Are Alpha-2D Adrenoceptor Subtypes Involved in Rat Mydriasis Evoked by New Imidazoline Derivatives: Marsanidine and 7-Methylmarsanidine?

Joanna Raczak-Gutknecht¹, Teresa Frąckowiak¹, Antoni Nasal¹, Anita Kornicka², Franciszek Sączewski², and Roman Kaliszan¹

Abstract
The imidazoline compounds may produce mydriasis after systemic administration to some species (rats, cats, and mice). In mydriatic activity of imidazolines, α₂-adrenoceptor subtype(s) seems to be involved. In this study, the pupil dilatory effect evoked by 2 newly synthesized imidazoline derivatives—α₂-adrenoceptor agonists: marsanidine and 7-methylmarsanidine—was compared. The compounds were tested alone as well as in the presence of α₂-adrenoceptor antagonists (nonselective, yohimbine, and selective toward the following α₂-adrenoceptor subtypes—α₂A-2-[(4,5-dihydro-1H-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1H-isoindole maleate (BRL44408), α₂B-2-[2-(2-methoxyphenyl)piperazin-1-yl]ethy]-4,4-dimethyl-1,3-(2H,4H)-isoquinolinidione dihydrochloride (ARC239), α₂C-JP1302, α₂D-2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1H-imidazole hydrochloride [RX821002]). The agonists were studied in male Wistar rats and were administered intravenously in cumulative doses. The antagonistic compounds were given in a single dose before the experiment with marsanidine or 7-methylmarsanidine. Pupil diameter was measured with stereoscopic microscope equipped in green light filter. Marsanidine and 7-methylmarsanidine exerted marked mydriatic effects. BRL44408, JP1302, and ARC239 did not cause significant parallel shift to the right of the dose–effect curves obtained for both imidazolines. In case of yohimbine and RX821002, the marked parallel shifts of dose–response curves were observed, with the antagonistic effects of RX821002 more pronounced. In vivo pharmacodynamics experiment suggests that α₂D-adrenoceptor subtype is mainly engaged in mydriatic effects evoked in rats by imidazoline derivatives, in particular by clonidine.

Keywords
rat eye mydriasis, α₂-adrenoceptors subtypes, α₂-adrenergic imidazoline agents, marsanidine, 7-methylmarsanidine, clonidine

Introduction
The compounds having imidazol(in)e moiety (the so-called “clonidine-like” agents) are showing a variety of pharmacological activities, such as hypotension, bradycardia, sedation, analgesia, and mydriasis.¹,² These effects can be explained by the affinity of imidazol(in)es to the α-adrenergic and imidazoline receptors. It has been proved that α₂-adrenergic agonists of imidazol(in)e structure evoke mydriasis in laboratory animals (rats, mice, and cats) after systemic application.³,⁴ Further studies lead to the conclusion that pupillary dilation produced by these compounds is mediated via the stimulation of the brain α₂-adrenoceptors located in the Edinger-Westphal nucleus where they inhibit parasympathetic tone to the iris.⁵,⁶ Christensen et al⁷ and Hey et al⁸ demonstrated in experiments on both anesthetized and conscious rats that these α₂-adrenergic receptors are located postsynaptically to noradrenergic neurons.

Based on molecular biological and radioligand receptor binding techniques, α₂-adrenoceptors are divided into 4

¹ Department of Biopharmaceutics and Pharmacodynamics, Medical University of Gdańsk, Gdańsk, Poland
² Department of Chemical Technology of Drugs, Medical University of Gdańsk, Gdańsk, Poland

Corresponding Author:
Antoni Nasal, Department of Biopharmaceutics and Pharmacodynamics, Medical University of Gdańsk, Al. Gen. J. Hallera 107, 80-416 Gdańsk, Poland. Email: antonasa@gumed.edu.pl

