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Research Article
Binding of Amitriptyline to Adenosine A_1 or A_{2a} Receptors Using Radioligand Binding Assay

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Abstract

Background and Objective: Tricyclic antidepressants such as amitriptyline (AMT) may result in life-threatening cardiovascular toxicities. Previous studies showed that AMT-induced cardiovascular toxic effects were prevented/reversed by selective adenosine receptor (AR) antagonists. This study aimed to examine whether AMT mediates its cardiovascular effect through binding to ARs and for this purpose measured the binding affinity of AMT to A_1-or A_{2a}-ARs. **Materials and Methods:** Membranes expressing the A_1- or A_{2a}-ARs were labeled with their specific radioactive ligands ([^{3}H]-cyclopentyl-1,3-dipropylxanthine and [^{3}H]CGS21680, respectively). The displacement of the radioligand binding was determined in the presence of different concentrations of AMT or the selective adenosine receptor antagonists for A_1-AR and A_{2a}-AR, 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) and [8-(3-Chlorostyryl) caffeine (CSC), respectively. The student's t-test was used to compare the differences of two groups. **Results:** The bound A_{2a}-AR radioligand was completely displaced by AMT and the K_i value was calculated [half-maximal inhibitory concentration (IC_{50}): 51.42$\pm$15.87 μM and K_i: 4.8$\pm$0.11 μM, $p<0.05$]. High concentrations of AMT (10^{-4} and 10^{-3}M) inhibited radioligand binding to the A_1-AR, which was nearly 25% ($p<0.05$). **Conclusion:** AMT showed significant binding to the A_{2a}-AR, which might play an important role in its pharmacological and toxicological effects. Finally, the toxicity of high AMT concentrations may be mediated through the A_1-AR.

Key words: Adenosine, amitriptyline, A_1-adenosine receptor, A_{2a}-adenosine receptor, radioligand, ligand binding

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Among antidepressant agents, tricyclic antidepressants (TCAs) such as opipramol and amitriptyline (AMT) are the ones that most frequently cause drug poisoning[1,2]. The cardiovascular signs of TCA poisoning are predominantly hypotension, dysrhythmias and cardiac conduction abnormalities[3-6]. The effects of adenosine are mainly mediated by its actions on the A_1 and A_2 receptors in the cardiovascular system. The activation of A_1-adenosine receptors (A_1-ARs) inhibits the heart and causes negative chronotropic, inotropic and dromotropic effects. The activation of A_{2a}-adenosine receptors (A_{2a}-ARs) reduces the mean arterial pressure by causing the relaxation of arterial smooth muscle cells[7,8].

In the previous *in vivo* toxicity model, AMT-induced hypotension and QRS prolongation were prevented/reversed by selective adenosine receptor (AR) antagonists[9]. In an isolated rat aorta preparation, it was demonstrated that A_{2a}-ARs might be responsible for AMT-induced vasorelaxation[10]. In addition, in a rat isolated heart model, the AMT-induced QRS prolongation was shortened by a selective A_1-AR antagonist[11]. In isolated rat atria, AMT-induced electrophysiological changes such as a reduction of the maximum rise in the slope of the action potential duration were prevented by a selective A_1-AR antagonist[12]. Previous studies as mentioned above have stated that the A_1-AR or A_{2a}-ARs may be responsible for AMT-induced cardiovascular toxicity but the interaction between AMT and ARs has not been demonstrated.

Therefore, the goal of the present study was to examine the affinities and binding properties of AMT to ARs to determine the involvement of these receptors in AMT-induced poisoning.

MATERIALS AND METHODS

This study was supported by the Scientific and Technological Research Council of Turkey [TUBITAK, Grant Number: 107S251]. The project was approved by the Animal Care and Use Committee of Dokuz Eylul University School of Medicine.

Drugs: Amitriptyline (AMT), selective A_1-ARs antagonist 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) and selective A_{2a}-ARs antagonist 8-(3-Chlorostyryl)caffeine (CSC) were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). The rat cell membranes and the specific radioligands were obtained from Perkin Elmer (Boston, MA USA)[13].

