DE MÁB).

The guinea pigs were guillotined and then left atria were quickly removed and mounted at 10 mN resting tension in 10 mL vertical organ chambers containing Krebs solution. Atria were paced by platinum electrodes (3 Hz, 1 ms, twice the threshold voltage) by means of a programmable stimulator and power amplifier. The contractile force was characterized by the amplitude of the isometric twitches.

2.2.3. Groups and protocols for the *ex vivo* study

The atria were divided into seven groups (CPA NBTI and its control; CPA FSCPX, FSCPX+NBTI and their control; adenosine FSCPX; adenosine NBTI and after FSCPX+NBTI) (n = 6-10) according to the seven experimental protocols of the present study. The protocols were the same as those used for our previous investigations, except for a rearrangement of protocol numbering and introduction of a new protocol (CPA FSCPX+NBTI), which has not been used yet in the previous *ex vivo* studies and which was based on the detection of the interaction between FSCPX and NBTI in the *in silico* study. In this group, the E/c curve was plotted with CPA in a nucleoside transport-inhibited system with a reduced receptor population. With this addition, we were able to determine the surplus interstitial adenosine concentration expressed as CPA in a FSCPX and NBTI co-treated system, thus, in contrast to the previously used method, by comparing the corrected curve (generated with RRM) with the control adenosine E/c curve, we were able to obtain more accurate information about the extent of the receptor reserve.

2.2.4. Evaluation of the *ex vivo* curves

The effect (defined as a percentage decrease in the initial contractile force), obtained from the experiments, was plotted against concentration of agonists administered.

To empirically characterize the E/c curves, we fitted the Hill equation for both individual and averaged E/c curves. Hill parameters (E_{max}, EC_{50}, n) of the individual E/c curves were used for the statistical analysis. Hill parameters of some averaged E/c curves were applied for the mathematical correction of some other E/c curves.

2.2.5. Quantification of the distortion produced by NBTI in the CPA E/c curves according to the original and the new method

NBTI increases the interstitial concentration of endogenous adenosine in the atrium under well-oxygenated conditions. Thus, our E/c curves constructed in the presence of NBTI were distorted by a surplus interstitial adenosine concentration that was developed already before the construction of the E/c curve. To quantify this extra adenosine concentration, RRM was performed.

Determination of the c_x (CPA concentration that is equieffective with the surplus interstitial adenosine concentration accumulated by NBTI) belonging to the NBTI treated CPA group (that characterizes the effect of NBTI treatment on the interstitial concentration of endogenous adenosine) was a part of both the original and improved forms of our receptor reserve-estimating method, while assessment of c_x related to the FSCPX-NBTI co-treated CPA group (characterizing the effect of FSCPX-NBTI co-treatment on the interstitial level of endogenous adenosine) appeared first in the improved version, because the effect of FSCPX on endogenous interstitial adenosine has not been known to date.

2.2.6. Correction of distorted effect values of adenosine E/c curves with new method

The effect values of adenosine E/c curves biased by NBTI were corrected by means of c_x (CPA concentration that is equieffective with the surplus interstitial adenosine concentration accumulated by NBTI) values obtained from CPA E/c curves distorted by NBTI. First, the effect (E_x) belonging to c_x was determined using the Hill equation.

When E_x was calculated for the correction of the averaged, only NBTI-treated adenosine E/c curve, c_x belonging to the averaged solely NBTI-treated CPA E/c curve and empirical parameters of the averaged control CPA E/c curve was substituted into the Hill equation. In turn, when E_x was computed for correcting the averaged, FSCPX-NBTI co-treated adenosine E/c curve, c_x related to the averaged FSCPX-NBTI co-treated CPA E/c curve and empirical parameters of the averaged only FSCPX-pre-treated CPA E/c curve were written into the equation (because the in silico study have shown that FSCPX affects interstitial adenosine levels). From the distorted effects and their corresponding E_x values, corrected effects were computed by RRM.

2.2.7. Data analysis

According to the recommendation, concentrations in the equations used for curve fitting were expressed as common logarithms. Normality of data was checked using Shapiro-Wilk normality test. Two data sets, if passed the normality test, were compared with unpaired t test. If not, Mann-Whitney U test was used. More than two data sets were compared using one-way

ANOVA (with Geisser-Greenhouse correction) followed by Tukey post-testing (herein, all data sets undergone multiple comparison passed the normality test).