Creative Commons CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 3.0 License (http://www.creativecommons.org/licenses/by-nc/3.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).
subtypes—\(\alpha_2A\), \(\alpha_2B\), \(\alpha_2C\), and \(\alpha_2D\)—\(^9,10\) which are responsible for different physiological processes. The \(\alpha_2A\) subtype has been identified at first in human platelets and in rabbit spleen.\(^11,12\) The \(\alpha_2B\) subtype was found in rat tissues (lung and kidney)\(^11,13\) and the \(\alpha_2C\) subtype in the opossum kidney cell line.\(^14\) The \(\alpha_2D\) subtype was reported in rat submaxillary gland and in bovine pineal gland.\(^12,15\) However, \(\alpha_2A\) and \(\alpha_2D\) receptors have very similar structure and expectingly are the species orthologs.\(^16\) It was suggested that \(\alpha_2A\) subtype is present in human and pig, while \(\alpha_2D\)-adrenoceptors is present in rat, mouse, guinea pig, and cow.\(^17,18\) According to Lanier et al\(^9\) and Link et al, rodent \(\alpha_2A\) subtype was defined as \(\alpha_2D\) on the basis of very similar structure and ligand binding profile.\(^16\) However, it was also found that the residue in the position 201 in the human species seems to be an important feature that differentiates \(\alpha_2A\) from \(\alpha_2D\) pharmacology.\(^16,17\) In addition, some authors underline the lower affinity of yohimbine for the \(\alpha_2D\) subtype adrenoceptor.\(^17\)

The question arises which subtype(s) of postsynaptic brain \(\alpha_2\)-adrenergic receptors could be engaged in mydriatic activity of imidazoline compounds. Based on the results of both radioligand binding and functional studies, it has been postulated by Heal et al\(^8\) that postsynaptic \(\alpha_2\)-adrenergic receptors, localized in the rat cortex and Edinger-Westphal nucleus, are predominantly of \(\alpha_2D\) subtype.

The aim of the present study was an in vivo assessment in anesthetized rats of the effects on pupil diameter due to marsanidine and 7-methylmarsanidine—2 newly synthesized \(\alpha_2\)-adrenergic receptor agonists having an imidazoline moiety in their structure.\(^18\) The well-established mydriasis model according to Koss\(^3\) was applied. The activity of marsanidine and 7-methylmarsanidine was compared to clonidine, a reference imidazoline drug stimulating brain \(\alpha_2\)-adrenoceptors. All agents were studied as administrated alone and after the pre-treatment with yohimbine—a “classical” nonselective antagonist of \(\alpha_2\)-adrenoceptors. To test pharmacologically, whether the \(\alpha_2D\)-adrenergic receptor is (or not) solely involved in mydriatic effects of marsanidine and 7-methylmarsanidine, the separate experiments were carried out in the presence of the known selective antagonists of individual \(\alpha_2\)-adrenoceptor subtypes—2-[(4,5-dihydro-1H-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1H-isindole maleate (BRL44408; \(\alpha_2A\)),\(^3,10\) 2-[2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl]-4,4-dimethyl-1,3-(2H,4H)-isoquinolindione dihydrochloride (ARC239; \(\alpha_2B\)),\(^11\) and JP1302 (\(\alpha_2C\)).\(^21\) Additionally, 2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1H-imidazole hydrochloride (RX821002), classified as a compound preferably blocking \(\alpha_2D\)-adrenoceptors, was included in the project.\(^22\)

**Aim**

The aim of the study was the comparison of the pupil dilatory effects evoked in rats by a model imidazoline drug—clonidine—and 2 newly synthesized imidazoline compounds: marsanidine and 7-methylmarsanidine. The compounds were tested alone and also in the presence of \(\alpha_2\)-adrenergic receptor antagonists. The aim of the study was also the pharmacological evaluation of the role of \(\alpha_2\)-adrenergic receptor subtype(s) in mydriatic effects evoked by compounds studied using \(\alpha_2\)-adrenergic receptor antagonists.

**Materials and Methods**

**Animals**

The studies were performed in male Wistar rats weighing 200 to 300 g. The rats were anesthetized with urethane 1.5 g/kg intraperitoneally.

This study was carried out in accordance with the recommendations of “National ethics committee for animal researches in Poland.” The protocol was approved by the “National ethics committee for animal researches in Poland.”

**Imidazoline Derivatives**

Clonidine hydrochloride, BRL44408 maleate, JP1302 dihydrochloride, ARC239 dihydrochloride, and RX821002 hydrochloride were purchased from Tocris (Bristol, United Kingdom). Yohimbine hydrochloride was obtained from Sigma-Aldrich (St Louis, Missouri); marsanidine and 7-methylmarsanidine hydrochlorides were synthesized by Prof. F. Sączelewski at the Department of Chemical Technology of Drugs, Medical University of Gdańsk. All substances studied were dissolved in 0.9% NaCl solution.

**Rat Eye Pupil Diameter Measurement**

Pupil diameter measurement was carried on by adapting the Koss method.\(^3\) Measurements were performed using a stereoscopic microscope (MST 132 Lab TK PZO, Warszawa, Poland) equipped with a scale and an external light source. A green filter was used to eliminate the reaction of the pupil on the light and also to enhance the image contrast of the iris. All experiments were performed in a darkened room at fixed light conditions. Pupil diameter was measured with an accuracy of 0.10 mm at the maximum width of the pupil. The initial value of the pupil diameter, before the administration of 0.9% NaCl solution and studied drugs, was about 0.70 ± 0.5 mm.