Membranes: For the binding experiments, rat cell membranes expressing 2.4 pmol mg^{-1} protein of A_1-ARs (ES-010-M400UA) and 5.0 pmol mg^{-1} protein of A_{2a}-ARs (RBHA2AM) were used.

Binding studies: The binding affinity of AMT, for A_1-AR and A_{2a}-AR was determined by using a radioligand binding competition assay[13].

A_1-ARs binding: The A_1-AR-expressing cell membranes (40 µg membrane protein/tube) were incubated and labeled with specific radioactive ligand, 2.5 nM of [^{3}H]-cyclopentyl-1,3-dipropylxanthine ([3H]-DPCPX, ART 0520) in the binding buffer (25 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 100 mM NaCl) for 60 min at 27°C. In separate tubes including A_1-AR-expressing cell membranes (40 µg membrane protein), the displacement of the 2.5 nM of [^{3}H]-DPCPX binding were determined in the presence of different concentrations ranging between 10^{-12}-10^{-2} M AMT or in the presence of different concentrations ranging between 10^{-13}-10^{-4} M of the selective A_1-AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) in the binding buffer (25 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 100 mM NaCl) for 60 min at 27°C.

A_{2a}-ARs binding: The A_{2a}-AR-expressing membranes (40 µg membrane protein/tube) were incubated and labeled with specific radioactive ligands, 26.3 nM of [^{3}H]CGS21680 (ART 1671) in the binding buffer (50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid [EDTA]) for 90 min at 25°C. In separate tubes including A_{2a}-AR-expressing cell membranes (40 µg membrane protein), the displacement of [^{3}H]CGS21680 binding was determined in the presence of different concentrations ranging between 10^{-12}-10^{-2} M AMT or in the presence of different concentrations ranging between 10^{-12}-10^{-5} M of the selective A_{2a}-AR antagonist 8-(3-chlorostyryl) caffeine (CSC), in the binding buffer (50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid [EDTA]) for 90 min at 25°C.

Radioactivity measurement: The reactions were terminated by rapid filtration of the tubes using a cell harvester and Whatman GF/C filters. The filters were washed 4 times with 4 mL of ice-cold binding buffer. The filter-bound radioactivity was measured using a beta-counter (1450 Microbeta WALLAC Trilux, Perkin Elmer, Turku, Finland) after incubation with the scintillation solution and all assays were conducted in duplicate.

Statistical analysis: All data were expressed as the Mean±SEM of three independent experiments; in each experiment duplicate determinations were performed. The binding experiments were analyzed using 'LIGAND' programme to obtain the equilibrium dissociation constant (Kd)[14]. Statistical analyses were performed using SPSS software (IBM SPSS Statistics 20.0, Chicago, IL). Statistical differences between two groups were tested by the use of a Student's t-test. A p-value <0.05 was considered statistically significant.

RESULTS

To determine the potential binding of AMT to the A_1-AR, cell membranes expressing this receptor were labeled with a specific ligand, [^{3}H]-DPCPX. The binding characteristics of AMT to A_1-AR were determined through the analysis of the displacement of [^{3}H]-DPCPX binding by different concentrations of AMT (10^{-12}-10^{-2} M). The displacement of [^{3}H]-DPCPX binding by different concentrations of unlabeled-DPCPX (10^{-13}-10^{-4} M, a specific ligand for the A_1-AR) was also measured as a control binding experiment to compare with AMT binding. The DPCPX completely displaced the binding of [^{3}H]-DPCPX but only high concentrations (10^{-4} and 10^{-3} M) of AMT inhibited the radio ligand binding of [^{3}H]-DPCPX (A_1-AR binding);approximately 25% inhibition of the total specific binding was obtained ($p<0.05$, Fig. 1).

