doi: 10.4149/gpb_2024033

The *ex vivo* effects of hypoxanthine-tricyclano, a synthetic adenosine analogue, on rat left and right atria

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Abstract. Hypoxanthine-tricyclano is a synthetic adenosine analogue, in which adenine and ribose have been replaced by hypoxanthine and a morpholino-derived tricyclic moiety, respectively. We investigated whether hypoxanthine-tricyclano could influence atrial inotropy and/or chronotropy, two important functions regulated by the A_1 receptor, the main adenosine receptor type of the supraventricular myocardium. Paced left atria and spontaneously beating right atria, isolated from male, 30–35 weeks old, Wistar rats, were used. The ino- and chronotropic effects of adenosine and hypoxanthine-tricyclano (separately and together) were assessed in the absence and presence of 8-cyclopentyl-1,3-dipropylxanthine (CPX), a selective, orthosteric, reversible A_1 adenosine receptor antagonist. We found that adenosine exerted a strong negative inotropic effect (similar in left and right atria). However, hypoxanthine-tricyclano elicited a moderate positive inotropic effect (also similar in all atria). In right atria, adenosine evoked a robust negative chronotropic effect, whereas hypoxanthine-tricyclano produced a slight positive chronotropy. CPX blunted the effects of both adenosine and hypoxanthine-tricyclano, although this antagonism was strong (and significant) for adenosine, while smaller (and non-significant) for hypoxanthine-tricyclano. Both effects of hypoxanthine-tricyclano were easily surmountable with adenosine. Thus, hypoxanthine-tricyclano may act as a week, orthosteric, reversible, inverse and low-affinity agonist of the A_1 receptor, although alternative mechanisms of action cannot be excluded.

Key words: Adenosine — Hypoxanthine-tricyclano — Rat — Atrium (*ex vivo*) — A_1 adenosine receptor — Inotropic effect — Chronotropic effect

Introduction

Proper functioning of the cardiovascular system is essential for maintaining good health and quality of life. Cardiovascular diseases claim 3,9 million lives in Europe *per* year, which accounts for 45% of all deaths. Death rates of ischemic heart disease and stroke are higher in Central and Eastern Europe than those in other regions of this continent (Wilkins et al. 2017: http://www.ehnheart.org/images/CVD-statistics-report-August-2017.pdf).

Purinergic transmission, including the adenosinergic one, is one of the most ancient and general regulatory systems in the living organisms. Adenosine, the main endogenous agonist of the adenosine receptors (Fredholm et al. 2001,

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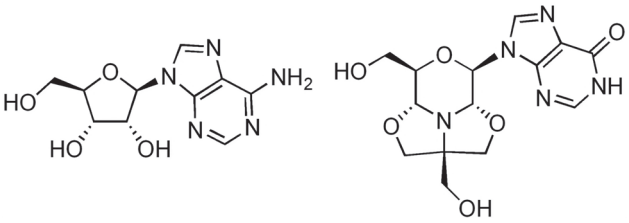

Figure 1. Chemical structure of adenosine (left) and hypoxanthine-tricyclano (right) (Kicsak 2018; Kicsak et al. 2018).

2011), exerts its effects through several signaling pathways contributing to protective and reparative processes (Fredholm et al. 2001, 2011; Geldenhuys et al. 2017; Sousa and Diniz 2017; Reiss et al. 2019; IJzerman et al. 2022).

However, adenosine itself is only suitable for the acute treatment of some supraventricular arrhythmias and acute myocardial infarction, as its therapeutic value is limited by its short half-life (Rankin et al. 1992; Szentmiklosi et al. 2015; Batra et al. 2021). Another disadvantage of using adenosine is the excessively broad spectrum of its effects, due to the ubiquitous occurrence of adenosine receptors (Dhalla et al. 2003). Nevertheless, adenosine can serve as a lead compound ("template") for the development of new drugs targeting adenosine receptors.

