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

# Investigation of pharmacons influencing the atrial adenosinergic system using the receptorial responsiveness method (RRM)

BY: Gábor Viczján

SUPERVISOR: Dr. Rudolf Gesztelyi


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Supervisor: Prof. Rudolf Gesztelyi, PhD

Doctoral School of Nutrition and Food Sciences, University of Debrecen

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| Head of the Defense Committee: | Prof. Ildikó Bácskay, PhD |
| Reviewers: | Anikó Pósa, PhD |
| | Tamás Röszer, PhD |
| Members of the Defense Committee: | Prof. Ildikó Bácskay, PhD |
| | Anikó Pósa, PhD |
| | Tamás Röszer, PhD |
| | Prof. Zsuzsanna Helyes, DSc, MHAS |
| | István Lekli, PhD |

The PhD Defense takes place at Lecture Hall of Building “A”, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
13:00, October 30, 2023.

*Experiments are the only means of knowledge at our disposal. The rest is poetry, imagination.*

*Max Planck*

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# 1 Introduction and objectives

## 1.1 The purinergic signaling

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 $A_3$. Reviews are available about the physiology and pathophysiology and the therapeutic potential of purinergic signaling. There are both short-term purinergic signaling in neurotransmission, neuromodulation, and secretion and long-term (trophic) purinergic signaling in cell proliferation, differentiation, and death in development and regeneration. Reviews on various aspects of purinergic signaling in cardiac physiology and pathophysiology have been published, including: physiological roles of cardiac P2X and P2Y purinoceptors, roles of adenosine in health and disease, effects of ATP and adenosine on coronary myocytes, purine degradation pathways in the myocardium, myocardial nucleotide transport, nonadrenergic, noncholinergic neural control of the atrial myocardium, vagal cardiovascular reflexes, and genetic modulation of adenosine receptor function.

All four ARs and many P2Rs including are ubiquitously expressed in the cardiovascular system and involved in the regulation of cardiovascular function. Existing evidence has shown that all four ARs are expressed in cardiomyocytes, endothelium and vascular smooth muscle cells.

$A_1R$ and $A_3R$ are negatively coupled to adenylyl cyclase via the $G_{i/o}$ protein subunits, and activation of those receptors results in a reduction in cAMP levels, whereas $A_{2A}R$ and $A_{2B}R$ are positively coupled to adenylyl cyclase through $G_s$, and activation of those receptors increases cAMP levels. $A_1R$ mediates the negative chronotropic and dromotropic actions of adenosine and anti-β-adrenergic actions in the heart. Activation of $A_{2A}R$ results in cardiac contraction indirectly through modulating the $A_1R$-mediated antiadrenergic effects, while $A_{2B}R$ mediates direct contractile effects without affecting β-adrenergic or $A_1R$-mediated antiadrenergic effects in the heart. Activation of $A_3R$ may induce cardiomyocyte apoptosis.

## 1.2 Adenosine, adenosine analogues and receptors

Adenosine is a nucleoside consisting of an adenine and ribose. Adenosine is a purine nucleoside that plays a special role in the nucleic acid metabolism by modulating a variety of physiological and especially pathological processes throughout the body 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 *in vivo* half-life (<1 s).

The adenosine receptors are G protein-coupled receptors with seven transmembrane domains. Three types of adenosine receptors are distinguished: $A_1$, $A_2$ and $A_3$. Within $A_2$, there are two subtypes: $A_{2A}$ and $A_{2B}$. CPA ($N^6$-cyclopentyladenosine) and CHA ($N^6$-cyclohexyladenosine) are synthetic adenosine analogues that act as selective, biologically stable (enzyme-resistant, especially CHA) and high-efficacy (especially CPA) full agonists for the $A_1$ adenosine receptor ($A_1$ receptor).

The $A_1$ receptor is the predominant adenosine receptor type of the myocardium that mediates several protective processes including negative tropic effects (primarily on the supraventricular myocardium). In the isolated, paced left atrium, the direct (i.e. detectable without a previous enhancement of the contractile force) negative inotropic effect, characteristic of the atrial muscle, can serve as a well-measurable and reliable output of the $A_1$ receptor function.

ENT1, an important adenosine transporter in the cardiac muscle, can influence the distribution and levels of various nucleotides and exogenous nucleotide analogues. Adenosine is a quickly metabolizing substrate, net formation and elimination of which occur in the interstitium and cell interior, respectively. Therefore, the adenosine transport is directed into the cells (e.g. endothelium and cardiomyocytes), furthermore its inhibition elevates the interstitial adenosine concentration. The significance of adenosine transport through ENT1 in the regulation of the interstitial adenosine level (and thereby in the adenosine-induced protective processes) is reflected by the repressed expression and activity of ENT1 during long-term ischemia. The consequently elevated interstitial adenosine concentration enhances the adenosinergic signaling because of the cell-surface localization of the orthosteric binding site of

adenosine receptors.

Nitrobenzylthioinosine derivatives (e.g. NBMPR and NBTI) are selective inhibitors of ENT1. Nitrobenzylthioinosines substantially modify the tissue distribution of molecules that are transported by ENT1 to a significant extent. Consistently, nitrobenzylthioinosines can drastically transform the concentration-effect (E/c) curve of adenosine receptor agonists through two mechanisms that we can distinguish as a general and a specific one

The general modifying effect of nitrobenzylthioinosines affects the E/c curve of all adenosine receptor agonists because it is mediated by a change in the interstitial level of endogenous adenosine resulted from the blockade of the transmembrane adenosine flux. In the heart, ENT1 blockade usually increases the interstitial concentration of endogenous adenosine, since, under physiological conditions, the adenosine flux is directed into the cell interion. This general influence of nitrobenzylthioinosines is manifested in a decrease of $E_{max}$ and increase of $EC_{50.}$

FSCPX is known as a selective, irreversible $A_1$ receptor antagonist. In earlier studies, however, we observed that a pretreatment with FSCPX seemed to paradoxically increase the direct negative inotropic response to adenosine and CPA, but only when NBTI was also present in the system. To solve this “FSCPX paradox”, we hypothesized that FSCPX pretreatment inhibited the general, but not the specific, effect of NBTI, probably by blunting the interstitial adenosine production.

Until the writing of the present work, this hypothesis has been based on *ex vivo* data obtained from one single animal model (isolated and paced guinea pig left atrium) with the use of adenosine, CPA, FSCPX and NBTI. To properly evaluate these data and to test their interpretation, a simple but highly specialized mathematical model was previously developed and then applied. As a mechanism of FSCPX paradox, we supposed that FSCPX might inhibit one (or some) enzyme(s) participating in the interstitial formation of adenosine. This supposition enables that FSCPX only interferes with the general modifying effect of NBTI, which affects the interstitial level of endogenous, but not exogenous, adenosine.

Differentiation between endogenous and exogenous adenosine is justified by the fact that, when analyzing E/c curves in a traditional manner, an increase in the interstitial concentration of the endogenous adenosine has the opposite effect on the

E/c curve of an adenosine receptor agonist as an increase in the interstitial level of the exogenous adenosine has on its own E/c curve. Nitrobenzylthioinosines, by preventing adenosine from intracellular elimination, elevate the interstitial level of adenosine, irrespective of its origin. It should be noted that most of synthetic $A_1$ receptor agonists (including CHA and CPA), as compared to adenosine, are greatly resistant to adenosine-handling enzymes, therefore their concentration is only slightly affected by nitrobenzylthioinosines (in the time window of our experiments). Consequently, the biologically stable synthetic agonists are suitable for examining exclusively the general E/c curve modifying effect of nitrobenzylthioinosines, while exogenous adenosine enables the investigation of the specific E/c curve modifying effect.

