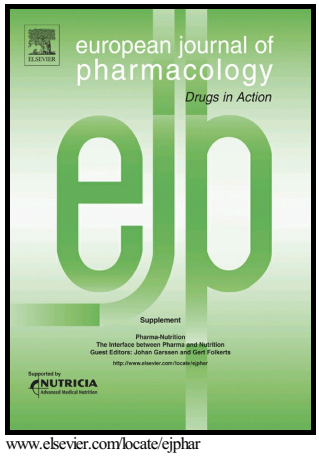

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New 2-Aminothiazoline derivatives lower blood pressure of spontaneously hypertensive rats (SHR) via I_1-imidazoline and alpha-2 adrenergic receptors activation.

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Abstract

2-Aminothiazolines share an isosteric relationship with imidazolines and oxazolines with antihypertensive activity mainly mediated by the imidazoline I_1-receptor. In the present work, we have prepared five aminothiazolines, following a previously described synthetic pathway. Aminothiazolines derived from dicyclopropylmethylamine (**ATZ1**) and cyclohexylamine (**3**) are unprecedented in the literature. Competitive radioligand

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assay was carried out with all synthetic compounds, and the I_1 receptor affinity in comparison to rilmenidine in PC12 cells was determined. Surprisingly, the rilmenidine isoster (**ATZ1**) showed no I_1-receptor interaction. Diethyl (**ATZ4**) and 2-ethyl-hexylamine (**ATZ5**) derivatives bind to the receptor with 11.98 and 10.94 nmol/l, respectively. These compounds were selected for in vivo experiments. Both compounds reduced the blood pressure of spontaneously hypertensive rats (SHR). The hypotensive effect of these compounds was abrogated in the presence of α_2 adrenergic (yohimbine) and I_1 (efaroxan) receptor antagonists suggesting that both aminothiazolines bind to the adrenergic and imidazoline receptors. Lipinski's descriptors of the synthesized aminothiazolines were calculated and are similar to the known imidazoline I_1 receptor ligands. 3D-Similarity between **ATZ5** and agmatine, the natural imidazoline receptor ligand, was also observed.

Keywords: Imidazoline Receptor, Aminothiazoline, Hypertension, PC12 Cells, Binding Assay

1. Introduction

The activation of α_2 (adrenergic) and I_1 (imidazoline) receptors in the central nervous system generates a hypotensive response, making these receptors an important target for the development of antihypertensive drugs (Nikolic et al., 2008). Clonidine belongs to the class of antihypertensive drugs with central action, which promotes inhibition of the sympathetic activity. Currently, its activity is attributed both to the stimulus of α_2 adrenergic receptors in the nucleus tractus solitarius and receptors located in the rostral ventrolateral medulla (RVLM). However, stimulation of α_2 receptors located at locus coeruleus is responsible for significant side effects, such as sedation (De

Sarro et al., 1987). The binding sites in the RVLM, called imidazoline receptors, are regions that specifically recognize imidazolines or similar structures, both in the brain and peripheral system, and some of them are involved in blood pressure regulation. As result of the stimulus of I_1 receptors in the RVLM, a decrease in the release of norepinephrine (NE) from the storage vesicles (pre-synaptic neurons) and epinephrine in the adrenal medulla, there is a decrease in the blood pressure (Bousquet and Feldman, 1999; Ernsberger, 2000; Zhang and Abdel-Rahman, 2005). Hence, there is a growing interest in developing drugs that preferentially activate the the imidazoline receptors while exhibiting low or no activity at the α_2 receptors (Fellmann et al., 2013; Wasilewska et al., 2014; Krasavin, 2015). These findings resulted in the development of a second generation of central antihypertensive drugs, represented by the commercially available moxonidine and rilmenidine (Van Zwieten, 1999; Bousquet et al., 1984; Bousquet, 2001). The chemical structures of these compounds, clonidine and 2-aminothiazolines are shown in Fig. 1A, in which the isosteric relationship of their heterocyclic moieties is highlighted. Despite the structural similarity, thiazolines have not been studied as potential antihypertensive agents, except for some 3-methyl-2-thiazolidinimine derivatives (Rokach et al., 1980). Thus, we evaluated N-substituted 2-aminothiazolines (Fig. 1B), as potential ligands for I_1receptors and hypotensive agents. Besides the 2-aminothiazoline **ATZ1**, a sulfur analogue of rilmenidine, we prepared four more *N*-substituted-2-aminothiazolines **ATZ2-5** to study their I_1-receptor affinity. Compounds that presented good affinity for the I_1-receptor were selected for the *in vivo* evaluation of their effects on arterial blood pressure of spontaneous hypertensives rats (SHR).

Fig.1.

2. Materials and Methods

2.1. Chemistry

Compounds **ATZ2, 4** and **5** were obtained as previously reported (Ferreira et al., 2013) and are available in our laboratory. The syntheses of compounds **ATZ1** and **3** are first reported in this work and followed the same protocol of others. The synthetic pathway to achieve these compounds involves the following steps: preparation of isothiocyanates from the appropriated amine and carbon disulfide followed by treatment of isothiacyanates with ethanolamine led to the corresponding thioureas, which were converted into the desired aminothiazolines, by *S*-cyclization process, as detailed in the supplementary material.