By modifying adenosine, an almost unlimited number of new molecules can be acquired. In addition, certain chemical structures less similar to adenosine can also act on adenosine receptors. Some of these synthetic compounds have been reported to be kinetically and/or dynamically superior to adenosine, showing slower elimination and/or selectivity for certain adenosine receptor types or subtypes, and/or exhibiting antagonist, partial agonist, biased agonist or allosteric modulator activity. Adenosine receptor agonists and enhancers can exert anti-ischemic, antiarrhythmic, vasodilatory, analgesic, antidiabetic, neuroprotective, anti-inflammatory, antifibrotic, immunomodulatory, antiproliferative, antineoplastic and antiviral effects (Szentmiklosi et al. 2011, 2015; Lubecka-Pietruszewska et al. 2014; Burnstock and Pelleg 2015; Deng et al. 2016; Cronstein and Sitkovsky 2017; Prejs et al. 2019; Franco et al. 2021; McNeill et al. 2021; IJzerman et al. 2022; Jacobson et al. 2023; Kutryb-Zając et al. 2023; Perfilova et al. 2023; Vincenzi et al. 2023).

Regarding the inherent protective mechanisms of the cardiomyocytes, the A_1 type of adenosine receptors (A_1 receptor) seems to be one of the most important actors (Headrick et al. 2011, 2013; Guieu et al. 2021). Thus, the A_1 receptor is a promising target for new drug candidates to improve the condition of the heart and to treat certain heart diseases. Several selective A_1 receptor agonists entered clinical trials, including adenosine analogues, e.g. tecadenoson (to convert paroxysmal supraventricular tachycardia to sinus rhythm: Ellenbogen et al. 2005), selodenoson (to slow heart rate in atrial fibrillation: Kiesman et al. 2009) and trabodenoson (to treat ocular hypertension and primary open-angle glaucoma: Spinozzi et al. 2021), furthermore compounds only remotely resembling adenosine, such as capadenoson (to treat angina pectoris: Tendera et al. 2012) and neladenoson (to treat chronic heart failure: Meibom et al. 2017). Further diseases and pathological conditions thought to be improved by A_1 receptor activation are ischemic heart disease, acute myocardial infarction, cardiac fibrosis, myocardial hypertrophy, cardiac remodeling and certain other supraventricular arrhythmias (Szentmiklosi et al. 2011, 2015; Dinh et al. 2017; Geldenhuys et al. 2017; Batra et al. 2021; Jacobson et al. 2023; Kutryb-Zając et al. 2023; Perfilova et al. 2023; Vincenzi et al. 2023).

Hypoxanthine-tricyclano is a synthetic adenosine analogue (Kicsak 2018: https://dea.lib.unideb.hu/dea/handle/2437/249958 (PhD thesis); Kicsak et al. 2018), in which both characteristic moieties of adenosine have been replaced: adenine with hypoxanthine, and ribose with a fused tricyclic molecule derived from morpholino (Fig. 1). Hypoxanthine is a natural purine base (formed by deamination of adenine), the original hydroxyl group of which can undergo keto-enol tautomerization (Nguyen et al. 1992). The synthetic morpholinos belong to the azanucleosides possessing a nitrogen-containing ring (Summerton and Weller 1997). Hypoxanthine-tricyclano has been developed to test its possible antiviral activity in the future (Kicsak 2018; Kicsak et al. 2018).

However, until the time of this writing, no selective A_1 receptor agonists have been fully introduced into clinical use for the treatment of heart diseases, because their benefits are limited by their side effects and the lack of sufficient specificity for their therapeutic target (Rueda et al. 2021; Spinozzi et al. 2021; Jacobson et al. 2023; Kutryb-Zając et al. 2023; Perfilova et al. 2023; Vincenzi et al. 2023). So, the task of worldwide research is to overcome these challenges.

Besides to synthesize new molecules, it is also expedient to investigate the potential cardiovascular effects of molecules developed for other purposes. Accordingly, the aim of the present study was to explore whether hypoxanthine-tricyclano, due to its structural similarity to adenosine, influences inotropy and/or chronotropy of the left and/or right atrium. In the atrial (but not ventricular) myocardium, both inotropy and chronotropy are strongly regulated by the A_1 receptor (Belardinelli et al. 1995; Geldenhuys et al. 2017; Sousa and Diniz 2017).

Materials and Methods

Chemicals and solutions

The chemicals used in the study were hypoxanthine-tricyclano, produced by the Department of Pharmaceutical

Chemistry, Faculty of Pharmacy, University of Debrecen (Debrecen, Hungary), furthermore adenosine and 8-cyclopentyl-1,3-dipropylxanthine (CPX), both purchased from Sigma (St. Louis, MO, USA).