Clonidine, marsanidine, and 7-methylmarsanidine were administered to the rats through the femoral vein at a volume of 1 mL/kg in cumulative doses (1, 3, 5, 10, 30, 50, 100, 300, 500, 1000 μg/kg) at 5-minute intervals. Yohimbine (1.5 mg/kg), BRL44408 (1 mg/kg), JP1302 (1 mg/kg), ARC239 (0.5 mg/kg), and RX821002 (0.05 mg/kg) were given intravenously 10 minutes before starting the administration of a series of agonist in cumulative doses.

The results (mean of 5 experiments) are shown in the form of curves illustrating the dependence of mydriatic effect (in millimeters) on the logarithmically increasing dose (μg/kg) of clonidine, marsanidine, and 7-methylmarsanidine, given to the animals alone and in the presence of antagonists.
Data Analysis

Dose–mydriatic effect curves were constructed applying GraphPad Prism, version 6.00 for Windows, GraphPad Software (La Jolla, California). The doses of imidazoline agents studied, which produced 50% maximum mydriatic effect, the dose causing 50% of maximum effect (ED50), were also calculated by nonlinear regression analysis with the use of this program. These data were presented with 95% confidence intervals and the number of degrees of freedom (df) given in parentheses.

Antagonistic potencies of yohimbine, BRL44408, ARC239, JP1302, and RX821002 were expressed as a pA2 defined as the negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of the agonist needed to elicit the original submaximal response obtained in the absence of antagonist. GraphPad Prism was used for calculation of pA2 values applying Gaddum-Schild model with 95% confidence intervals and the number of df. Molecular dynamics calculations were applied when attempting to identify molecular descriptors of imidazolines determining interactions with hypothetical receptors. Unfortunately, our efforts failed. One-way analysis of variance was used to compare the mydriatic effect among the 6 groups (the clonidine and clonidine with each of 5 antagonists studied). Post hoc Tukey test was performed to compare the difference of effects between the groups at a significance level of P < .05.

Results

Intravenous administration of clonidine, marsanidine, and 7-methylmarsanidine in increasing doses 1 to 1000 μg/kg resulted in sigmoid mydriatic dose–response curves (Figures 2–4). The pupil dilation was rapid in onset within the first minute after injection to rats and was sustained for the duration of the experiment. The rank order of potency of the imidazoline agents studied was 7-methylmarsanidine > clonidine > marsanidine (Table 1). Maximal pupillary dilations observed, Emax, were 3.52 ± 0.10, 3.63 ± 0.09, and 3.97 ± 0.10, respectively.

When rats were pretreated with α2-adrenoceptor antagonist: yohimbine, BRL44408, ARC239, JP1302, and RX821002, dose-dependent pupillary dilation curves observed for clonidine, marsanidine, and 7-methylmarsanidine were shifted in parallel fashion to the right. The maximal mydriatic responses of the agents under study were indistinguishable from the effects of these imidazole compounds alone, indicating competitive antagonism (Figures 2–4). The corresponding ED50 and pA2 values are collected in Table 1.
As shown in Figure 1, RX821002, the preferential antagonist of \( \alpha_2 \)-adrenergic receptor, is the most potent inhibitor of clonidine-induced mydriasis in rat model. This compound causes a parallel shift to the right of the dose–mydriatic effect curve for clonidine. The calculated ED_{50} values are 524.1 (485.0-566.4) \( \mu \)g/kg and 11.23 (11.01-11.46), respectively (Table 1). Also, the pupillary response curve for clonidine was competitively antagonized by yohimbine in a dose-related fashion, but this effect was less pronounced. The calculated ED_{50} for clonidine + yohimbine is 34.79 (32.89-36.80) and pA\(_2\) value equals 6.66 (6.54-6.79; Table 1). BRL44408, ARC239, and JP1302—the selective antagonists of \( \alpha_2A \), \( \alpha_2B \), and \( \alpha_2C \) subtypes of \( \alpha_2 \)-adrenoceptor, respectively—have no significant effects on mydriasis produced by cumulative doses of clonidine. The corresponding ED_{50} values for clonidine pretreated with BRL44408, ARC239, and JP1302 are close to ED_{50} value for clonidine alone (Table 1). The antagonistic potencies (pA\(_2\)) could not be calculated because of the overlapping curves of the dose–mydriatic effects, obtained for clonidine in the presence of BRL44408, ARC239, and JP1302 (Figure 1).