2.2. Calculations of physicochemical properties

The partition coefficient, represented by its logarithm (Log P), was calculated *on line* (Tesco, 2005) by the software ALOGPS (VCCLAB, 2005). The optimization of 3D structures of agmatine and aminothiazolines were carried out by using Gaussian 09 (Frisch et al., 2009). Polar surface area and molecular volume are obtained by Molinspiration software.

*2.3. Determination of the I_1 receptor affinity for the synthesized compounds **ATZ1-5** in comparison to rilmenidine in PC12*

A modified protocol for competitive radioligand binding assay previously reported (Greney et al., 2000) was used to determine the I_1 receptor affinity for the investigated compounds using PC12 cell membranes.

2.3.1. Cell culture

PC12 cells, which express I_1 receptor, were cultured in 75-cm^2 flasks at 37°C with 10% CO_2 in F12 Kaighn's medium supplemented with 15% Horse Serum (HS),

2.5% fetal bovine serum (FBS) and 1% Anti-Anti (100x). After removing the medium, cells at confluence were frozen in the flasks at -20°C until use to prepare membranes.

2.3.2. Membrane preparations

Frozen PC12 cells were scraped into cold Tris-HEPES buffer (5 mM Tris-HEPES, pH 7.7, 0.5 mM EDTA, 0.5 mM EGTA, and 0.5 mM MgCl2) and homogenized with a Potter homogenizer. After centrifugation at 35,000 *rpm* for 20 min, the pellet was washed in cold Tris-HEPES buffer and centrifuged again. Pellets were re-suspended in Tris-HEPES buffer at 4 mg protein/ml and used immediately for binding assays.

2.3.3. Binding assay

[^{125}I] paraiodoclonidine (PIC) was used as the radioligand to detect the imidazoline receptors in the PC12 cell membranes (B_{max} = 20 fmol/mg of protein). Incubations were initiated by the addition of membranes (40 μg of protein/80 μl final volume) and were carried out at 25°C for 30 min. For competition assay, increasing concentrations of compounds (10^{-7} to 10^{-2} M) were added with 0.5 nM [^{125}I] PIC (corresponding to the *K*D value of the radioligand). To stop the incubation, samples were filtered very quickly through GF/B glass fiber filters, incubated for 3 h in 0.03% polyethylenimine with a Brandel harvester, and filters washed twice with 3 ml of 50 mM cold TrisHCl buffer, pH 7.7. Radioactivity retained on the filters was determined in a Minaxi gamma counter. All compounds were dissolved in DMSO (100%) in stock solution and diluted as needed to concentrations 10^{-7} to 10^{-2} M. The values of the inhibition constants (K_i nM) are calculated from the respective IC_{50} values by applying

the Cheng-Prusoff equation using GraphPad Prism 5 software. Each value is mean of 5-16 experiments.

2.4. Arterial blood pressure measurement

Animals were housed under a 12:12 h light-dark cycle conditions with maintained temperature (24°C). All animal procedures and the experimental protocols were approved by the Ethical Principles in Animal Research adopted by Brazilian College for Animal Experimentation (COBEA; No. 3341-1). Four-month-old SHRs, provided by CEMIB-UNICAMP, were anesthetized by sodium thiopental (40 mg/kg, i.p). The right femoral artery and left femoral vein were cannulated for the measurement of arterial blood pressure and drug administration, respectively (Antunes et al., 1996; El-Mas and Abdel-Rahman, 2001). Arterial pressure was measured via a pressure transducer and the signal converted by a bridge amplifier coupled with PowerLab (ADInstruments, Sydney, Australia). Mean arterial pressure (MAP) was recorded with LabChart 7 software (ADInstruments). Drugs used were noradrenaline, clonidine, yohimbine, efaroxan (Sigma, St. Louis, MO, USA), as well as compounds **ATZ4** and **5**.

2.4.1. Effect of combining ATZ5 with clonidine or yohimbine

Blood pressure was allowed to stabilize for at least 15 min before administration of any drug or vehicle. All drugs were diluted in albumin/saline (0.3% v/v) solution, and injected intravenously as a single bolus and were washed in with a further 100 µl of saline. First, we injected the vehicle and then noradrenaline (2.5 µg/kg), followed by **ATZ5** (300 µg/kg) and clonidine (10 µg/kg). Arterial pressure was allowed to return to the baseline level before the subsequent injections were conducted. In separate groups

of rats, the tested-compound was administered following α_2-adrenergic blockade with yohimbine (2.5 mg/kg) (Allard et al., 1995). Results are expressed as the mean values ± standard error of means of 3-8 rats per group. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post-test. In all analyses, a *P*-value < 0.05 was considered significant. All procedures were performed using Graph Pad Prism 5.0 (Graph Pad Software Inc., San Diego, CA, USA).