Adenosine and hypoxanthine-tricyclano were dissolved in modified Krebs-Henseleit buffer (hereinafter referred to as Krebs solution) at 36°C. CPX was dissolved in dimethyl sulfoxide (DMSO). All stock solutions were prepared at a concentration of 10 mM. The adenosine and hypoxanthine-tricyclano stock solutions were diluted with Krebs solution. The composition of Krebs solution was (in mM): NaCl (118), KCl (4.7), $CaCl_2$ (2.5), NaH_2PO_4 (1), $MgCl_2$ (1.2), $NaHCO_3$ (24.9), glucose (11.5) and ascorbic acid (0.1). Ascorbic acid, in the concentration used here as an antioxidant, did not affect the pH of the buffer (about 7.4, after a few minutes of carbogenization, see below).

Atria and measurements

The left and right atria were isolated from male, 30–35 weeks old, Wistar rats weighing 400–500 g. The animal use protocols were approved by the Committee of Animal Research, University of Debrecen, Hungary (25/2013 DEMÁB and 5/2020/DEMÁB).

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

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

Both the left and right atria were mounted at 10 mN resting tension in 10 ml vertical organ chambers (Experimetria TSZ-04; Experimetria Kft, Budapest, Hungary), filled with Krebs solution, gassed ("carbogenized") with 95% O_2 and 5% CO_2 (36°C; pH 7.4). In the case of the right atria, the rest of the supraventricular myocardium (other than the tissue of the two appendages) floated in the bathing medium alongside the suspended right atrial appendage. The importance of this tissue arose from the fact that it contained the sinoatrial node responsible for the spontaneous beating.

The left atria were paced with platinum electrodes (3 Hz, 1 ms, twice the threshold voltage) using a programmable stimulator (Experimetria ST-02; Experimetria Kft, Budapest, Hungary) and power amplifier (Experimetria PST-02; Experimetria Kft, Budapest, Hungary). The right atria were allowed to work spontaneously.

The isometric twitches of atria were measured by a transducer (Experimetria SD-01; Experimetria Kft, Budapest, Hungary) and strain gauge (Experimetria SG-01D; Experimetria Kft, Budapest, Hungary), and recorded by a polygraph (Medicor R-61 6CH Recorder; Medicor Művek, Budapest, Hungary).

Protocols

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

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

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

Evaluation of data

To assess inotropy, the distance between the lower (showing the minimal tension) and upper (indicating the maximal tension) envelopes of the recorded consecutive isometric twitches of atria was considered as the contractile force. The inotropic effect (E_{in}) was calculated from the change of the contractile force:

$$E_{in} = \frac{F - F_0}{F_0} \times 100\%$$

where F is the contractile force measured after the administration of the given agent dose, and F_0 is the initial contractile force (which was measured before the administration of the first agent dose). If the atrium weakened (in the presence of the given agent used to generate the E/c curve), the smallest contractile force seen after the administration of the given agent dose was taken into account. In contrast, if the atrium strengthened (in the presence of the given agent used to

construct the E/c curve), the largest contractile force detected after the administration of the given agent dose was chosen.

To assess chronotropy, we used the frequency of the mechanical activity of the right atria (as "beating rate" (BR)), which corresponds to the heart rate of an intact animal (or, rather, of an isolated whole heart). The beating rate was determined using the speed of the polygraph to transmit the paper:

$$BR = 8.82\frac{mm}{s} \times \frac{x}{50\ mm} \times 60\frac{s}{min}$$

where 8.82 mm/s is the speed of the chart recorder paper, and x is the number of twitches on a 50 mm long record. If the right atrium slowed down (in the presence of the given agent used to construct the E/c curve), the 50 mm part of the record showing the lowest frequency was selected. If the atrium speeded up (in the presence of the given agent used to generate the E/c curve), the 50 mm record part with the highest frequency was chosen.

The chronotropic effect (E_{ch}) was then computed from the initial "beating rate" (BR_0) and the "beating rate" developed in the presence of the given agent concentration (BR):