Curve fitting and statistical analysis were performed with GraphPad Prism 7.04 for Windows (GraphPad Software Inc., La Jolla, CA, USA), while other calculations were made by means of Microsoft Excel 2016 (Microsoft Co., Redmond, WA, USA).

3. Results

3.1. *In silico* results

E/c curves of agonist A (representing adenosine) and B (modelling CPA), simulated in a system with naive receptor population (without IA), exhibited shape and position similar to E/c curves of adenosine and CPA (respectively), constructed using our previous data measured in FSCPX-naive guinea pig atria.

The TI (modelling NBTI) treatment shifted the E/c curve of agonist A to the left and slightly diminished the maximal effect, just like a 10 μmol/L NBTI treatment did with the adenosine E/c curve. Due to the need to harmonize several factors during the simulation, this effect came out somewhat smaller in the simulated model than in the biological one. The astonishing effect of 10 μmol/L FSCPX and 10 μmol/L NBTI co-treatment on the adenosine E/c curve (i.e., upon ENT1 blockade, the irreversible A_1 receptor antagonist appeared to increase the maximal effect of adenosine; Figure 2A), however, was not reproducible *in silico* under the assumption that TI elicits its action irrespectively of the IA treatment. In contrast, when we introduced an interaction between IA and TI at the level of the transmembrane transport of agonist A, the relative position of the three E/c curves could be made similar to that seen in the biological model. More specifically, we had to hypothesize that a prior IA treatment slightly blunted the effect of TI.

The TI treatment shifted the E/c curve of agonist B to the left and moderately decreased its maximal effect as compared to the simple unbiased and IA-naive E/c curve of agonist B (representing the control curve), similarly to the CPA E/c curves generated in the presence and absence of 10 μmol/L NBTI. Furthermore, RRM provided an estimate for the surplus agonist A concentration (in response to transport inhibition) that was almost the same as the predefined value ($3.86 \cdot 10^{-7}$ versus $4 \cdot 10^{-7}$).

After the correction for the biasing effect of the simulated and real agonist accumulation under agonist transport inhibition, the relative position of E/c curves exhibited the same rearrangement in the *in silico* and *ex vivo* models. At saturating agonist A concentrations, effect values of the IA and TI co-treated E/c curve were exceeded by effect values of the TI treated (and IA-naive) E/c curve, effect values of these curves being practically the same. This result corroborates the finding of our previous studies, i.e., the A_1 receptor reserve pertaining to the direct negative inotropic effect of adenosine is substantially great in the guinea pig atrium.

The corrected TI-treated (and IA-naive) E/c curve practically overlapped its corresponding unbiased E/c curve (that served as a built-in control for the correction), as expected. In contrast, the corrected IA and TI co-treated E/c curve markedly surpassed its unbiased counterpart (the built-in control) at small and medium concentrations of agonist A, due to modeled interference between IA and TI.

3.2. *Ex vivo* results

Adenosine concentration-dependently decreased the contractile force of all atria. Empirical parameters of the first adenosine E/c curves, provided by fitting of the Hill equation, did not show any significant differences among the experimental groups. This observation indicates the homogeneity of atria used for this investigation.

Consistent with our earlier observations, NBTI treatment markedly pushed down the CPA E/c curve (decreasing E_{max}) and shifted it to the right (increasing $logEC_{50}$), as compared to its control CPA curve. Also in agreement with our previous findings, FSCPX pre-treatment caused a moderate rightward shift of the CPA E/c curve (only increasing $logEC_{50}$ in comparison with its control CPA curve). However, combination of FSCPX and NBTI made the CPA E/c curve almost the same as the solely FSCPX-pre-treated one, aside from a slight, non-significant reduction of E_{max} and Hill coefficient. Thus, FSCPX pre-treatment almost entirely abolished all effects of NBTI on the response to CPA.

The surplus interstitial adenosine accumulated by NBTI was found to be equieffective with 100.2 nM, while the surplus interstitial adenosine accumulated by FSCPX and NBTI co-treatment with only 6,73nM CPA. Thus, FSCPX pre-treatment reduced the concentration of the extra interstitial adenosine produced by NBTI to less than a tenth in the isolated guinea pig atrium.