**In the present study, our goal was to test our hypothesis about the inhibitory effect of FSCPX on one (or a few) adenosine-forming enzyme(s) in the interstitium.** It was an obvious strategy to first investigate the possible involvement of ectonucleotidases in the FSCPX paradox. Ectonucleotidases play a key role in the extracellular generation of adenosine. In the heart, the two most important ectonucleotidases are CD39 (ecto-apyrase) and CD73 (ecto-5'-nucleotidase), which together catalyze three consecutive steps: decomposition of ATP, via ADP, to AMP (plus two inorganic orthophosphate ions) by CD39, furthermore breakdown of AMP to adenosine (and one inorganic orthophosphate ion) by CD73. First, we carried out enzyme inhibitor assays aiming to directly explore the effect of FSCPX on CD39 and/or CD73. Next, we continued the investigation with various indirect approaches to get closer to answering the question.

## 1.3 Relationship between cannabidiol and the adenosinergic system

Cannabidiol (CBD), the most extensively studied non-intoxicating constituent of the plant *Cannabis sativa*, differs from $\Delta^9$-tetrahydrocannabinol (THC), the best-known phytocannabinoid, only in the cleavage of a six-membered, oxygen-containing ring. CBD is widely labelled as a non-psychoactive compound, while others, due to its anxiolytic, antipsychotic and antidepressant effects, consider CBD to be psychoactive but non-intoxicating (does not produce cannabinoid tetrad, furthermore it does not cause addiction). The main source of CBD is hemp, which is a collective noun referring to one of the three major cultivar groups of *Cannabis sativa.*

In 2018, CBD was approved to treat Lennox-Gastaut syndrome and Dravet syndrome, two kind of child epylepsy. The list of well-evidenced and putative molecular targets of CBD in the human body contains more than fifty enzymes, ion channels, receptors and transporters, interaction with which enables CBD to exert antiinflammatory, anticancer, neuroprotective, anticonvulsant, anxiolytic, antipsychotic, antidepressant, antidiabetic and even antiobesity effects.

Anticancer mechanisms of CBD has been proved by several *in vitro* and *in vivo* findings (depending on cancer type and dose). CBD promoted cancer cell apoptosis independently of CB1 and CB2 receptors. The mechanism of this is not yet fully understood, but it seems that it can be - at least partially - related to the increase in the production of oxygen-based free radicals produced in cancer cells.

CBD is also promising in the treatment of neuroinflammatory diseases and other nervous system diseases (e.g. anxiety, epilepsy, schizophrenia). Due to its antioxidant, anti-inflammatory and neuroprotective effects, it has also been used in the treatment of Alzheimer's disease. CBD moderates the phosphorylation of tau protein (phosphorylation of glycogen synthase kinase decreases, thereby releasing the Wnt/β-catenin pathway), inhibits acetylcholinesterase and also reduces the accumulation of amyloid-beta (Aβ). These beneficial effects are presumably not only cannabinoid (CB) receptor-mediated processes. CBD acts as an agonist on the PPARγ receptor, the activation of which has an anti-inflammatory effect and reduces the amount of Aβ deposits.

According to research related to the immune system, in Wistar rats a dose of 2.5 mg/kg body weight of CBD for 14 days did not cause lymphopenia, in fact, the number of natural killer (NK) cells even increased), while a dose of 5 mg/kg body weight already reduced the number of lymphocytes. Based on these, it seems that CBD weakens the specific immune response, while non-specific immunity becomes more effective, which mainly favors antitumor and antiviral activity.

In male Wistar rats, CBD reduced the effects of stress on behavior and the functioning of the cardiovascular system, the background of which was the inhibition of the hypothalamic-pituitary-adrenal (HPA) axis and the enhancement of 5-$HT_{1A}$ receptor signaling. In male Sprague-Dawley rats exposed to mild, chronic stress, CBD treatment inhibited anxiogenic and depressive behavior by acting on CB1 and CB2

receptors. These results confirm the involvement of the endocannabinoid system in the creation of the antidepressant-like effects of CBD.

CBD may reduce obesity by negatively modulating the CB1 receptor. In addition to the accumulation of fat, obesity is associated with a condition similar to chronic, low-grade inflammation ("metaflammation"). CB1 and CB2 receptors are also found in visceral and subcutaneous adipose tissue, which can be promising anti-inflammatory and anti-obesity targets.

CBD delayed the development of diabetes mellitus (DM) in NOD (non-obese diabetes-prone) mice. The explanation for this may be that it changed the proportion of T lymphocytes ($T_{h2}$ came to the fore instead of $T_{h1}$, which produces inflammatory cytokines), and increased the secretion of anti-inflammatory cytokines (e.g. IL-10). High blood sugar causes increased oxidative stress, which causes adhesion molecules to appear on the endothelium that lead to a harmful immune response. CBD has been proven to reduce the number of these adhesion molecules, thereby reducing the risk of atherosclerosis. In mice with diabetic cardiomyopathy, both pre- and post-treatment with CBD reduced fibrosis and cell death, myocardial dysfunction, inflammation and oxidative stress. In a hyperglycemic environment, CBD moderated the production of both oxygen- and nitrogen-based free radicals.

CBD also affects the adenosinergic system. According to research, the two most important targets of CBD for myocardial adenosinergic signaling are ENT1 (proven in neurons, macrophages, retina and brain microglial cells), and the $A_1$ adenosine receptor (supposed in the heart). However, until now, the contribution of these pathways to the resultant effect of CBD remained unknown. **Therefore, the aim of our second study, which forms the basis of this thesis, was to reveal the main mechanism by which CBD exerts its effects on the myocardial adenosinergic signaling.** To address the problem of discriminating between adenosinergic activations caused by $A_1$ adenosine receptor agonism and adenosine transport blockade, we applied a self-developed method enabling the correction of a concentration-effect (E/c) curve for the distortion caused by a surplus (and neglected) agonist concentration.

Based on the possible therapeutic benefits of CBD in both type 1 and type 2 diabetes mellitus that affect the heart as well, the essential experiments presented in this paper were carried out on isolated, paced atria of obese type Zucker Diabetic Fatty

(ZDF) rats. The obese ZDF rat is a widely used animal model for the type 2 diabetes mellitus, a disease occurring with increasing frequency and being a major cause of blindness, kidney failure, heart attack, stroke, lower limb amputation and - in general - premature mortality worldwide. A “diseased” animal model may have greater translational potential as the results obtained this way can be more reliably applied to solving clinical problems. This is in accordance with the observation that some protective mechanisms, verified in a healthy state, do not prevail under certain pathological conditions.

# 2 Materials and methods

## 2.1 Methods related to the examination of FSCPX

### *2.1.1 In vitro enzyme inhibitor assays*

#### 2.1.1.1. Materials

Applied chemicals and kits: dimethyl-sulfoxide (DMSO), 8-cyclopentyl-1,3-dipropylxanthine (CPX), 8-cyclopentyl-$N^3$-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-$N^1$-propylxanthine (FSCPX), sodium polyoxotungstate (POM-1; as a part of the CD39 Inhibitor Screening Assay Kit, see below), disodium $N^6$-benzyl-α,β-methyleneadenosine-5'-diphosphate (PSB-12379), CD39 Inhibitor Screening Assay Kit and CD73 Inhibitor Screening Assay Kit.

CPX and DMSO were purchased from Merck KGaA (Darmstadt, Germany). FSCPX was manufactured by Santa Cruz Biotechnology, Inc. (Heidelberg, Germany) and distributed by BIO-Kasztel, Ltd. (Budapest, Hungary). Kits (including POM-1) were produced by BPS Bioscience (San Diego, CA, USA) and distributed by THP Medical Products Vertriebs GMBH (Vienna, Austria). PSB-12379 was manufactured by Tocris Bioscience (Bristol, UK) and distributed by Bio-Techne R&D Systems, Ltd. (Budapest, Hungary). Redistilled water was used to dissolve and to dilute POM-1, and to dilute the pre-dissolved PSB-12379. FSCPX and CPX were dissolved in dimethyl-sulfoxide (DMSO), and then they were diluted with redistilled water (when needed).