In case of 7-methylmarsanidine, similar situation occurred (Figure 2). Mydriatic effect produced by cumulative doses of this imidazoline agent was strongly antagonized by pretreatment with RX821002 (ED_{50} = 18.11 [16.44-19.94], pA\(_2\) = 6.99 [6.81-7.17]) and less when yohimbine was used (ED_{50} = 6.54 [5.89-7.24], pA\(_2\) = 5.66 [5.41-5.92]; Table 1). BRL44408, ARC239, and JP1302 had no significant effects on pupillary dilation evoked by 7-methylmarsanidine. The ED_{50} values obtained for 7-methylmarsanidine in the presence of BRL44408, ARC239, and JP1302 are close to the value for 7-methylmarsanidine alone (Table 1). Similarly as in the case of clonidine, it was impossible to calculate the values of pA\(_2\) for 7-methylmarsanidine studied in the presence of BRL44408, ARC239, and JP1302 due to the overlapping of dose–pupillary dilation effect curves (Figure 1).

Pretreatment with RX821002 caused a marked parallel shift to the right of the marsanidine mydriasis curve (Figure 3). The potency of yohimbine to inhibit marsanidine-induced mydriasis is lower. The corresponding pA\(_2\) values are 8.34 (8.18-8.49) and 6.02 (5.79-6.24), respectively (Table 1). The antagonists of \( \alpha_2 \)-adrenoceptor subtypes, ARC239 (\( \alpha_2B \)) and JP1302 (\( \alpha_2C \)), produced slight parallel shifts to the right of the marsanidine pupillary dilation curves. The antagonistic potencies (pA\(_2\)) of these compounds do not statistically differ as compared to pA\(_2\) value calculated for marsanidine + yohimbine. BRL44408 practically did not shift the marsanidine dose–response curve; therefore, its pA\(_2\) value was not calculated (Table 1).

**Discussion**

Imidazol(ine) agents, classified as \( \alpha_2 \)-adrenoceptors ligands, may interact with different subtypes of this receptor (\( \alpha_2A \), \( \alpha_2B \), \( \alpha_2C \), and \( \alpha_2D \) subtype). However, the role of individual \( \alpha_2 \)-adrenergic receptor subtypes in physiological processes is still not satisfactorily elucidated. As it was demonstrated in studies of many researchers, adrenoceptors of \( \alpha_2A \) subtype could play a significant role in hypotension and bradycardia\( ^{25,26} \) as well as in antinociceptive activity.\( ^{27,28} \) This receptor subtype seems to be responsible also for sedative and hypothermic effects.\( ^{27} \) It is also postulated that \( \alpha_2A \)-adrenoceptors take part in presynaptic inhibition of noradrenaline release in nerve endings at high stimulation frequencies (while release of this neurotransmitter on lower frequencies is regulated rather by \( \alpha_2C \)-adrenoceptors).\( ^{29} \)
Stimulation of \(\alpha_{2B}\)-adrenoceptor subtype in vascular smooth muscle evokes increase of blood pressure and counteracts the hypotensive effect of \(\alpha_{2A}\)-receptors stimulation in the central nervous system.\(^{30}\) The adrenoceptors of \(\alpha_{2C}\) subtype are located mainly in the central nervous system and could be involved (beyond \(\alpha_{2A}\)-receptors) in the regulation of transmitter release.\(^{29}\) Although their role in hemodynamics is still not fully understood, \(\alpha_{2C}\)-adrenoceptors seem to mediate venous vasoconstriction\(^ {31}\) and also may mediate other peripheral actions, for example, play a role in hypothermic effect, secondary to the prominent role of the \(\alpha_{2A}\)-subtype.\(^ {32}\)

Commonly applied in vitro radioligand binding method to study an affinity of newly synthesized imidazoline analogs of potential pharmacological activity to \(\alpha_{2}\)-adrenergic/imidazoline receptors is obviously not sufficient to decide whether the agent studied could be regarded as receptor agonist or antagonist. To obtain actual information about the pharmacological properties of imidazoline ligands, in vivo tests are necessary. The rat eye mydriasis model\(^ {33}\) has many advantages as compared to the other pharmacological tests (eg, clonidine-induced reduction of motor activity in mice\(^ {33}\) in allowing to evaluate the interactions of potential imidazoline ligands with brain \(\alpha_{2}\)-adrenergic receptors). The most important is that the pharmacodynamics experiment could be performed in vivo in a whole animal, with an individual imidazoline compound injected in a wide range of doses (from few \(\mu\)g/kg to several mg/kg). This method is simple and reproducible. Moreover, it provides an opportunity to test not only both the \(\alpha_{2}\)-agonistic and \(\alpha_{2}\)-antagonistic properties of imidazolines studied but also to exclude potential ability of the ligands to interact with \(\alpha_{1}\)-imidazoline receptors because this type of receptor is not practically involved in the mediation of central mydriasis in rats.\(^ {34,35}\)