2.4.2. Effect of efaroxan on cardiovascular effects of ATZ4 and ATZ5

In this experiment, we investigated the effect of imidazoline (I_1) receptor blockade with efaroxan (10 μg/kg; Bock et al., 1999), on the cardiovascular effects of compounds **ATZ4** or **5** (300 μg/kg). Each rat served as its own control and received one of the two compounds before and after efaroxan. Adequate time was allowed to permit full recovery of MAP and HR to baseline after the first injection. At the conclusion of the experiment, all animals were killed with sodic thiopental anesthesia (100 mg/kg, i.p). Results are expressed as the mean values ± S.E.M of changes in MAP after the injections. For each compound, a number of four to six rats were used. Statistical analysis was performed likewise as mentioned in the previous sub-item.

3. Results

Building on the availability of amines in our laboratory, we decided to study a series of the aminothiazolines. **ATZ2**, **4** and **5** are available in our laboratory from a previous work (Ferreira et al., 2013).

3.1. Preparation of compounds ATZ1 and 3

ATZ1 and **3** were prepared from dicyclopropylmethylamine and cyclohexylamine, respectively. Experimental procedure of each synthetic step is available in the supplementary material.

3.1.1. N-(Dicyclopropylmethyl)-4,5-dihydro-1,3-thiazol-2-amine (**ATZ1**)

White solid; Mp 123-124 °C.

IR (KBr, υ_{max}): 3443, 3194, 3078, 3003, 2941, 2860, 1607, 1547, 1319, 1248, 1020, 1041, 829, 642, 608 cm^{-1};^{1}H NMR (250 MHz, $CDCl_3$): δ 3.94 (t, *J* = 7.3 Hz, 2H), 3.27 (t, *J* = 7.4 Hz, 2H), 2.97 (t, *J* = 7.1 Hz, 1H), 0.55-0.24 (m, 8H), 0.92 (st, *J* = 6.8, 2H); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 161.3 (C), 61.1(CH), 59.9 (CH_2), 34.9(CH_2), 15.5(CH), 2.8 (CH_2), 1.9 (CH_2); HRMS **(**EI TOF**):** Calculated for $C_{10}H_{16}N_2S$: 196.1034; Found M^+: 196.1024

3.1.2. N-cyclohexyl-4,5-dihydro-1,3-thiazol-2-amine (**ATZ3**)

White solid; Mp: not determined due the decomposition (> 100° C)

IR (KBr, υ_{max}): 3184, 2997, 2930, 2854, 1609, 1547, 1450, 1321, 1246, 1153, 1097, 1030, 891, 648, 453 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.00 (t, *J* = 7.4 Hz, 2H), 3.66 (br s, 1H); 3.52 (tt, *J* = 3.9 and 10.5 Hz, 1H); 3.30 (t, *J* = 7.4 Hz, 2H); 2.03 (m, 2H); 1.71 (m. 1H); 1.59 (m, 1H); 1.36 (m, 3H); 1.17 (m, 3H); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 160.9 (C), 60.5 (CH_2), 53.9 (CH), 35.3 (CH_2), 33.9 (CH_2), 25.1 (CH_2), 25.8(CH_2). HRMS (ESI$^+$ TOF): Calculated for $C_9H_{17}N_2S^+$: 185.1107; Found M^+: 185.1149.

3.2. Physicochemical properties

The results for calculated logarithm of the partition coefficient and Lipinski's descriptors (Lipinski, 1997) of the synthesized aminothiazolines and known I_1-imidazoline receptor are summarized in Table 1. Log *P* values of the synthesized

compounds **ATZ1-5** range from 2.06 to 3.86, whereas for the known I_1-receptor ligands this range is -0.91 to 3.67. All synthesized compounds**ATZ1-5** have three hydrogen bond donating groups (HBD) and one acceptor (HBA). These numbers are the same of clonidine, rilmenidine, efaroxan and 4-iodoclonidine.

Table 1. Lipinski's descriptors of I_1-Receptor Imidazoline receptor and Aminothiazolines

Compound	**Log *P*[a]**	**HBD[b]**	**HBA[c]**	**MW[d]**
Clonidine	2.53	1	3	230
Moxonidine	1.46	1	5	240
Rilmenidine	1.57	1	3	180
LNP 911	3.67	1	2	334
Benazoline	2.53	1	2	196
Tracizoline	1.92	1	2	172
AGN 192403	2.23	1	1	153
Idazoxan	1.01	1	4	204
Efaroxan	2.17	1	3	216
Agmatine	-0.91	4	4	130
Harmane	2.71	1	2	182
4-Iodoclonidine	3.55	1	3	153
ATZ 1	2.06	1	3	196
ATZ 2	2.39	1	3	206
ATZ 3	2.20	1	3	184
ATZ 4	2.15	1	3	172

ATZ 5	3.86	1	3	214

[a]Average from ALOGPS; [b] Hydrogen Bond Donors;[c] Hydrogen Bond Acceptors; [d] Molecular weight

3.3. Imidazoline receptor affinity for Aminothiazolines ATZ1-5

The investigated compounds displaced the specific binding of [^{125}I] PIC in PC12 cell membranes, except for **ATZ3** as shown in the Fig. 2. Relative K_i values, compared to rilmenidine (2.62 nM) are also presented.