As expected, NBTI caused a significant depression and substantial sinistral displacement in the adenosine E/c curve, as compared to its control adenosine curve (although the decrease of E_{max} was greater than previously observed). Also in line with expectations, FSCPX pre-treatment shifted the adenosine E/c curve to the right as compared to its control adenosine curve, approximately to an extent as it did with the CPA E/c curve. At the same time, the FSCPX-NBTI co-treatment produced an adenosine E/c curve, whose E_{max} practically equaled E_{max} of the control as well as solely FSCPX-pre-treated adenosine E/c curves, while $logEC_{50}$ of which was similar to $logEC_{50}$ of the exclusively NBTI-treated adenosine E/c curve. So, FSCPX pre-treatment appeared to inhibit some but not all the effects of NBTI on the response to adenosine.

In the present investigation, the extent of this interference was unexpectedly large. FSCPX pre-treatment significantly augmented the response to both CPA and adenosine not only at high concentrations but also at medium ones, when contrasted the FSCPX-NBTI co-treated E/c curves with the solely NBTI-treated ones. Nevertheless, FSCPX pre-treatment did not significantly affect the extensive leftward shift of the adenosine E/c curve caused by NBTI. As a result, the FSCPX-NBTI co-treated CPA E/c curve and the FSCPX-pre-treated one almost coincide, whereas the FSCPX-NBTI co-treated adenosine E/c curve and the FSCPX-pre-treated one are furthest apart.

Another way to show this interference, if we contrast the differences between the NBTI-treated E/c curves and the corresponding control E/c curves with the differences between the FSCPX-NBTI co-treated E/c curves and the corresponding FSCPX-pre-treated E/c curves. In this manner, atria possessing similar operable A_1 receptor population can be compared. Regarding the CPA E/c curves, NBTI alone decreased E_{max} by about 16.7% and produced a 10-fold shift of the E/c curve to the right. In contrast, after FSCPX pre-treatment, NBTI decreased E_{max} only by 2.9% and did not shift the E/c curve. In the case of the adenosine E/c curves, NBTI alone reduced E_{max} by 19.8% and caused a 15-fold shift of the E/c curve to the left. After FSCPX pre-treatment, however, NBTI decreased E_{max} by 3.6% (similarly to results with CPA), while produced a huge, about 158-fold shift to the left.

The corrected, solely NBTI-treated adenosine E/c curve substantially exceeded its uncorrected (conventionally evaluated) counterpart. The maximum of the corrected NBTI-treated adenosine E/c curve reached that of its control (the naïve adenosine E/c curve). The starting point of the corrected NBTI-treated adenosine E/c curve was the effect evoked by the surplus interstitial adenosine produced by NBTI in the absence of FSCPX. When computed using the improved method, the corrected FSCPX-NBTI co-treated adenosine E/c curve started deep beneath the corrected NBTI-treated adenosine E/c curve but reached practically the same maximum as its control curve and the corrected NBTI-treated adenosine E/c curve did. In agreement with our previous finding, this indicates the existence of a great receptor reserve for the direct negative inotropic effect of adenosine in the guinea pig atrium. This finding shows that results obtained from the original method overestimate the receptor reserve in question but only in a small compass.

4. Discussion

4.1. Interpretation of the *in silico* results

The *in silico* study had provided an evidence that our qualitative method can reliably assess receptor reserve for adenosine, an endogenous agonist with rapid metabolism, in our recently published experimental setting (isolated and paced guinea pig left atrium).

At the same time, our findings imply that FSCPX treatment weakens the inhibitory action of NBTI on the transport (and thereby elimination) of adenosine. As a consequence, the adenosine E/c curve generated under NBTI and FSCPX co-treatment, after a correction with our method in the recently published manner, probably overestimates the effect of adenosine at small and medium concentrations.