#### 2.1.1.2. Protocol

Level of inhibition of CD39 (ecto-apyrase) and CD73 (ecto-5'-nucleotidase) elicited by POM-1, PSB-12379, FSCPX and CPX was determined by means of malachite green colorimetric method, compliant with instructions of the manufacturer of assay kits used.

For both kits, 14 sorts of mixtures were prepared for the 96-well microplates: negative control (blank), negative control with 1% DMSO, negative control with 10% DMSO, positive control, positive control with 1% DMSO, positive control with 10% DMSO, inhibitor control with 20 μM POM-1, inhibitor control with 200 μM POM-1,

inhibitor control with 0.1 μM PSB-12379, inhibitor control with 1 μM PSB-12379, inhibitor test of 10 μM FSCPX (in 1% DMSO), inhibitor test of 100 μM FSCPX (in 10% DMSO), inhibitor test of 10 μM CPX (in 1% DMSO) and inhibitor test of 100 μM CPX (in 10% DMSO).

To quantify the inorganic orthophosphate produced by CD39 and CD73 (and thereby to characterize enzyme activities), the absorbance at 630 nm was measured with Varioskan LUX Multimode Microplate Reader obtained from Thermo Fisher Scientific (Waltham, MA, USA).

### *2.1.2 Ex vivo functional assays*

#### 2.1.2.1. Materials

As a bathing medium for all preparations, modified Krebs-Henseleit buffer (Krebs solution) was used. The following agents were used: adenosine, $N^6$-cyclohexyladenosine (CHA), $N^6$-cyclopentyladenosine (CPA), *S*-(4-nitrobenzyl)-6-thioinosine (NBMPR), *S*-(2-hydroxy-5-nitrobenzyl)-6-thioinosine (NBTI), FSCPX and PSB-12379. Although abbreviations "NBMPR" and "NBTI" are often used as synonyms, herein they refer to different structures. Agents were purchased from Merck KGaA (Darmstadt, Germany), except for FSCPX and PSB-12379 (see above). Adenosine was dissolved in 36 °C Krebs solution. CHA and CPA were dissolved in ethanol:water (1:4) solution (*v/v*). NBMPR, NBTI and FSCPX were dissolved in DMSO. All these stock solutions were adjusted to a concentration of 10 mM. Normal saline (0.9% *w/v* of NaCl) was used to dilute the pre-dissolved PSB-12379 (adjusting it to 1 mM concentration). Adenosine, CHA and CPA stock solutions were diluted with Krebs solution.

#### 2.1.2.2. Animals and Groups

The animal use protocols were approved by the Committee of Animal Research, University of Debrecen, Hungary (5/2020/DEMÁB). Male Wistar rats and male Hartley guinea pigs weighing 400-500 g and 500–700 g, respectively, were used. The animals were guillotined and then 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). Left 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 isometric twitches, measured by a transducer and strain gauge. Since we monitored the decrease in resting contraction force, we determined the direct negative inotropic effect, which offers a simple and highly reliable way to test myocardial $A_1$ adenosine receptor function when measured on an isolated, stimulated left atrium in a classical organ bath system.

When investigating the potential interaction of FSCPX and NBMPR, the rat left atria were randomly divided into five groups: Control for NBMPR group, NBMPR group, Control for FSCPX & FSCPX+NBMPR group, FSCPX group and FSCPX+NBMPR group.

To explore a potential interaction between PSB-12379 and NBTI, the guinea pig left atria were randomly divided into six groups: Control (for CPA) group, NBTI (for CPA) group, PSB (for CPA) group, PSB+NBTI (for CPA) group, Control+NBTI+PSB (for Ado) group and Control+PSB+NBTI (for Ado) group.

During the investigation of the different FSCPX administration regimens, the rat and guinea pig left atria were randomized into four groups: DMSO (5 cycles) group, FSCPX (1 cycle) group, FSCPX (2 cycles) group and FSCPX (5 cycles) group.

#### 2.1.2.3. Protocols

In the organ chambers, all atria (isolated from both species) were first allowed to equilibrate in Krebs solution for 25 min, next they were subjected to 100 μM adenosine for 2 min (as a priming), afterwards a wash-out was made with Krebs solution for 20 min. Next, a cumulative E/c curve was constructed with adenosine, followed by another 20-min long wash-out period (using Krebs solution). From this stage of the experiments, the different protocols continued different ways.

When investigating the potential interaction between FSCPX and NBMPR, each rat left atrium underwent one of five protocols, the same as those were used in a previous study, excepting three modifications. Herein, CHA was applied instead of

CPA; NBMPR was used instead of NBTI; and some protocols performed in were omitted from this investigation (where adenosine was used as a main agonist).

Specifically, each atrium underwent an *in vitro* treatment as follows: the Control for NBMPR group received 10 μL DMSO for 15 min; the NBMPR group was subjected to 10 μM NBMPR (administered with 10 μL DMSO) for 15 min; the Control for FSCPX & FSCPX+NBMPR group received 10 μL DMSO for 45 min followed by a 60-min wash-out, next 10 μL DMSO was administered for 15 min; the FSCPX group received 10 μM FSCPX (with 10 μL DMSO) for 45 min followed by a 60-min wash-out, and then 10 μL DMSO was added for 15 min; the FSCPX+NBMPR group received 10 μM FSCPX (with 10 μL DMSO) for 45 min followed by a 60-min wash-out, next 10 μM NBMPR (with 10 μL DMSO) was administered for 15 min. Finally, without washing out the last administered agent (and/or solvent), a cumulative CHA E/c curve was generated in all rat atria.

When investigating the potential interaction between PSB-12379 and NBTI, each guinea pig left atrium was subjected to one of six protocols, similar to those described in the previous paragraph. Main differences are as follows: return to NBTI instead of NBMPR; return to adenosine and CPA instead of CHA; use of protocols, where adenosine was the main agonist, again; and use of PSB-12379 to replace FSCPX (with modifications of wash-out and incubation periods consistent with this latter interchange). So, the Control (for CPA) group received 10 μL DMSO for 15 min; the NBTI (for CPA) group got 10 μM NBTI for 15 min; the PSB (for CPA) group received 10 μL DMSO and 3 μM PSB-12379 for 15 min; the PSB+NBTI (for CPA) group got 3 μM PSB-12379 for 15 min, and 10 μM NBTI for further 15 min; the Control+NBTI+PSB (for Ado) group received 10 μM NBTI for 15 min; and the Control+PSB+NBTI (for Ado) group got 10 μL DMSO and 3 μM PSB-12379 for 15 min. Next, for groups labelled with “for CPA”, a cumulative CPA E/c curve was generated (and their protocols ended there), whereas for groups with “for Ado” in their names, a cumulative adenosine E/c curve was constructed (the second one for these atria). Afterwards, both groups labelled with “for Ado” received 3 μM PSB-12379 for 15 min, and then 10 μM NBTI for further 15 min. Finally, a third cumulative adenosine E/c curve was generated in these two groups. Thus, overall, groups Control+NBTI+PSB (for Ado) and Control+PSB+NBTI (for Ado) provided four kinds of adenosine E/c curve: a control one (first curve), an NBTI- and a PSB-12379-treated

one (second curve), and a PSB+NBTI-treated one (third curve).