$$E_{ch} = \frac{BR - BR_0}{BR_0} \times 100\%$$

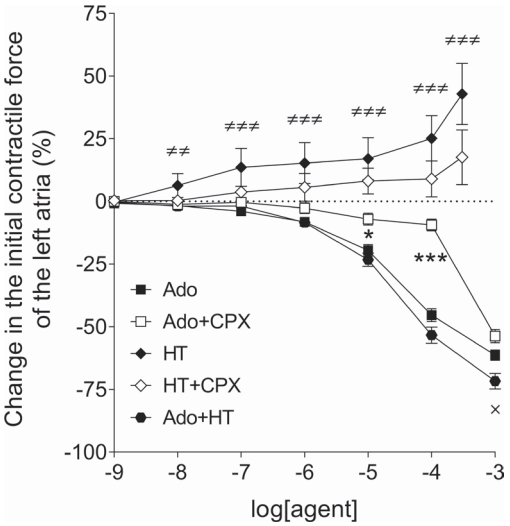

Figure 2. The direct inotropic effect of adenosine and hypoxanthine-tricyclano on isolated, paced left rat atria. The *x*-axis shows the common logarithm of the molar concentration of agents used for the concentration-effect curves, while the *y*-axis denotes the effect (expressed as the percentage change in the initial contractile force). 0% indicates the initial contractile force, −100% shows the cessation of the atrial mechanical activity, and 75% denotes an increase of the initial contractile force by 75% (i.e. 1.75 times). The symbols indicate the responses to the agents averaged within the groups (± SEM). For an explanation of group abbreviations, see the Protocols subsection in the Materials and Methods. SEM, standard error of the mean. $^{\neq\neq}$ $p < 0.01$, $^{\neq\neq\neq}$ $p < 0.001$ HT *vs.* Ado; * $p < 0.05$, *** $p < 0.001$ Ado+CPX *vs.* Ado; $^{\times}$ $p < 0.05$ Ado+HT *vs.* Ado.

Statistical analysis

The Gaussian distribution of the data was investigated with D'Agostino-Pearson test and Shapiro-Wilk test. Two data sets, both following normal distribution, were compared with unpaired t test (if the F-test indicated significantly different variances, with Welch's correction). To compare two data sets, from which at least one showed non-normal distribution, Mann-Whitney U test was used. To compare more than two data sets with normal distribution, one-way ANOVA and Tukey's post-test were performed. More than two data sets with non-normal distribution were compared using Kruskal-Wallis test and Dunn's post-test. Statistical analysis was performed with GraphPad Prism 8.4.2 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

Results

Inotropic response of the atria

As expected, adenosine, an orthosteric, reversible adenosine receptor agonist, exerted a strong, concentration-dependent, direct negative inotropic effect, which showed no statistically significant difference between the left and right atria. CPX, a selective, orthosteric, reversible A_1 receptor antagonist, significantly antagonized the effect of adenosine at 10 and 100 μM adenosine concentrations in both the left and right atria, and even at 1 mM adenosine concentration in the right atria (Fig. 2 and 3).

In contrast, hypoxanthine-tricyclano evoked a moderate, concentration-dependent, direct positive inotropic effect that did not differed significantly between the left and right atria. It should be mentioned that the scatter around the mean effects of hypoxanthine-tricyclano was substantially greater than that of adenosine. CPX appeared to decrease the effect elicited by hypoxanthine-tricyclano, but this decrease did not reach the level of statistical significance (Fig. 2 and 3).

The presence of hypoxanthine-tricyclano (in a high, about 411 μM concentration) slightly increased the response to adenosine that was statistically significant in the case of 1 mM adenosine when administered to the left atria (Fig. 2).

Chronotropic response of the right atria

Our results for the chronotropic response were consistent with those obtained for the inotropic one. Adenosine

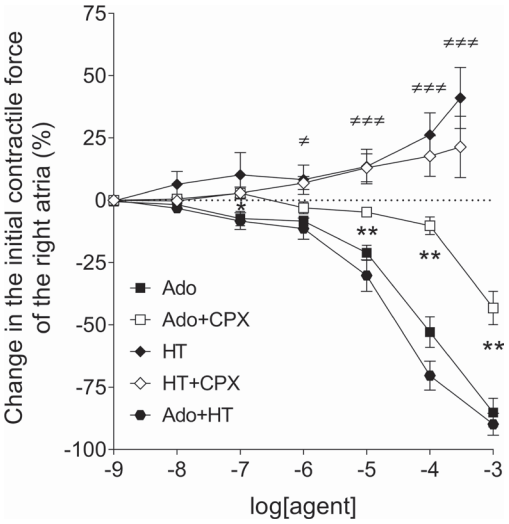

Figure 3. The direct inotropic effect of adenosine and hypoxanthine-tricyclano on isolated, spontaneously beating right rat atria. The *x*-axis shows the common logarithm of the molar concentration of agents used for the concentration-effect curves, while the *y*-axis denotes the effect (expressed as the percentage change in the initial contractile force). 0% indicates the initial contractile force, –100% shows the cessation of the atrial mechanical activity, and 75% denotes an increase of the initial contractile force by 75% (i.e. 1.75 times). The symbols indicate the responses to the agents averaged within the groups (± SEM). For an explanation of group abbreviations, see the Protocols subsection in the Materials and Methods. SEM: standard error of the mean. $^{\neq}$ $p < 0.05$, $^{\neq\neq\neq}$ $p < 0.001$ HT *vs.* Ado; ** $p < 0.01$ Ado+CPX *vs.* Ado.