The control, (solely) FSCPX-treated and (solely) NBTI-treated adenosine and CPA E/c curves could be easily reproduced. However, to reproduce the position of the adenosine E/c curve subjected to FSCPX and NBTI co-treatment relative to other adenosine E/c curves, it had to be assumed that the IA treatment (simulating FSCPX treatment) weakens the effect of TI (simulating NBTI), thereby it enables a bigger influx of agonist A (representing adenosine) into the cells as compared to the case of TI treatment alone.

This (newly proposed) effect of IA treatment was taken into account on the level of both exogenous and endogenous agonist A. Consequently, the corrected IA and TI co-treated E/c curve of agonist A diverged from its corresponding unbiased E/c curve, whish served as the "built-in control" for the validation of our method to assess receptor reserve. On the final parts of the corrected and unbiased IA and TI co-treated E/c curves (i.e., at the highest concentrations of exogenous agonist A), only a minor difference was experienced between the corrected and unbiased effect values. Therefore, conclusions drawn based on the relative position of final parts of corrected E/c curves in earlier works can be considered to be still appropriate.

In summary, our method, presented in this and earlier studies, enables the qualitative assessment of receptor reserve for adenosine, an endogenous agonist for which no reliable receptor reserve value could be determined previously. Hence, this method may contribute to the accumulation of useful data regarding receptor reserve for other endogenous agonists with short half-lives.

4.2. Interpretation of the *ex vivo* results

The main experimental finding of the present study is that a pre-treatment with FSCPX, a chemical widely considered to be a selective and irreversible A_1 receptor antagonist,

selectively influences the different effect components of NBTI, a selective nucleoside transport inhibitor, that are apparent on E/c curves of adenosine and CPA, two A_1 receptor agonists, in the isolated guinea pig atrium. While FSCPX pre-treatment considerably counteracts the depressive (E_{max}-decreasing) effect of NBTI on E/c curves of both adenosine and CPA, it does not significantly affect the extensive action of NBTI on EC_{50} of the adenosine E/c curve. Thus, our recent proposal (*in silico* results), that is, FSCPX blunts the effect of NBTI, should be refined in light of the new *ex vivo* results. It seems that FSCPX pre-treatment inhibits the effects of NBTI that are mediated by elevating the interstitial level of endogenous but not exogenous adenosine. Consequently, the target of FSCPX (other than the A_1 receptor) cannot be the ENT1 transporter or any other molecules that participate in the mediation of the effect of NBTI on the level of exogenous adenosine. Conversely, the target in question may be a (or some) molecule(s) that is (are) associated exclusively with the effect of NBTI exerted on the level of endogenous adenosine, for example, enzymes contributing to the interstitial adenosine formation.

If FSCPX were only a simple irreversible A_1 antagonist, the FSCPX+NBTI-treated CPA E/c curve would be shifted only to the right compared to the NBTI-treated CPA E/c curve (there would be a higher EC_{50} value, while the decreased E_{max} typical of a single NBTI treatment would have remained). In turn, when an FSCPX-NBTI co-treatment was applied in the case of an adenosine E/c curve, the E_{max}-decreasing effect of NBTI was only cancelled (similar to the experience with CPA), while the EC_{50}-decreasing effect of NBTI remained practically intact. This curve constellation further contradicts the prevailing concept being FSCPX only an irreversible A_1 receptor antagonist, moreover it also shows that the additional effect of FSCPX cannot be an equal inhibition of all effects of NBTI.

Taking all together, the target of FSCPX, to exert its effect other than A_1 receptor inactivation, should be a molecule that is related to the effect of NBTI that is mediated by increasing the interstitial level of endogenous adenosine. Enzymes contributing (exclusively or at least predominantly) to the interstitial adenosine formation are possible candidates. It can be speculated that FSCPX, which ruins the binding site for adenosine in the A_1 receptor, can do the same with the binding site of one (or some) enzyme(s) participating in the interstitial adenosine production.