When studying the different FSCPX administration regimens, each rat and guinea pig left atrium was subjected to one of four protocols. The DMSO (5 cycles) group received 10 μL DMSO for 45 min followed by a 75-min wash-out, while the other three groups were exposed to 10 μM FSCPX for 45 min followed by a 75-min wash-out. Importantly, the 45-min long incubation period was not interrupted in the FSCPX (1 cycle) group, whereas, in every other group, it was interrupted with one or more short but intense wash-out period(s) and subsequent readministration(s) of 10 μM FSCPX (with 10 μL DMSO) or 10 μL DMSO alone. In the FSCPX (2 cycles) group, the 45-min incubation was discontinued once (at 22-23 min) by making a wash-out and readdition of 10 μM FSCPX. In the FSCPX (5 cycles) group and DMSO (5 cycles) group, it was interrupted four times (every 8-9 minutes) with wash-out and readministration of 10 μM FSCPX and 10 μL DMSO alone, respectively. Finally, a cumulative CPA E/c curve was constructed in all atria.

#### 2.1.2.4. Characterization of the E/c 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 Methods related to CBD

### 2.2.1 Materials

The following chemicals were used: adenosine and $N^6$-cyclopentyladenosine (CPA), purchased from Sigma (St. Louis, MO, USA); a hemp extract oil rich in cannabidiol (CBD) and devoid of intoxicating psychoactive components, commercially available under the name “Vitality CBD Oral Drops/Spray 4800mg

Natural", ordered directly from the manufacturer (Vitality CBD Ltd, Birmingham, UK); sunflower oil, commercially available under the name "Vénusz", manufactured by Bunge CJSC (Martfű, Hungary).

CPA was dissolved in ethanol:water solution (1:4 v/v). Adenosine was dissolved in 36 °C Krebs solution. Both stock solutions were adjusted to a concentration of 10 mmol/L, and then were further diluted with Krebs solution. According to the manufacturer, the CBD-rich hemp extract in the product "vitality CBD" was dissolved in MCT (medium-chain triglyceride) oil. Before use, it was further diluted with sunflower oil in the required proportion.

### 2.2.2 Animal model and experimental groups

The animal use protocols were approved by the Committee of Animal Research, University of Debrecen, Hungary (5/2022/DEMÁB; April 14, 2022). Male, 10-week-old, lean type as well as obese type Zucker Diabetic Fatty (ZDF) rats were obtained from the AnimaLab Hungary Ltd. (Vác, Hungary), the distributor of Charles River Laboratories International Inc. (Wilmington, MA, USA). Until 6 months of age, the lean ZDF rats were maintained on conventional rat chow (S8106-S011 SM R/M-Z+H, purchased from the Toxi-Coop Ltd., Budapest, Hungary, the distributor of ssniff Spezialdiäten GmbH, Soest, Germany), while the obese ZDF rats were kept on a diabetogenic diet (Purina 5008 rat chow, obtained from the AnimaLab Hungary Ltd., according to the recommendation of Charles River Laboratories International Inc.).

The 6-month-old obese ZDF rats were randomized into two groups: the Obese ZDF group and the CBD-treated Obese ZDF group, while the lean ZDF rats formed the Lean ZDF group. In addition to continuing the previously introduced diets, animals in the Obese ZDF group received 0.2 mL of sunflower oil daily, via gavage, for four weeks, while animals in the CBD-treated Obese ZDF group received 60 mg/kg/day CBD, via gavage, in a 0.2 mL volume, for four weeks.

On the day before starting the administration of vehicle and CBD for the animals (in vivo treatments), the fasting blood glucose concentrations (in mmol/L) were (mean ± SEM): 5.9 ± 0.1, 19.1 ± 2.1 and 23.3 ± 1.4, while on the day after finishing the in vivo treatments, they were 5.6 ± 0.1, 19.3 ± 1.7 and 20.5 ± 1.7 in the

Lean ZDF, Obese ZDF and CBD-treated Obese ZDF groups, respectively. Furthermore, at the beginning of the in vivo treatments, the body weights of animals (in g) were (mean ± SEM): 388.8 ± 7.6, 364.5 ± 25.8 and 368.1 ± 14.2, whereas at the end of the in vivo treatments, these values changed to 413.2 ± 8.2, 403.5 ± 28.9 and 398.4 ± 17.6 in the Lean ZDF, Obese ZDF and CBD-treated Obese ZDF groups, respectively. Thus, the obese type ZDF rats developed an advanced stage type 2 diabetes mellitus, in which condition their body weight was already slightly smaller than that of the lean controls (despite the "obese" and "lean" type names).

### *2.2.3 Preparations and protocols*

Preparation of left atria in 2.1.2.2. as described in subchapter. Since there was no pretreatment with a contractility-influencing agent in this study either, the direct negative inotropic effect induced by adenosine receptor agonists was also measured here.

### *2.2.4 E/c curve correction method used for the present study*

If a neglected, surplus agonist concentration is present when an E/c curve is constructed with an agonist using the same signaling as the neglected agonist, then a distortion on this E/c curve (i.e. a virtual decrease in the effect) will develop. This E/c curve distortion is proportional to the magnitude of the surplus agonist concentration that enables the determination of this latter via curve fitting. This is the receptorial responsiveness method (RRM) that can be performed with several regression setting options. RRM forms the base of another method designed to correct an E/c curve for the distortion caused by the surplus agonist concentration. An E/c curve corrected this way has two informative parts: the initial one (if the first agonist concentration, used for the E/c curve, elicits very little or no effect), and the final one (in the case of a saturated E/c curve, i.e. a curve with well-defined top plateau). The initial part shows the effect value evoked (solely or mainly) by the surplus agonist concentration (because of which the correction is needed). In turn, the final part of the corrected E/c curve indicates the real maximal effect value that has been falsified on the uncorrected E/c curve.

### *2.2.5 Empirical characterization of the E/c curves*

All E/c curves were fitted to the Hill equation, a simple and reliable model of receptor function, that provided three empirical parameters to geometrically describe the E/c curves.

### *2.2.6 Quantifying the bias caused by CBD in the averaged CPA E/c curve*

CBD, by inhibiting the inward myocardial adenosine transport, was assumed to accumulate surplus adenosine in the atrial interstitium and thereby to distort the E/c curves generated in the CBD-treated Obese ZDF group. The concentration of this surplus interstitial adenosine was quantified with RRM using the combination of two independent settings: individual vs. global fitting, and ordinary vs. robust fitting. During individual fitting, the fitting of averaged CPA E/c curve of the CBD-treated Obese ZDF group we use the three empirical parameters of the averaged CPA E/c curve of the Obese ZDF group. When fitting globally, the averaged CPA E/c curves of both groups mentioned above were simultaneously fitted this time with variable empirical parameters. For every other regression setting, the default option was chosen.

### *2.2.7 Correction of effects of all averaged E/c curves biased by CBD*

The effect values of the averaged CPA and adenosine E/c curves of the CBD-treated Obese ZDF group were corrected using $c_x$ obtained with individual and ordinary fitting (similarly to our previous E/c curve corrections. First, the effect value belonging to this $c_x$ was determined using the Hill equation

From this $E_x$ and the distorted effect values of the averaged CPA and adenosine E/c curves of the CBD-treated Obese ZDF group, corrected effect values were computed using. The corrected effect values reflect the combined effect of the surplus interstitial adenosine caused by CBD and the agonist (CPA or adenosine) administered during the generation of the given E/c curve. Nonetheless, the corrected effect values were plotted against solely the administered agonist concentrations since the exact value of the surplus interstitial adenosine produced by CBD could not be determined.

## 2.3 Data analysis

Each atrium was required to meet three criteria in order to qualify for inclusion in the further evaluation: (i) the resting contractile force had to reach 1 mN before the adenosine E/c curve; (ii) the mechanical activity of the paced atrium had to be regular; (iii) the response to 10 μM adenosine was required to be within the mean ± 2 SD range (if more than one adenosine E/c curves occurred, this requirement applied to the first one). The mean and SD were computed using atria meeting the first two criteria.

Gaussian distribution of data and homogeneity of variances were tested with the Shapiro-Wilk test and Brown-Forsythe test, respectively. Since all datasets showed Gaussian distribution and homogenous variances, ordinary one-way ANOVA followed by Tukey post-testing was performed for comparison. The difference of means was considered significant at $p < 0.05$.