elicited a strong, concentration-dependent, direct negative chronotropic effect on the spontaneously beating right atria that could significantly be inhibited with CPX. In turn, hypoxanthine-tricyclano exerted a minor direct positive chronotropic effect. CPX seemed to reduce this positive chronotropy, but the extent of this (non-significant) effect did not reach that seen with inotropy. Furthermore, the presence of hypoxanthine-tricyclano (about 411 μM) slightly (and non-significantly) enhanced the response to adenosine (Fig. 4).

Discussion

The present study is the first that provides evidence about the biological activity of hypoxanthine-tricyclano, a synthetic adenosine analogue (Kicsak 2018; Kicsak et al. 2018). Hypoxanthine-tricyclano has been found to exert a moderate direct positive inotropic effect in both the left and right atrium, furthermore, consistent with this, it proved to evoke a minor direct positive chronotropic effect in the right atrium. Both effects appeared in part to be antagonized by CPX (an orthosteric, reversible A_1 receptor antagonist), however, this antagonism was not found to be statistically significant (Fig. 2–4). This uncertainty forms the major limitation of this study, because antagonizability by CPX (or a similar selective antagonist) provides the strongest form of functional evidence that an agent, which can evoke one or some effect(s) characteristic of the A_1 receptor, is an orthosteric agonist of this receptor.

Our experiments were conducted on atria, because the atrial myocardium expresses predominantly the A_1 type of the adenosine receptors and can respond to A_1 receptor agonists directly, i.e. without prior sympathomimetic stimulation (Belardinelli et al. 1995; Geldenhuys et al. 2017; Sousa and Diniz 2017). The use of an isolated atrium mounted in a classic organ bath system (Weston et al. 2022), together with the avoidance of any pretreatment affecting the myocardial function (Gesztelyi et al. 2003), enables the acquisition of reliable and meaningful data, of course with some limitations, about the function of the atrial adenosinergic system, which is predominantly related to the A_1 receptor (Belardinelli et al. 1995; Kapicka et al. 2003).

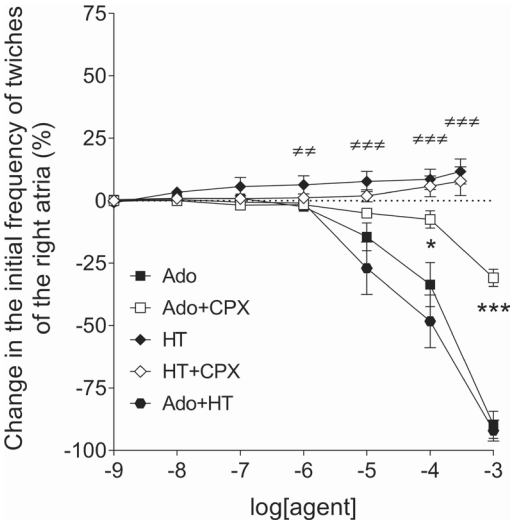

Figure 4. The direct chronotropic effect of adenosine and hypoxanthine-tricyclano in the right atria. The *x*-axis shows the common logarithm of the molar concentration of agents used for the concentration-effect curves, while the *y*-axis denotes the effect (expressed as the percentage change in the initial spontaneous frequency of the contractions of the isolated rat right atria). 0% indicates the initial frequency, –100% shows the cessation of the atrial mechanical activity, and 75% denotes an increase of the initial frequency by 75% (i.e. 1.75 times). The symbols indicate the responses to the agents averaged within the groups (± SEM). For an explanation of group abbreviations, see the Protocols subsection in the Materials and Methods. SEM: standard error of the mean. $^{\neq\neq}$ $p < 0.01$, $^{\neq\neq\neq}$ $p < 0.001$ HT *vs.* Ado; * $p < 0.05$, *** $p < 0.001$ Ado+CPX *vs.* Ado.