Fig. 2.

Except for **ATZ3**, I_1 receptor showed affinity for the synthesized compounds as well as rilmenidine. Compounds**ATZ4** and **5** presented the lowest K*i* values: 11.98 and 10.94 nM, respectively.

3.4. Effects of yohimbine on arterial blood pressure changes by ATZ5 and clonidine

Blood pressure recordings of two different groups of SHRs are presented in Figs. 3A and B. Basal MAP in SHR was 163 ± 5.1 mmHg. Intravenous administration of vehicle produced no significant variations in MAP. After intravenous injections of either compound **ATZ5** or clonidine there was an increase in arterial blood pressure of 6.4 ± 1.8 and 37.9 ± 5.6 mmHg (n = 5), respectively, which was followed by significant reductions in MAP (Fig. 3A). In a separate group of SHR, the blockade of α_2-adrenergic receptor with yohimbine (2.5 mg/kg, i.v) abolished the hypotensive response caused by **ATZ5** or clonidine (Figs. 3B, C).

Fig. 3

3.5. Effects of efaroxan on arterial blood pressure changes by ATZ4 and 5

Baseline value for MAP of SHR was 174.0 ± 5.5 mmHg (n = 12). As seen in Fig. 4, intravenous administration of vehicle had no significant effect on MAP (2.5 ±

0.8 mmHg). However, the administration of compound **ATZ5** induced significant ($P < 0.05$) hypotensive response (-10.3 ± 1.0 mmHg). Similarly, **ATZ4** significantly ($P < 0.05$) reduced MAP (-10.8 ± 3.3 mmHg). These hypotensive responses were short lived and MAP fully recovered to the baseline within a few minutes (Fig. 4). The blockade of imidazoline I_1 receptors by efaroxan significantly ($P < 0.05$) attenuated the hypotensive responses caused by **ATZ4** or **5** (-3.9 ± 0.9 and -4.02 ± 1.2 mmHg, respectively; Fig. 4).

Fig. 4.

5. Conclusions

The I_1-receptor affinity for the five aminothiazolines sharing a bioisosteric relationship with rilmenidine, a known selective I_1-agonist, were evaluated by a competition experiment using the radioligand [^{125}I]-p-iodoclonidine in PC12 cell membranes. Except for compound **ATZ3**, all compounds exhibited affinity to the I_1-receptor. **ATZ4** and **5** showed the lowest K*i* values. To our disappointment, the synthesized compounds were not exclusively selective for I_1-receptor because the antihypertensive effect of compound **ATZ4** or **5** was blocked by efaroxan or yohimbine. Based on these results, we concluded that the mechanism of MAP reduction involves the activation of both α_2 and I_1 receptors, a biological characteristic known for clonidine as well as rilmenidine. The limited number of compounds along with the difference in the I_1-receptor affinity by compounds sharing molecular similarity precluded conducting a full structure-activity relationship (SAR) study.

Additional information

Authors have no conflit of interest.

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Fig. 1. (A) Structures of clonidine, rilmenidine, moxonidine and 2-aminothiazoline, highlighting the imidazoline, oxazoline, pyrrolidine and thiazoline isosteric moieties, respectively. (**B**) Structures of 2-compounds **ATZ1-5**.

Fig. 2. Plots of binding (CPM) versus Log of the aminothiazolines concentration (nM) and rilmenidine**.**

Fig. 3: Representative blood pressure recordings of the pressor response caused by noradrenaline (NOR) and the reductions in mean arterial pressure (MAP) caused by compound ATZ**5** (300 µg/kg) or clonidine (10 µg/kg) in anesthetized spontaneously hypertensive rats. **B**. Pretreatment with yohimbine (2.5 mg/kg, i.v) significantly attenuated the responses elicited by compound ATZ5 or clonidine. **C**. Bar graphs showing the ΔMAP responses in different groups. Values are expressed as mean ± S.E.M (n = 3 – 8). **P* <. .05; ***P* < .01 versus vehicle (one-way ANOVA, Tukey's post-test).