Curve fitting and statistical analysis were performed with GraphPad Prism 8.4.3 (686) 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 *Results related to FSCPX*

DMSO, present in the wells even in 10% (*v/v*), did not significantly influence the activity of CD39 and CD73. POM-1, an inhibitor of E-NTPDase1-3 enzymes and antagonist of some P2 purinergic receptors, significantly but incompletely reduced the activity of CD39. Interestingly, POM-1 also inhibited CD73, at least to the same extent as it inhibited CD39. (Starting from the fact that the level of inhibition barely increased by increasing the POM-1 concentration from 20 μM to 200 μM, this could be the maximal inhibitory effect of POM-1 for both enzymes in these assays.) In turn, PSB-12379, an inhibitor recently developed for CD73, significantly decreased the activity of CD73, which action was selective (did not cover to CD39) and complete at 1 μM. However, neither FSCPX nor CPX appeared to significantly affect the activity of either CD39 or CD73, despite the high concentrations administered.

We investigate the interaction between FSCPX and NBMPR on rat left atrium. Adenosine, the non-selective, physiological adenosine receptor agonist, concentration-dependently decreased the resting contractile force of rat left atria in all groups used for this investigation. Responses to adenosine did not differ significantly, at any concentrations, among the groups (handled uniformly at this stage of the experiments). This result denotes the homogeneity of the groups regarding the main adenosinergic mechanisms. CHA, a selective, synthetic $A_1$ receptor full agonist, also reduced the resting contractile force of rat left atria in a concentration-dependent manner. Responses to CHA did not differ significantly between the two control groups, indicating that the difference in the duration of their protocols did not significantly influence the effect of CHA on the atria. (As the protocols using FSCPX were considerably longer than the other ones, two control protocols, a long (Control for FSCPX & FSCPX+NBMPR) and a short one (Control for NBMPR), were elaborated for the proper comparison with protocols using and not using FSCPX. Regarding the response to CHA after different *in vitro* treatments, we found that when administered alone, both FSCPX and NBMPR blunted the effect of CHA in the rat left atrium in a statistically significant manner: FSCPX pushed the CHA E/c curve considerably to the right without reducing the maximal response, while NBMPR moderately decreased the

response to CHA (seemingly including the maximal response). However, a pretreatment with FSCPX made the effect of NBMPR on the response to CHA negligible: the CHA E/c curves in the FSCPX and FSCPX+NBMPR groups practically coincided. These results provided by rat atria corroborate the results of our previous investigation using guinea pig atria. Although sole effects of FSCPX and nitrobenzylthioinosines on the response to the synthetic $A_1$ receptor agonists, regarding their magnitude (but not direction), exhibit considerable differences between the rat and guinea pig models, it is clear that FSCPX pretreatment reduced the effect of nitrobenzylthioinosines in atria of both species.

We investigate the interaction between PSB-12379 and NBTI on guinea pig left atrium. Regarding the first adenosine E/c curves of the groups (before any *in vitro* treatment), adenosine concentration-dependently decreased the resting contractile force of guinea pig left atria. The response to adenosine did not differ significantly among the groups at any concentrations, proving the homogeneity of the groups in terms of the adenosinergic mechanisms. Similarly, when compared the third adenosine E/c curves of groups Control+NBTI+PSB (for Ado) and Control+PSB+NBTI (for Ado), responses to adenosine did not differ significantly at any concentrations. Thus, the previous treatments (with NBTI or with PSB-12379) did not disturb the outcome of the final co-treatment with PSB-12379 and NBTI. (In the absence of prior knowledge, it was unclear whether a previous NBTI or PSB-12379 treatment could influence a subsequent NBTI and PSB-12379 co-treatment. Thus, the co-treatment with NBTI and PSB-12379 was performed after both NBTI and PSB-12379 treatments. Accordingly, these groups were named Control+NBTI+PSB (for Ado) and Control+PSB+NBTI (for Ado) groups, respectively.) CPA, a selective, synthetic $A_1$ receptor full agonist, also decreased the resting contractile force of guinea pig left atria in a concentration-dependent manner. The effect of NBTI was similar to that of NBMPR in the rat atrium and practically the same as our earlier results with NBTI in the guinea pig atrium. When added alone, PSB-12379, a selective inhibitor of CD73 with no apparent effect on the $A_1$ receptor, did not significantly influence the effect of CPA, in contrast to FSCPX. However, PSB-12379 significantly blunted the effect of NBTI on the response to CPA, just like FSCPX did. The only difference between the "anti-NBTI" effects of PSB-12379 and FSCPX was that the former was weaker than the latter. Similar to the results with CPA, PSB-12379, administered alone, did not

significantly affect the effect of adenosine. Importantly, also consistent with the results with CPA, PSB-12379 significantly inhibited the effect of NBTI on the response to adenosine; more precisely, it inhibited the so-called general E/c curve modifying effect of NBTI. Thus, PSB-12379, when co-administered with NBTI, moderately but statistically significantly increased the response to adenosine as compared to the adenosine E/c curve generated in the presence of NBTI alone. Also, in line with the results with CPA, this effect of PSB-12379 was weaker than that of FSCPX. In summary, these results demonstrate that PSB-12379, which restricted the interstitial adenosine formation by blocking its final step, produced the same kind of transformation in the CPA and adenosine E/c curves generated in the presence of NBTI as the pretreatment with FSCPX did in these curves, of course in addition to the consequences of $A_1$ receptor blockade. This finding has provided indirect evidence for an inhibitory effect of FSCPX, a verified irreversible $A_1$ receptor antagonist, on the interstitial adenosine production in the heart (or, at least, in the rat and guinea pig left atrium). Starting from our experimental conditions, this additional effect of FSCPX appeared also to be irreversible.

We investigate the influence of different administration regimens on the effect of FSCPX on rat and guinea pig left atria. Adenosine concentration-dependently diminished the resting contractile force of left atria, which diminution was greater for guinea pigs than for rats. The effect of adenosine did not differ significantly, at any concentrations, among the groups related to the same species. As the different groups received the same treatment until this stage of these experiments, this result indicates the homogeneity of the groups, within the same species, regarding the adenosinergic mechanisms. In addition, a greater sensitivity of the (Hartley type) guinea pig atrium to adenosine (in terms of the direct negative inotropy) was demonstrated as compared to the (Wistar type) rat atrium. As expected, CPA reduced the resting contractile force of atria in a concentration-dependent manner. The response of guinea pig left atria to CPA was spectacularly greater than that of rat ones. The FSCPX pretreatment produced a substantial dextral displacement of the CPA E/c curves with no diminution of the maximal effect (in comparison with the DMSO-pretreated control CPA E/c curves), in both species. However, the magnitude of this antagonistic effect exerted by FSCPX statistically significantly depended on the number of administrations of FSCPX to the bathing medium of atria. During the single addition of FSCPX, the

antagonistic effect was the weakest, upon both species, in comparison with cases when FSCPX was administered more than once (not exceeding a concentration of 10 μM in the organ baths at any time. Regarding the rat atria, increase of number of administrations of FSCPX from two to five did not enhance the irreversible antagonism elicited by FSCPX. In contrast, in the guinea pig atria, when FSCPX was administered five times (of course not exceeding the 10 μM concentration), the antagonism was the strongest among the FSCPX-pretreated samples. Importantly, even quintuple administration of FSCPX ("FSCPX regimen with five cycles") was not able to reduce the maximal effect of CPA, in both species. For the rat atria, fitting of the CPA E/c curve data (averaged within the groups) to the Hill model provided moderately different $E_{max}$ and Hill coefficient (n) values with substantially different $logEC_{50}$ values. As FSCPX is a verified irreversible antagonist for the $A_1$ receptor, this result is indicative of a great $A_1$ receptor reserve for the direct negative inotropic effect of CPA in the atrial myocardium of both species. Starting from this observation, we also used global fitting with shared $E_{max}$ and n parameters to illustrate the influence of the different FSCPX administration regimens. Regarding the rat atria, the effect of FSCPX could only be enhanced by increasing the number of FSCPX administrations until two, probably due to reaching the maximal effect of FSCPX in this system. In turn, with regard to the guinea pig atria, the more cycles in the FSCPX administration regimen of a group existed, the bigger the $logEC_{50}$ value of the given group was. These observations are consistent with the location of the CPA E/c curves of the different FSCPX-pretreated groups.