To determine whether AMT binds to the A_{2a}-AR, cell membranes expressing this receptor were labeled with an A_{2a}-AR-specific ligand, [^{3}H]CGS21680. The binding properties AMT to the A_{2a}-AR were determined through the analysis of the displacement of [^{3}H]CGS21680 binding with different concentrations (10^{-12}-10^{-2} M) of AMT. We also measured the displacement of [^{3}H]CGS21680 binding with different concentrations of CSC (10^{-12}-10^{-5} M, an A_{2a}-AR-spesific ligand), as a control binding experiment to compare with the AMT binding. The binding of [^{3}H]CGS21680 to the A_{2a}-AR was completely displaced by the specific ligand CSC and AMT (half-maximal inhibitory concentration [IC_{50}]: 51.42±15.87 μM) and the calculated K_i value for AMT was 4.8±0.11 μM as shown in Fig. 2 ($p<0.05$).

DISCUSSION

This is the first study to evaluate the affinity and binding properties of amitriptyline (AMT), a tricyclic antidepressant, to adenosine receptors by using a radioligand binding assay. The present study showed that while AMT (10^{-4} and 10^{-3} M), at high concentrations, binds approximately 25% of A_1-AR, it totally inhibits the binding of [^{3}H]CGS21680 suggesting a significant binding property to A_{2a}-AR with a Ki value of 4.8±0.11 μM.

ARs play important role in several physiopathological processes ranging from vascular function to metabolic control and from neuromodulation to immune regulation. Four AR subtypes have been cloned: A_1, A_{2a}, A_{2b}ve A_3. These receptors are widely expressed in heart, brain, lung, blood vessels, platelets and many other organs and cells. AR agonists and antagonists have potential therapeutic utility[15,16]. Drugs such as dipyridamole and methotrexate act by

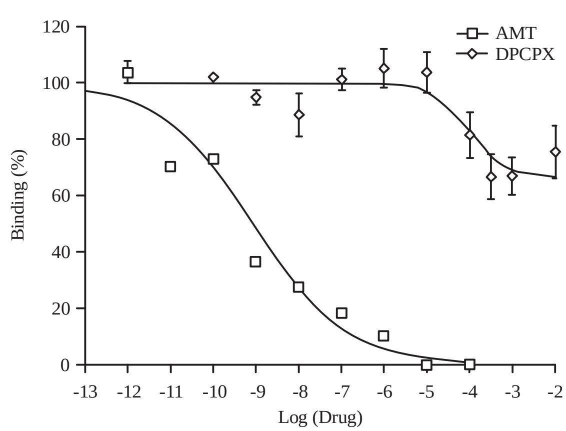

Fig. 1: Affinity of amitriptyline to adenosine A_1 receptors (AMT, Amitriptyline, DPCPX: 8-Cyclopentyl-1-1, 3-dipropylxanthine, selective A_1 receptor antagonist)
All data were expressed as the Mean±SEM

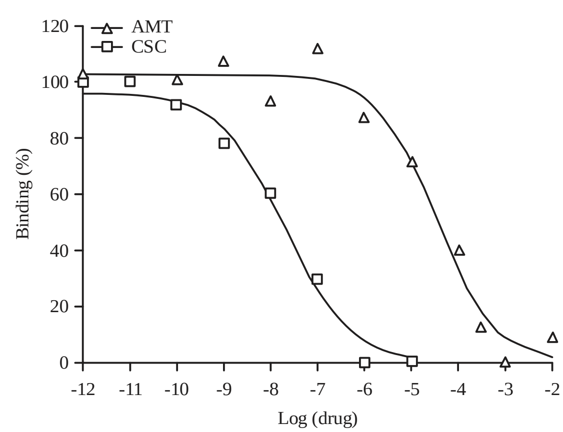

Fig. 2: Affinity of amitriptyline to adenosine A_{2a} receptors (AMT: Amitriptyline, CSC: 8-(3-Chlorostyryl) caffeine, selective A_{2a} receptor antagonist)
All data were expressed as the Mean±SEM

of enhancing activation of ARs[6,17]. Moreover methylxanthines such as theophylline has bronchodilator and anti-inflammatory actions as an antagonist at AR[18].