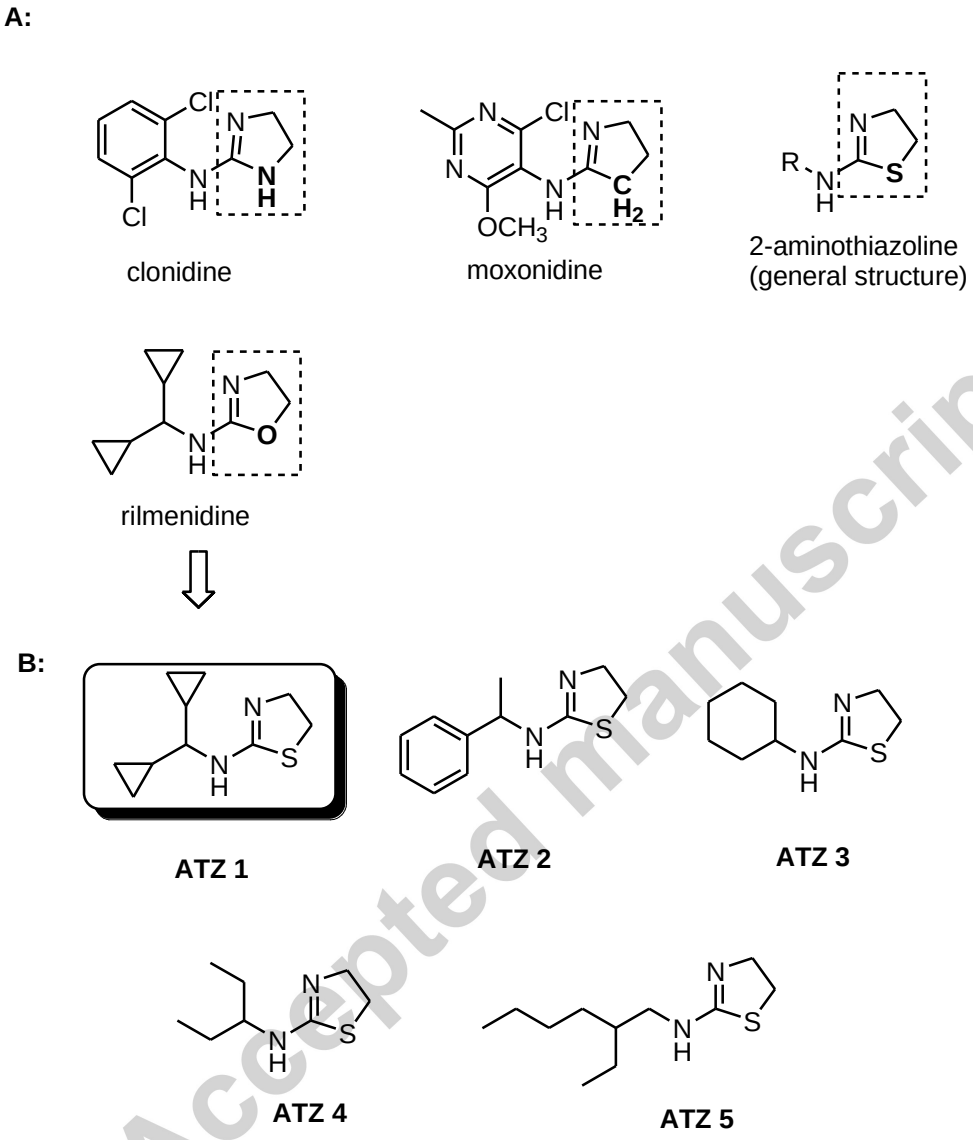
Fig. 4. Representative blood pressure recordings showing the effect of compound ATZ**5** (300 µg/kg; **A**) or compound ATZ**4** (300 µg/kg; **B**) before and after efaroxan (10 µg/kg, i.v.). **C.** Changes in mean arterial pressure (ΔMAP) in animals treated with vehicle, **ATZ4** or **ATZ5** in the absence or presence of efaroxan. Bars represent the means ± S.E.M for n = 4-6 animals. $^{*}P < 0.05$ compared with vehicle; $^{\#}P < 0.05$ compared with ATZ **5**; $^{\phi}P < 0.05$ compared with ATZ **4**.

Fig. 5. The most important ligands for imidazoline receptors**.**

Fig. 6. Hypothetical construction of the aminothiazoline **5** from the structure of agmatine.

Fig. 7. A. 3D optimized geometry of agmatine (top), **ATZ5** (center) and aminothiazoline **ATZ4** (bottom); **B**. Superimposed structures of agmatine (magenta), aminothiazoline **5** (green).

Fig. 1.



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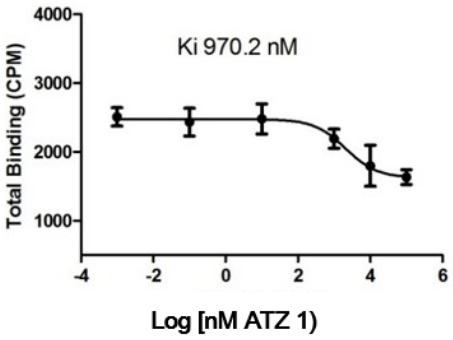