## 3.2 *Results related to CBD*

Regarding the response to CPA and adenosine, we found that both CPA and adenosine evoked a concentration-dependent decrease in the atrial contractile force in all groups. However, while CPA, a relatively stable, poorly transported, synthetic $A_1$ adenosine receptor agonist, elicited the weakest response in the CBD-treated Obese ZDF group, adenosine, the quickly metabolized and transported physiological adenosine receptor agonist, evoked the strongest effect just in this group. In the Lean ZDF group, the behavior of atria towards CPA and adenosine was comparable to that observed in the Obese ZDF group (received vehicle treatment).

Investigation related of surplus interstitial adenosine produced by CBD showed that the distortion seen on the averaged CPA E/c curve of the CBD-treated Obese ZDF group (shortly: the CBD-treated CPA E/c curve) as compared to the averaged CPA E/c curve of the Obese ZDF group (shortly: the intact CPA E/c curve) was evaluated with RRM, a method based on regression. The regression, performed with individual or global fitting in combination with ordinary or robust fitting, provided four $c_x$ values as surrogates for the surplus interstitial adenosine caused by CBD, expressed as CPA concentrations being equieffective with it. In line with our expectation, the four $c_x$ values were within a narrow range, although one of them (resulted from global and ordinary fitting) was considerably smaller than the others. From the three $c_x$ values being close to each other, we selected the one that was obtained with individual and ordinary fitting for further use. In addition, two further $c_x$ values were yielded by the global fitting. These additional $c_x$ values served as an internal control for the curve fitting, with zero as an expected value (because of the absence of CBD).

The corrected effects of the CPA and adenosine E/c curves were investigated on CBD-treated rat atria. The corrected CBD-treated CPA E/c curve started from an about 25% effect value, indicating a considerably strong response that can be attributed to the surplus interstitial adenosine caused by CBD. Thus, CBD produced a “quarter-effective” adenosine concentration ($EC_{25}$) in the atrial interstitium of the obese ZDF rats. The initial effect value of the corrected CBD-treated adenosine E/c curve was somewhat above 25% since the uncorrected initial effect value was unusually great, about 11%. As expected, the final part of the corrected CBD-treated CPA E/c curve did not hold extra information in comparison with the final part of the intact CPA E/c curve. At low and medium concentrations, the corrected CBD-treated CPA E/c curve ran above the intact CPA E/c curve, whereas at high concentrations, they practically reached the same maximum. The result that the corrected and the intact CPA E/c curves shared approximately the same maximum meant that the data used were reliable. In contrast, the corrected CBD-treated adenosine E/c curve considerably exceeded the intact adenosine E/c curve at all concentrations including the highest ones. Thus, the real maximal effect of the CBD-treated adenosine E/c curve was greater than the maximal effect of the intact adenosine E/c curve, indicating that the CBD treatment augmented the maximal response to adenosine. Unfortunately, as the exact values of the interstitial adenosine concentration in the microenvironment of the

myocardial $A_1$ adenosine receptors remained unknown, the corrected effect values could only be plotted against the exogenous adenosine concentrations in the bathing medium (that could be computed).

# 4 Discussion

## 4.1 Conclusions related to FSCPX

By providing a body of indirect evidence, results of the present study have corroborated our earlier hypothesis that FSCPX has an additional action besides the $A_1$ adenosine receptor antagonism, through which it decreases the interstitial adenosine level. Furthermore, FSCPX seems to exert its effects in the cell membrane, in which it may accumulate after administration, while its fraction remaining dissolved in a water-based solution rapidly decomposes. As CD39 (ecto-apyrase) and CD73 (ecto-5'-nucleotidase) enzymes are membrane-bound (just like the $A_1$ receptor), CD39 and CD73 have remained the most likely possible targets for the additional action of FSCPX. Incidentally, we have found that POM-1, thought to be an inhibitor of E-NTPDase1-3 enzymes (including E-NTPDase1 *a.k.a.* CD39) and antagonist of some P2 purinergic receptors, inhibits CD73 as well.

Our *ex vivo* results are predominantly based on evaluating E/c curve transformations, from which conclusions have been drawn regarding the underlying mechanisms. FSCPX pushed the E/c curve of both CPA and adenosine to the right (in comparison with the appropriate Control E/c curves) that reflects the loss of $A_1$ receptors, consistent with the irreversible $A_1$ receptor antagonist nature of FSCPX. NBTI (the nitrobenzylthioinosine derivative used) pushed the E/c curve of CPA to the right and decreased the maximal effect. Since nitrobenzylthioinosines do not antagonize the $A_1$ receptor, the above-mentioned phenomenon indicates an effect that diminished the response of the $A_1$ receptors (expressed by the E/c curves) in some other manner. This is the so-called general E/c curve modifying effect of NBTI that has been attributed to the interstitially accumulated endogenous adenosine produced by the inhibition of the inward adenosine transport elicited by NBTI. The effect of NBTI on the adenosine E/c curve is somewhat more complex because it results from two components: the general E/c curve modifying effect and a specific one, which enhances the $A_1$ receptor response by increasing the amount of exogenous adenosine (via inhibition of adenosine transport that prevents exogenous adenosine from the intracellular elimination).

If the effects of FSCPX and NBTI were simply added together, the E/c curves

reflecting the co-action would be shifted to the right from the NBTI-treated E/c curves, but this is not the case. Instead, the FSCPX+NBTI E/c curves show a sinistral displacement from the NBTI-treated E/c curves and exceed them (irrespective of the agonist used), indicating a significant interaction between effects of FSCPX and NBTI. Based on *ex vivo* and *in silico* pieces of evidence, this interaction was finally attributed to a previously unknown effect of FSCPX, by which it can reduce the general E/c curve modifying effect of NBTI. The possible mechanism of this additional effect of FSCPX is the main topic of the present study.

Accordingly, the essence of our *ex vivo* investigations was a reverberation between the shape of E/c curves and the molecular causes determining it: interpreting an E/c curve transformation based on the known mechanisms of action of agents used and, conversely, assuming a mechanism of action from E/c curve transformations observed. During this process, we built up our final conclusion from the acquired knowledge step by step.

In earlier studies dealing with E/c curves generated in isolated and paced guinea pig left atria, we found that FSCPX paradoxically increased the maximal response to adenosine and CPA, in the presence of NBTI. In the background of this paradoxical phenomenon, we hypothesized that FSCPX might blunt the activity of one or some interstitial adenosine-forming enzyme(s), most likely CD39 and/or CD73, the two main ectonucleotidases in the heart. The aim of the present study was to verify or reject this hypothesis, preferably by providing direct evidence for or against the enzyme inhibitory activity of FSCPX.

In the present study, direct evidence for the ectonucleotidase inhibitory action of FSCPX would have been the inhibition of CD39 and/or CD73 by FSCPX during a reliable *in vitro* inhibitor assay. However, when using kits containing the enzymes and their verified and putative inhibitors in an aqueous solution, neither CD39 nor CD73 were inhibited by FSCPX. The cause of this outcome can be the fast degradation of FSCPX (in 2-3 min) in aqueous solutions. Additionally, CPX, a selective and reversible antagonist of the $A_1$ receptor, the initial structure for the synthesis of FSCPX, also failed to inhibit CD39 and CD73. As CPX, apart from its poor water solubility, seems to be sufficiently stable in water, it can be concluded that this part of FSCPX itself does certainly not responsible for any ectonucleotidase inhibitory action.