5. Summary

In our *in silico* study underlying the present short thesis, we reproduced previous concentration-effect (E/c) curves generated in isolated, paced guinea pig left atria with adenosine and CPA, two A_1 adenosine receptor agonists, in order to validate our qualitative method for receptor reserve estimation that was developed to determine the A_1 adenosine receptor reserve for the direct negative inotropic effect of adenosine. In living tissues, adenosine has a short half-life due to its quick metabolism that makes investigations dealing with adenosine difficult in *in vivo* and *ex vivo* systems. Our results confirmed reliability of our method and its potential significance during screening assays of drugs with agonist properties. In addition, results of our computer simulations have suggested that FSCPX and NBTI, two chemicals used in the original *ex vivo* study, may interact with each other at the level of their effects: and FSCPX may attenuate effects of NBTI on the E/c curves of A_1 receptor agonists and thereby our method to estimate receptor reserve may slightly overestimate A_1 receptor reserve. To the best of our knowledge, this observation has no antecedents in the literature, so we developed an ex vivo experiment to support our hypothesis.

In our *ex vivo* investigation underlying the present short thesis, we constructed again the ex vivo adenosine and CPA E/c curves simulated in the study described above, according to an extended protocol. In light of our results, we had to refine our previous hypothesis about the interaction between FSCPX and NBTI: FSCPX pretreatment does not blunt the effects of NBTI mediated by an inhibition of intracellular elimination of exogenous adenosine, it rather blunts the effects of NBTI mediated by an increase in interstitial concentration of endogenous adenosine (before the construction of the E/c curves). Thus, FSCPX, in addition to the permanent inactivation of the A_1 receptor, possesses another mechanism of action that remained uncover to date. According to our hypothesis to address this phenomenon, FSCPX may permanently inhibit an (or some) enzyme(s) contributing to the formation of interstitial adenosine. Through this mechanism, FSCPX may moderate the interstitial accumulation of endogenous adenosine caused by NBTI and all the effects of this on the E/c curves of A_1 receptor agonists. In addition, we confirmed our *in silico* hypothesis, i.e. the original version of our method to determine receptor reserve slightly overestimates receptor reserve. To resolve this problem, we improved our method, and the use of this new method is recommended in the future for receptor reserve estimation.

6. Acknowledgements

I owe thanks to professor Zoltán Szilvássy MD DSc, rector of our university, for allowing me to carry out my PhD work in his department (Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Debrecen).

I also thank my supervisor, Rudolf Gesztelyi MD PhD (Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Debrecen), for his help during my work.

I would like to thank the members of our working group: Nóra Lampé, Katalin Szabó, Gábor Viczján and Ignác Óvári PhD students, as well as assistants Ildikó Juhász and Anna Mária Dávid (Institute of Pharmacology and Pharmacotherapy, UD).

I would also like to thank the other employees of our institute: Béla Juhász, associate professor, as well as Marian Kozma, Dániel Priksz and Balázs Varga.

I would like to thank my family, especially my mother, who has always stood up for me and supported me.

Financial background for my PhD work was ensured by the grant "HU-MATHS-IN – Deepening the activities of the Hungarian Industrial Innovative Mathematical Services Network" (EFOP-3.6.2-16-2017-00015), supported by the European Union and the State of Hungary.

Further supports were received from NKFIH-1150-6/2019, GINOP-2.3.4-15-2016-00002, ED_18-1-2019-0028, EFOP-3.6.2-16-2017-00009 and ÚNKP-19-3 New National Excellence Program of the Ministry for Innovation and Technology (ÚNKP-19-3-I-DE-308), furthermore Project no. (TKP2020-IKA-04) has been implemented with the support provided from the National Research, Development and Innovation Fund of Hungary, financed under the 2020-4.1.1-TKP2020 funding scheme.

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Registry number: DEENK/74/2021.PL
Subject: PhD Publication List

Candidate: Tamás Dániel Erdei
Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Erdei, T. D.**, Szabó, A. M., Lampé, N., Szabó, K., Kiss, R., Zsuga, J., Papp, C., Pintér, Á., Szentmiklósi, J. A., Szilvássy, Z., Juhász, B., Gesztelyi, R.: FSCPX, a chemical widely used as an irreversible A1 adenosine receptor antagonist, modifies the effect of NBTI, a nucleoside transport inhibitor, by reducing the interstitial adenosine level in the guinea pig atrium.
Molecules. 23 (9), 1-17, 2018.
DOI: https://doi.org/10.3390/molecules23092186
IF: 3.06