The uncertainty around the effect of CPX can be explained in two ways. First, CPX antagonizes the effect of hypoxanthine-tricyclano, but the effect data of hypoxanthine-tricyclano have a lot of experimental scatter that makes it difficult to reach statistical significance. This option is supported by the fact that the inotropic effect of hypoxanthine-tricyclano is quite small, and its reduction is therefore difficult to investigate. Second, hypoxanthine-tricyclano acts through a pathway that does not involve the A_1 receptor. These options do not exclude each other and, in fact, together they might explain our results better than separately. Thus, we have concluded that hypoxanthine-tricyclano probably possesses orthosteric A_1 receptor agonist properties, but the existence of alternative mechanisms of action cannot be excluded.

Furthermore, since the activation of the atrial A_1 receptor by adenosine, the main endogenous agonist, results in negative ino- and chronotropic effects (Belardinelli et al. 1995; Fredholm et al. 2001, 2011), hypoxanthine-tricyclano, with its weak positive ino- and chronotropic effects (Fig. 2–4), can be considered an inverse agonist. Inverse agonism for the A_1 receptor is a rare but not unknown phenomenon, typically involving relatively weak effects (He et al. 2013; Lu et al. 2014).

It may seem surprising at first that hypoxanthine-tricyclano (at a high concentration) slightly enhanced the response to adenosine (Fig. 2–4). If hypoxanthine-tricyclano acts, at least in part, through the A_1 receptor, this phenomenon allows us to draw an important conclusion: hypoxanthine-tricyclano is a reversible ligand for the A_1 receptor with a substantially lower affinity than adenosine, so it can easily be removed from the receptor by adenosine. As a consequence of its weak binding to the receptor, hypoxanthine-tricyclano does not shift the adenosine E/c curve to the right (to a visible extent), even when present at a high concentration. Instead, due to its previously exerted positive inotropic action, hypoxanthine-tricyclano increases the negative inotropic response capacity of the atria (i.e. the possibility of reducing the contractile force) and thereby enhances the response to adenosine (as a resultant effect).

Interestingly, ribavirin, the only selective A_1 receptor agonist drug approved by the FDA, is an antiviral medicament (Kutryb-Zając et al. 2023), while hypoxanthine-tricyclano was originally intended to be tested as an antiviral agent (Kicsak 2018; Kicsak et al. 2018).

As a further side note, although the changes in the right and left atrial contractility were consistent with each other, the experimental scatter observed for the right atrium was considerably greater than that found for the left atrium (Fig. 2 and 3). This denotes that the isolated left atrium with a constant-frequency pacing is a more reliable model to investigate agents with an inotropic effect than the isolated, spontaneously beating right atrium.

In summary, hypoxanthine-tricyclano, a synthetic adenosine analogue, has been found to elicit a moderate direct positive inotropic effect and a slight direct positive chronotropic action in the isolated rat atria. Based on our present results, hypoxanthine-tricyclano is probably a weak (low-efficacy), orthosteric, reversible, inverse and low-affinity agonist of the A_1 receptor. Besides this, alternative mechanisms of action in the background of this positive ino- and chronotropy cannot be excluded. Furthermore, possible sex-differences in the response to hypoxanthine-tricyclano should also be investigated in the future as they can occur in the adenosinergic system (Butler and Prendergast 2012). It should be mentioned that unique, rare, even whimsical features of a chemical agent may sometimes imply a greater pharmacological perspective than general, frequent, well-predictable properties. Moreover, unique and rare features (e.g. partial, inverse and biased agonism) are considered as possible breakthrough points for the development of useful adenosinergic drugs (Dinh et al. 2017; Perfilova et al. 2023; Vincenzi et al. 2023).

Conflict of interest. The authors declare that the research was implemented in the absence of any commercial or financial relationships that could be a potential conflict of interest.

Author contributions. All authors have contributed to this work in a significant extent.

Acknowledgements. This study was supported by the New National Excellence Program of the Ministry for Culture and Innovation from the National Research, Development and Innovation Fund/ providing personal support for GV (ÚNKP-23-4-I-DE-469) and for IO (ÚNKP-23-3-II-DE-85)/. Furthermore, this research was supported by the European Union and the State of Hungary under the grant number TKP2021-EGA-18 (implemented with the support provided by the Ministry for Culture and Innovation from the National Research, Development and Innovation Fund, financed under the TKP2021-EGA funding scheme).

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Received: May 13, 2024
Final version accepted: July 15, 2024