To resolve the contradiction between *ex vivo* efficiency and *in vitro* inefficiency of FSCPX, we have assumed that FSCPX, after administration, rapidly enters the lipid compartment of the tissues (probably mostly cell membranes), while its fraction remaining dissolved in aqueous solutions quickly decomposes. Thus, FSCPX might act on structures in/on the lipid compartment, such as membrane-associated proteins. Indeed, the $A_1$ receptor, CD39 and CD73 are all membrane-bound proteins. If this assumption is true, FSCPX cannot be examined with water-based kits.

At this point of the study, we returned to the *ex vivo* approach. First, we aimed to confirm the original phenomenon (FSCPX paradox), so we repeated the most reliable part of the original experiment (where a synthetic $A_1$ receptor agonist was applied) with some modifications: instead of CPA, NBTI and guinea pig, we used CHA, NBMPR and rat, respectively. These modifications served the purpose of reducing the influence of accidental properties of the previously used animal model and agents on the outcome.

We found that FSCPX paradox appeared in the rat model as well. To make this clear, the two nitrobenzylthioinosine-treated E/c curves (without and with an FSCPX pretreatment in the same animal model) had to be compared to their real controls. Thus, the solely NBMPR- and solely NBTI-treated E/c curves were contrasted with the appropriate Control E/c curves, whereas the FSCPX+NBMPR- and FSCPX+NBTI-treated E/c curves with the appropriate solely FSCPX-treated E/c curves (of course, within the same species). This comparison showed that the FSCPX pretreatment drastically decreased the effect of the given nitrobenzylthioinosine derivative on the E/c curve of the given synthetic $A_1$ receptor agonist. As this effect of NBMPR and NBTI is exclusively the so-called general E/c curve modifying effect that is mediated by an increase in the interstitial concentration of endogenous adenosine, it can be concluded that the FSCPX pretreatment can reduce the interstitial adenosine formation. Thus, results of this investigation (about the interaction of FSCPX with NBMPR in the rat atrium) corroborated our previous results displaying the FSCPX paradox.

As the next step, we aimed to investigate whether there is an interaction between an agent, which has been proven to restrict the interstitial adenosine production and is unable to antagonize the $A_1$ receptor, and a nitrobenzylthioinosine

derivative. Thus, this time we repeated our whole original experiment with the replacement of FSCPX with PSB-12379. We found that the presence of PSB-12379 transformed the CPA and adenosine E/c curves the same way as the FSCPX pretreatment did, apart from the dextral displacement of E/c curves caused by the $A_1$ receptor antagonist action of FSCPX that did not appear in response to PSB-12379. Namely, the general E/c curve modifying effect of NBTI, manifested in a decrease of $E_{max}$ and increase of $EC_{50}$, prevailed to a less extent, when PSB-12379 was also present. Interestingly, the pretreatment with 10 μM FSCPX had visibly a stronger influence on the NBTI-treated E/c curves than the presence of 3 μM PSB-12379, although even 1 μM PSB-12379 exerted maximal CD73 inhibitory effect in our *in vitro* investigation. It may be speculated that the highly lipid-soluble FSCPX can more efficiently inhibit the membrane-bound CD73 than the highly water-soluble PSB-12379. In summary, restriction of the interstitial adenosine formation mimicked the effect of FSCPX other than $A_1$ receptor antagonism.

Finally, we aimed to get evidence to support our assumption regarding the kinetic aspects of actions of FSCPX. For this purpose, we investigated the influence of increasing number of administrations on the efficiency of FSCPX in terms of $A_1$ receptor antagonism in the rat and guinea pig atria. We found that the effect of FSCPX could be enhanced by increasing the number of FSCPX administrations until two (in rat) and until five, the applied maximum (in guinea pig). This outcome supports our assumption on the priority role of cell membrane in the development of actions of FSCPX, and thereby provides a possible resolution for the contradiction between *ex vivo* efficiency and *in vitro* inefficiency of FSCPX in terms of the inhibitory action on CD39 and/or CD73. This kinetic property of FSCPX explains why mechanisms of action of this agent are difficult to explore.

Taking all together, instead of one overriding piece of direct evidence, we have collected three pieces of indirect evidence for the additional activity of FSCPX, by which it inhibits the interstitial adenosine-accumulating effect of nucleoside transport blockers. Declaring that clarification of the exact molecular mechanism of the action of FSCPX in addition to the irreversible $A_1$ receptor antagonism warrants further investigations, we propose that FSCPX has the ability to modify a membrane-bound target, as a result of which the interstitial adenosine production in the myocardium (or, at least, in the supraventricular one) narrows. Nevertheless, currently our experimental

approach is the only *ex vivo* system that has enabled the exposure and further investigation of the FSCPX paradox.

## 4.2 Conclusions related to CBD

To the best of our knowledge, the results of our studies on CBD, which form the basis of this thesis is the first study that has provided functional evidence about the adenosine transport inhibitory effect of CBD, a promising non-intoxicating phytocannabinoid, in the myocardium. The chronic oral administration of CBD to obese ZDF rats significantly increased the response of their isolated, paced left atria to adenosine, suggesting the presence of a long-term enhanced adenosinergic protection in the heart.

CPA evoked a significantly smaller response in the atria isolated from the CBD-treated obese ZDF rats than in the atria of the vehicle-treated ones. In contrast, adenosine elicited a significantly greater response in the CBD-treated atria than in the vehicle-treated ones, but only at low and medium concentrations. According to the earlier experiences of our work team, this pattern is typical of the condition when the myocardial adenosine transport is blocked. The reason for this is as follows: Upon sufficient oxygen supply, the myocardial adenosine transport is directed into the cells, so its inhibition increases the interstitial level of endogenous adenosine. This surplus interstitial adenosine, in part, uses up the response capacity of the adenosine receptors (prior to the generation of an E/c curve). Hence, a poorly transported adenosine receptor agonist (like CPA) cannot evoke an effect as great as that seen with intact transport. Contrary to this, when a quickly metabolized and transported adenosine receptor agonist (like adenosine) is administered, two opposing effects prevail: the above-mentioned distorting effect that virtually reduces the response, and another effect that really augments the response, since the transport blockade protects also this metabolizable and transportable exogenous agonist from the intense intracellular elimination

In the present study as regards the CBD-treated atria: we have found weaker responses to all CPA concentrations, while stronger responses to low and medium adenosine concentrations and unchanged responses to high adenosine concentrations

(in comparison with the vehicle-treated atria).

According to several studies, CBD was found to inhibit ENT1. This inhibitory effect was reported to be relatively strong and concentration-dependent, to evolve from relatively low, 100 nanomolar concentrations, and it was evidenced in neurons, macrophages, retinal and brain microglial cells, but not in the heart. Our present study is the first to provide functional evidence about the ENT1 inhibitory effect of CBD in the myocardium.

To properly exhibit and evaluate the ENT1 inhibitory effect of CBD, some adjustment on the E/c curves representing the CBD-treated condition was needed. As mentioned above, if neglected, the presence of a surplus interstitial adenosine biases the conventionally evaluated and plotted E/c curves of adenosine receptor agonists, namely it causes a virtual (and not real) decrease in the response to these agonists (that can be spectacular or barely noticeable, depending on the circumstances. To solve this problem, previously we elaborated a method, by which this E/c curve distortion can be corrected. With this method, it is possible to obtain E/c curve effect values that provide information on the magnitude of the cause of the distortion (looking at the initial part of the corrected E/c curve) and on the real receptorial responsiveness.