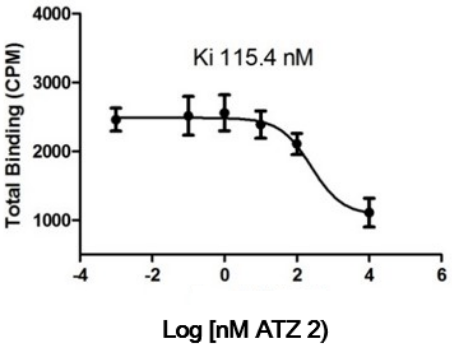

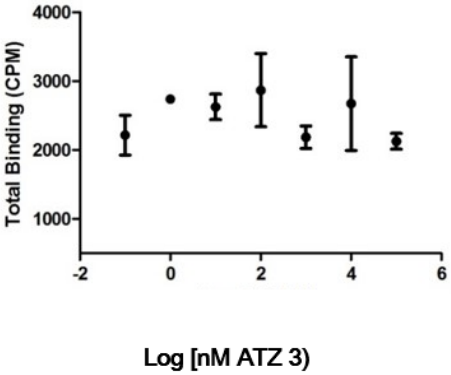

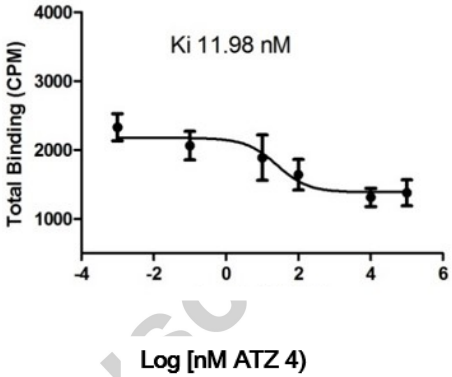

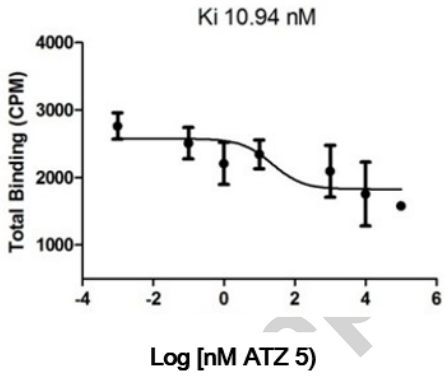

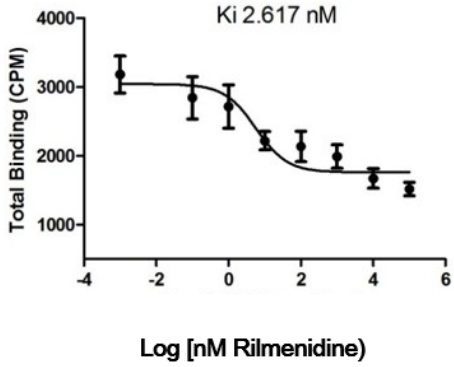

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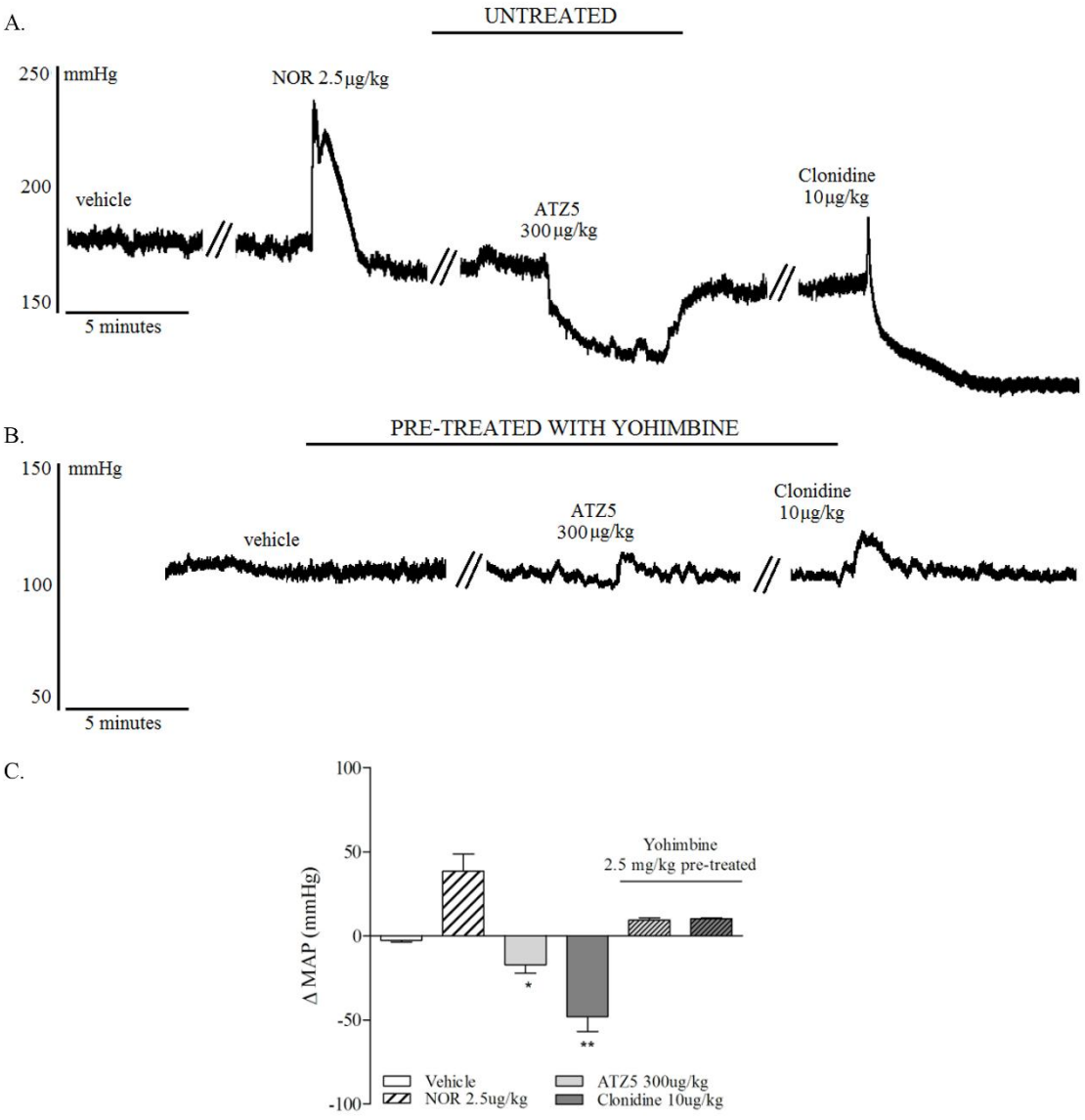