2. Zsuga, J., **Erdei, T. D.**, Szabó, K., Lampé, N., Papp, C., Pintér, Á., Szentmiklósi, J. A., Juhász, B., Szilvássy, Z., Gesztelyi, R.: Methodical Challenges and a Possible Resolution in the Assessment of Receptor Reserve for Adenosine, an Agonist with Short Half-Life.
Molecules. 22 (5), 1-17, 2017.
DOI: http://dx.doi.org/10.3390/molecules22050839
IF: 3.098

List of other publications

3. Lampé, N., Priksz, D., **Erdei, T. D.**, Bombicz, M., Kiss, R., Varga, B., Zsuga, J., Szerafin, T., Csanádi, Z., Balla, G., Balla, J., Szilvássy, Z., Gesztelyi, R., Juhász, B.: Negative Inotropic Effect of BGP-15 on the Human Right Atrial Myocardium.
J. Clin. Med. 9 (5), 1-18, 2020.
DOI: http://dx.doi.org/10.3390/jcm9051434
IF: 3.303 (2019)

4. 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.
Int. J. Mol. Sci. 20 (24), 6264-6277, 2019.
DOI: http://dx.doi.org/10.3390/ijms20246264
IF: 4.556

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5. Szabó, A. M., **Erdei, T. D.**, Viczján, G., Kiss, R., Zsuga, J., Papp, C., Pintér, Á., Juhász, B., Szilvássy, Z., Gesztelyi, R.: An Advanced in silico Modelling of the Interaction between FSCPX, an Irreversible A1 Adenosine Receptor Antagonist, and NBTI, a Nucleoside Transport Inhibitor, in the Guinea Pig Atrium.
Molecules. 24 (12), 1-16, 2019.
DOI: http://dx.doi.org/10.3390/molecules24122207
IF: 3.267

6. Zsuga, J., Móré, E. C., **Erdei, T. D.**, Papp, C., Kolozsváriné Harsányi, S., Gesztelyi, R.: Blind spot for sedentarism: redefining the diseasome of physical inactivity in view of circadian system and the irisin/BDNF axis.
Front. Neurol. 9, 1-13, 2018.
DOI: http://dx.doi.org/10.3389/fneur.2018.00818
IF: 2.635

7. Kemény-Beke, Á., Bodnár, B., **Erdei, T. D.**, Gesztelyi, R., Ujhelyi, Z., Bácskay, I.: Szempontok a megfelelő műkönnycsepp kiválasztásához a száraz szem betegség kezelésében.
Gyógyszerészet. 62, 722-725, 2018.

8. Papp, C., Pák, K., **Erdei, T. D.**, Juhász, B., Seres, I., Szentpéteri, A., Kardos, L., Szilasi, M., Gesztelyi, R., Zsuga, J.: Alteration of the irisin-brain-derived neurotrophic factor axis contributes to disturbance of mood in COPD patients.
Int. J. Chronic Obstr. Pulm. Dis. 12, 2023-2033, 2017.
IF: 2.917

9. Tajti, G., Pák, K., Képes, Z., **Erdei, T. D.**, Fodor, A., Mikáczó, A., Zsuga, J., Szilasi, M., Gesztelyi, R.: Asthma bronchiale-val kezelt betegek inzulinérzékeny és inzulinrezisztens-csoportjainak összehasonlítása.
Med. Thorac. 68 (3), 193-199, 2015.

10. Pák, K., Zsuga, J., Képes, Z., **Erdei, T. D.**, Varga, B., Juhász, B., Szentmiklósi, J. A., Gesztelyi, R.: The effect of adenosine deaminase inhibition on the A1 adenosinergic and M2 muscarinergic control of contractility in eu- and hyperthyroid guinea pig atria.
Naunyn Schmiedebergs Arch. Pharmacol. 388 (8), 853-868, 2015.
DOI: http://dx.doi.org/10.1007/s00210-015-1121-6
IF: 2.376

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11. Pák, K., Kiss, Z. M., **Erdei, T. D.**, Képes, Z., Gesztelyi, R.: Új lehetőség farmakológiai agonisták receptorközeli koncentrációjának becslésére: a receptoriális válaszkészség módszer (RRM). *Acta Pharm. Hung. 84*, 38-52, 2014.

Total IF of journals (all publications): 25,212
Total IF of journals (publications related to the dissertation): 6,158

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

01 March, 2021

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