The corrected CBD-treated CPA E/c curve started from an about 25% effect value, indicating that CBD accumulated circa the $EC_{25}$ value of adenosine in the microenvironment of the myocardial $A_1$ adenosine receptors (in terms of the direct negative inotropic effect). Furthermore, the final part of the corrected CBD-treated adenosine E/c curve shows a considerably stronger maximal response than that of the vehicle-treated counterpart. These results imply that the long-term oral CBD treatment maintains a continuous, moderately elevated basal adenosinergic activity in the heart, furthermore it enhances the response to exogenous adenosine.

Nevertheless, it should be noted that our correction method ascribes all distortions manifested in a smaller response to an extra, unaccounted agonist concentration, which is here the surplus interstitial adenosine concentration accumulated by CBD. However, because of the chronic presence of a surplus adenosine, the issue of downregulation and/or desensitization of the $A_1$ adenosine receptor should also be addressed. In a previous work, we found that the responsiveness of the myocardial $A_1$ adenosine receptor did not decrease in the time

window of our ex vivo experiments. Based on this, in our earlier studies dealing with the acute consequences of adenosine transport inhibition, no decrease in the (real) responsiveness of the $A_1$ adenosine receptor was considered. Consistent with this, the $A_1$ adenosine receptor was reported to be desensitized extremely slowly, even in the presence of significant amounts of full agonists for several weeks.

The four-week CBD treatment applied in the present study, however, was long enough to consider the possibility of the desensitization of the $A_1$ adenosine receptor. If there was some desensitization, the response to adenosine after the four-week CBD treatment may have been not as enhanced as it looks on the corrected adenosine E/c curve. The reason for this is that, upon desensitized receptors, the decrease in the response stems in part from the receptor desensitization, so the correction method overestimates the concentration of the surplus agonist that leads to an overcorrection of the distorted E/c curve. However, when we investigated the conventionally evaluated adenosine E/c curves, the long-term CBD treatment indisputably increased the response to adenosine at concentrations from 1 nmol/L to 10 μmol/L, an important range regarding cardioprotection. Thus, it can be concluded that the long-term oral CBD treatment significantly augmented the adenosinergic signaling of the heart even if the myocardial $A_1$ adenosine receptors underwent some downregulation and/or desensitization.

The observation that the presence of CBD in the heart led to $A_1$ adenosine receptor activation has raised the possibility that CBD might act as an $A_1$ adenosine receptor agonist. If so, CBD, persisting in the myocardium of the CBD-treated rats, had to act also as a surplus $A_1$ adenosine receptor agonist. Based on this, CBD, as a surplus $A_1$ adenosine receptor agonist, should have decreased the virtual response to both CPA and adenosine. However, in our present investigation, the in vivo CBD treatment decreased the response only to CPA, while it increased the response to adenosine. Thus, under our experimental conditions, CBD behaved as an adenosine transport blocker rather than an $A_1$ adenosine receptor agonist. This result may offer strong evidence in the debate concerning whether the adenosine transport blocker or the adenosine receptor agonist property dominates the effect of CBD on the cardiac adenosinergic signaling.

Finally, the results of the present study rest on the assumption that the in vivo

administered CBD was present in a sufficient amount in the atria to inhibit ENT1 during our ex vivo experiments. Our reason to assume this is the highly lipophilic nature of CBD. In our recent study, FSCPX, another highly lipophilic agent, was found to accumulate and persist in the lipid compartment of the isolated atria, where it was able to exert its effects for a long time. Thus, it is reasonable to presume that CBD, administered *in vivo*, can persist in the atria long enough to elicit its effect ex vivo on the transmembranous ENT1.

# 5 Summary

In the first part of our investigations, we examined a phenomenon called FSCPX paradox that has been detected during previous *ex vivo* studies of our work team. According to this paradox, FSCPX, a selective $A_1$ receptor antagonist, increased the maximal direct negative inotropic effect, shown in the E/c curves of $A_1$ receptor agonists, in the isolated, paced guinea pig left atrium, under nucleoside transport blockade. Based on *in silico* results, we hypothesized that FSCPX reduces the interstitial accumulation of endogenous adenosine during nucleoside transport inhibition, which may be due to an inhibition of CD39 and/or CD73, the two main enzymes responsible for the production of interstitial adenosine in the heart. We found three indirect pieces of evidence to prove our hypothesis: (i) we have confirmed the reproducibility of the FSCPX paradox in another animal model using another $A_1$ receptor agonist and another nucleoside transport inhibitor; (ii) we have confirmed the imitability of the FSCPX paradox using a CD73 inhibitor lacking of $A_1$ receptor antagonist property; (iii) we have confirmed the strong lipophilicity of FSCPX, which could be the reason for the failure of water-based CD39 and CD73 inhibitor assays, and it can also explain the *ex vivo* effectiveness of FSCPX in a way that it suggests that the fraction of FSCPX, which enters the membranes, is responsible for the blunting (antagonistic and inhibitory) effects. These three pieces of indirect evidence together are convincing enough to continue to assume that FSCPX inhibits the interstitial adenosine production.

In the second part of our investigations, we studied the effect of a long-term, oral CBD treatment on the adenosinergic system of isolated, stimulated left atria of obese-type ZDF rats. CBD reduced the direct negative inotropic effect of a stable, poorly transported $A_1$ receptor agonist; however, it increased the direct negative inotropic effect of the rapidly metabolized and transported adenosine. To the best of our knowledge, this result is the first evidence of the inhibitory effect of CBD on the adenosine transport in the myocardium, which suggests that, with long-term, oral CBD treatment, the heart can be kept under permanently enhanced adenosinergic protection even under the conditions typical of type 2 diabetes mellitus. Our results also support that the inhibitory property of nucleoside transport is mainly (or exclusively) responsible for inducing or enhancing adenosinergic activation, an effect evoked by CBD, and that it is unlikely that CBD has a direct $A_1$ receptor stimulatory property to a significant extent.

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## List of publications related to the dissertation

1. **Viczján, G.**, Szilágyi, A. T., Takács, B., Óvári, I., Szekeres, R., Tarjányi, V., Erdei, T. D., Teleki, V., Zsuga, J., Szilvássy, Z., Juhász, B., Varga, B., Gesztelyi, R.: The effect of a long-term treatment with cannabidiol-rich hemp extract oil on the adenosinergic system of the Zucker Diabetic Fatty (ZDF) rat atrium.
   *Front. Pharmacol. 13*, 1-12, 2022.
   DOI: http://dx.doi.org/10.3389/fphar.2022.1043275
   IF: 5.6

2. **Viczján, G.**, Erdei, T. D., Óvári, I., Lampé, N., Szekeres, R., Bombicz, M., Takács, B., Szilágyi, A. T., Zsuga, J., Szilvássy, Z., Juhász, B., Gesztelyi, R.: A Body of Circumstantial Evidence for the Irreversible Ectonucleotidase Inhibitory Action of FSCPX, an Agent Known as a Selective Irreversible A1 Adenosine Receptor Antagonist So Far.
   *Int. J. Mol. Sci. 22* (18), 1-21, 2021.
   DOI: http://dx.doi.org/10.3390/ijms22189831
   IF: 6.208

## List of other publications

3. **Viczján, G.**, Óvári, I., Erdei, T. D.: A kannabidiol és az adenozinerg rendszer kapcsolata.
   *Eü. Innov. Szle. 2* (2), 8-13, 2023.

4. Óvári, I., Szilágyi, V., **Viczján, G.**, Lampé, N., Bege, M., Borbás, A., Herczeg, P., Juhász, B., Gesztelyi, R., Erdei, T. D.: Egy újonnan szintetizált adenozin analóg, a hipoxantin-triciklánó hatása izolált patkány bal és jobb pitvaron.
   *Eü. Innov. Szle. 1* (1), 9-16, 2022.

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5. 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), 1-14, 2019.
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6. 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.
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