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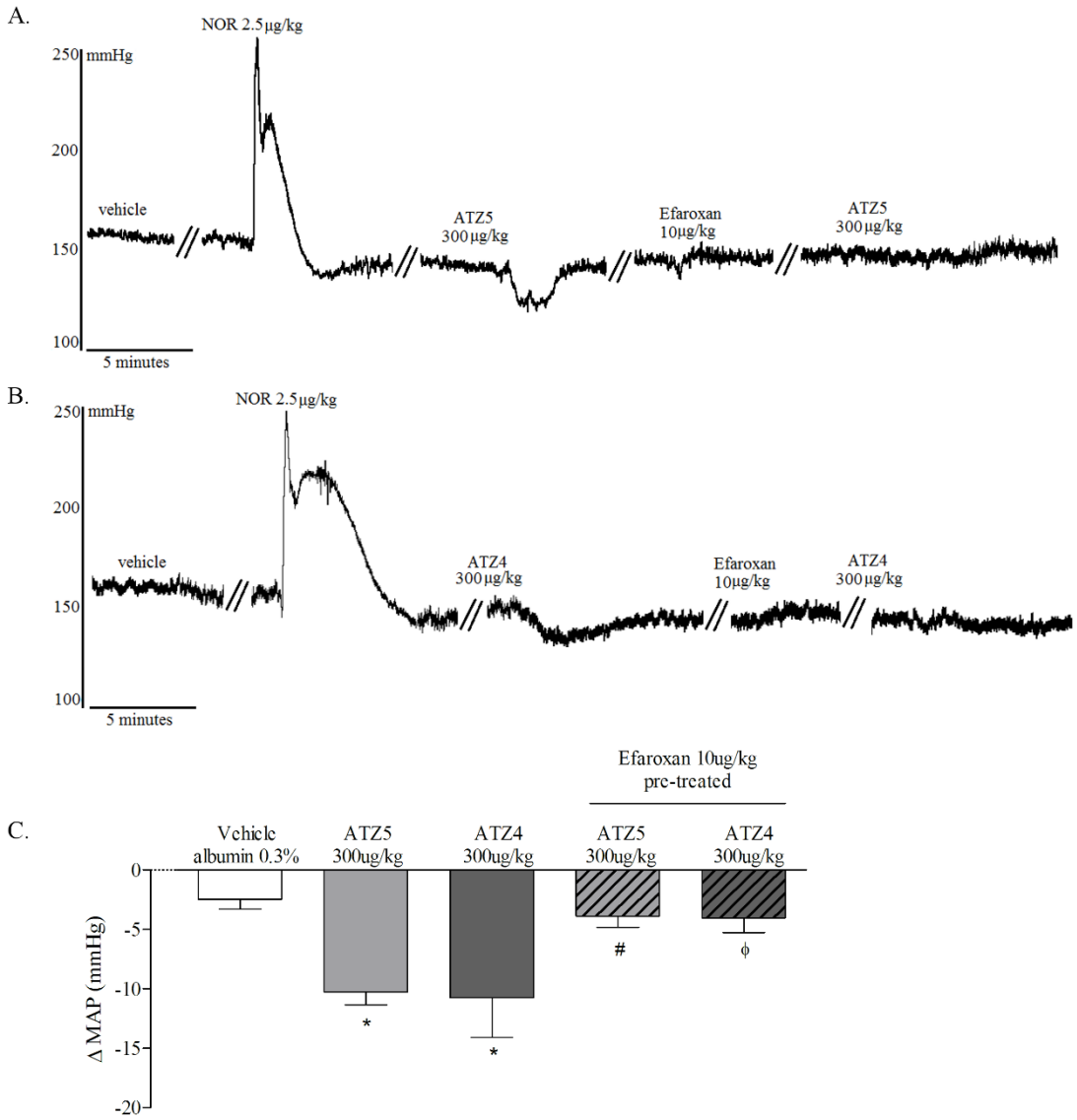