ARs are important pharmacological targets for the treatment of cardiovascular diseases. The heart predominantly expresses the A_1-AR in cardiomyocytes, atrial and ventricular cells and sinoatrial node cells, whereas the A_{2a}-AR localized in the vascular smooth muscle and endothelial cells[19,20]. The activation of the A_1-AR inhibits heart function and produces negative chronotropic, inotropic and dromotropic effects; the activation of A_{2a}-AR reduces the mean arterial pressure through the relaxation of vascular smooth muscle cells[7,8].

In previous studies, it was found that ARs might play a role in AMT-induced cardiovascular toxicity but the binding of AMT to ARs was not demonstrated[9-12]. A previous *in vivo* rat poisoning model showed that amitriptyline infusion produced 40-45% reduction of mean arterial pressure and prolonged QRS. It was shown that the hypotension and QRS prolongation induced by AMT was reversed and prevented by a selective A_1-AR antagonist (DPCPX)[9]. In a previous study demonstrated that amitriptyline (10^{-4} M) prolonged the QRS duration more than 150% in isolated rat heart model. This QRS prolongation induced by AMT was decreased by DPCPX treatment, which suggested that A_1-AR stimulation may have a role in AMT-induced QRS prolongation[11]. Furthermore, it was shown that DPCPX diminished the AMT (50 μM)-induced prolongation of action potential (AP) duration (APD_{50} and APD_{80}). In addition, DPCPX prevented the effects of AMT (1 and 50 μM) on the maximum rate of the rise in slope of the AP and the AMT (50 μM)-induced reduction of the maximum decay slope of AP in isolated rat atrium[12]. This present study indicated that AMT binds to A_1-AR only at high concentrations and suggested that A_1-AR may be responsible for the cardiovascular toxicity induced by AMT overdose.

The interaction between amitriptyline and A_1-AR was shown not only in cardiovascular system but also in central nervous system. Liu *et al*. showed that antinociceptive response induced with systemic application of amitriptyline was blocked by intrathecal or intraplantar administration of selective A_1-AR antagonist (DPCPX) in wild type mice. They demonstrated that this blockade was also seen in A_1-AR expressing +/+ mice but not in A_1-AR lacking -/- mice. They suggested that adenosine A_1-ARs contribute to amitriptyline-induced antinociception in both spinal and peripheral compartments[21].

Several studies also indicated that A_{2a}-AR might play a role in AMT-induced cardiovascular toxicity[9-10]. Kalkan *et al*.[9] showed that the hypotension and QRS prolongation induced by AMT poisoning in rats were reversed by the selective A_{2a}-AR antagonist (CSC). Furthermore, pretreatment with CSC also prevented the development of AMT-induced QRS prolongation and hypotension[9]. Kalkan *et al*.[10] showed that amitriptyline-inhibited 49.9% contractile response to noradrenaline (NA) at 1.8×10^{-5} M in the isolated rat aorta. Additionally, selective A_1-AR antagonist (DPCPX) increased amitriptyline-induced inhibition on contractile response to NA dose dependently and selective A_{2a}-AR antagonist (CSC) decreased the contractile response to NA only at 10^{-5} M. They suggested that A_{2a}-AR stimulation played a role in the vasodilation induced by AMT in the isolated rat aorta[10]. This study demonstrated the significant binding of AMT to the A_{2a}-AR and suggested that it may mediate the hypotensive effect associated with the therapeutic and toxic doses of AMT.

CONCLUSION

The results of this study showed that while AMT considerably binds to A_{2a}-AR, only high concentrations of it partially bind to A_1-AR. ARs, located on myocardial cells and arterial smooth muscle cells, may have a significant role in AMT mediated pharmacological and toxicological effects. A natural progression of these study results might be the development of new treatment strategies with A_1-AR and A_{2a}-AR antagonists for AMT poisoning. Future studies may also clarify the underlying molecular mechanisms of the intracellular signals triggered by AMT binding to the A_1-AR and A_{2a}-AR.

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