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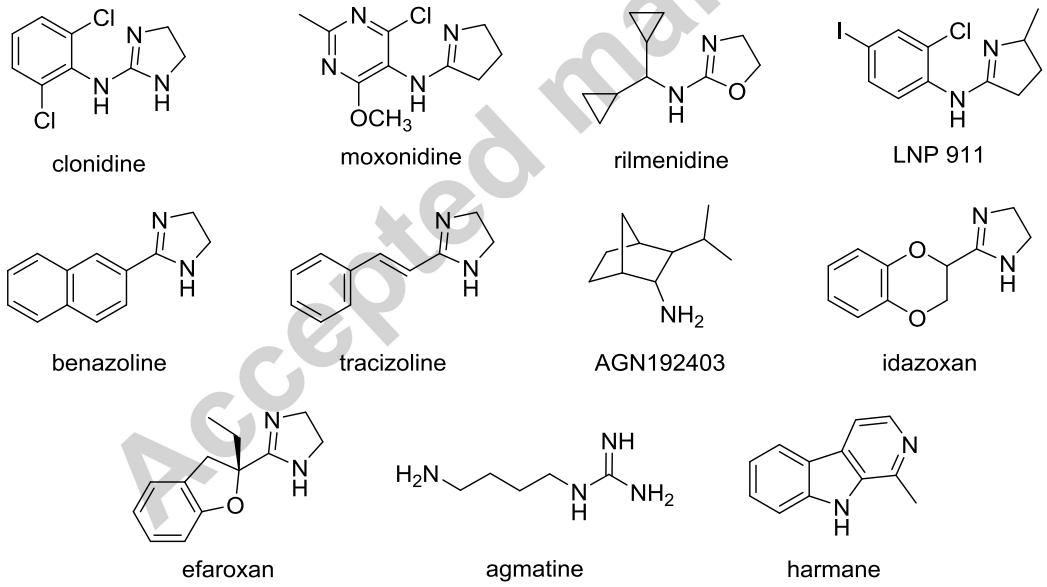

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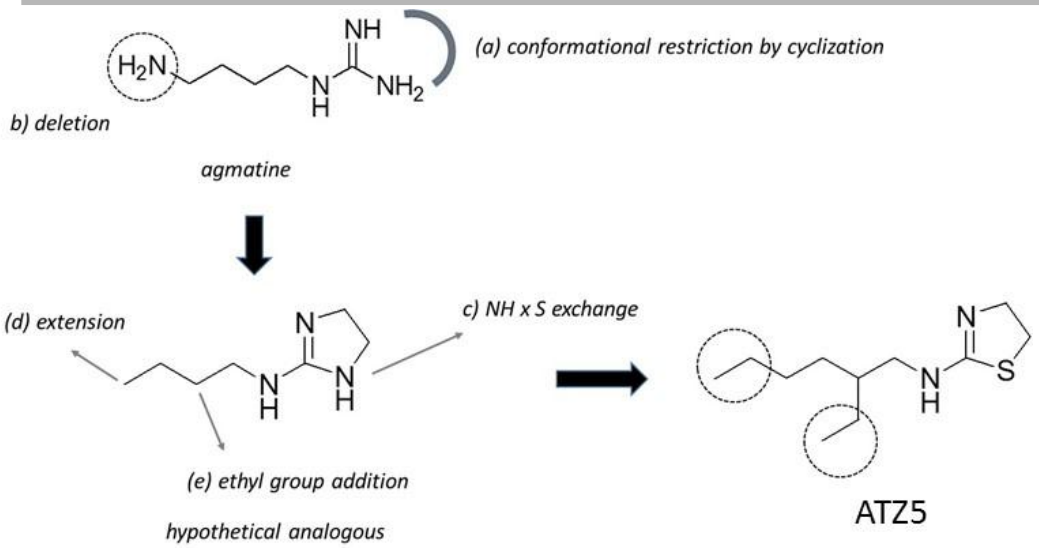

Fig.7A

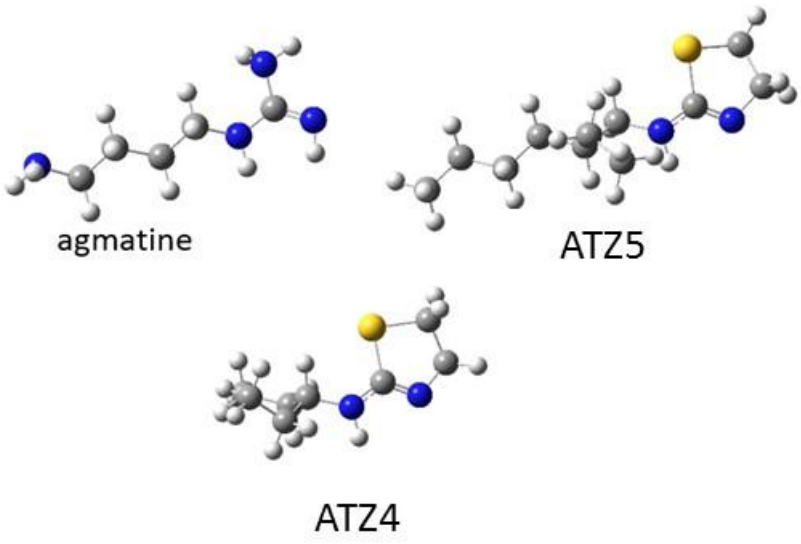

Fig. 7B