Marsanidine (1-[imidazolidin-2-yl]imino]indazole) and 7-methylmarsanidine (1-[imidazolidin-2-yl]imino]-7-methylindazole) are new imidazoline derivatives synthesized by Sączewski et al.\(^ {36}\) In radioligand studies performed on rat brain membranes, the first one proved to be a selective \(\alpha_{2}\)-adrenoceptor ligand (\(K_i = 14.05\) nM) having the \(\alpha_{2}/\alpha_{1}\) selectivity ratio 3879, while the second compound shows less affinity to \(\alpha_{2}\)-adrenergic receptor (\(K_i = 53.6\) nM) and its \(\alpha_{2}/\alpha_{1}\) selectivity ratio equals 7.2.\(^ {37}\) Both agents exert agonistic activity toward \(\alpha_{2}\)-adrenoceptors, lowering blood pressure and decreasing heart rate in experiments on anesthetized rats.\(^ {37,38}\)

The central antihypertensive agent clonidine shows “mixed” agonistic properties toward \(\alpha_{2}\)-adrenergic and \(\alpha_{1}\)-imidazoline receptors. Its radioligand binding affinity, \(pK_i\) values at the human \(\alpha_{2A}\) and \(\alpha_{2B}\)-receptors expressed in human embryonic kidney 293 cells are 7.21 and 7.16, respectively, while the corresponding \(pK_i\) value determined in vitro in human platelets equals 7.25. Clonidine injected intravenously to the rats evokes a dose-dependent pupil dilation at very low doses (from 1 \(\mu\)g/kg). Hence, this drug proved to be a good reference agent for other imidazolines having affinity to \(\alpha_{2}\)-adrenergic receptors.\(^ {3}\)

### Table 1. ED\(_{50}\) Values of Imidazoline Agents Studied in the Absence and in the Presence of Different \(\alpha_{2}\)-Adrenoceptor Antagonists as well as \(pA_2\) Values Calculated for Clonidine, Marsanidine, and 7-Methylmarsanidine in the Presence of Yohimbine, BRL44408, ARC239, JP1302, and RX821002.

| Compound                        | ED\(_{50}\), \(\mu\)g/kg | \(pA_2\) |
|---------------------------------|-------------------------|---------|
| Clonidine                       | 8.34 (7.55-9.18), \(df = 52\) | –       |
| Clonidine + yohimbine           | 34.79 (32.89-36.80), \(df = 52\) | 6.66 (6.54-6.79), \(df = 97\) |
| Clonidine + BRL44408            | 8.75 (8.17-9.38), \(df = 41\) | NC      |
| Clonidine + ARC239              | 5.56 (4.88-6.32), \(df = 52\) | NC      |
| Clonidine + JP1302              | 6.93 (6.58-7.30), \(df = 52\) | NC      |
| Clonidine + RX821002            | 524.1 (485.0-566.4), \(df = 47\) | 11.23 (11.01-11.46), \(df = 97\) |
| Marsanidine                     | 45.65 (39.60-52.63), \(df = 47\) | –       |
| Marsanidine + yohimbine         | 109.9 (84.2-143.4), \(df = 47\) | 6.02 (5.79-6.24), \(df = 77\) |
| Marsanidine + BRL44408          | 114.3 (89.38-146.1), \(df = 33\) | NC      |
| Marsanidine + ARC239            | 109.0 (71.93-165.2), \(df = 27\) | 6.55 (6.23-6.87), \(df = 57\) |
| Marsanidine + JP1302            | 68.2 (55.98-83.08), \(df = 47\) | NC      |
| Marsanidine + RX 821002         | 153.4 (131.1-179.6), \(df = 37\) | 8.34 (8.18-8.49), \(df = 67\) |
| 7-Methylmarsanidine             | 4.94 (4.28-5.93), \(df = 42\) | –       |
| 7-Methylmarsanidine + yohimbine | 6.54 (5.89-7.24), \(df = 37\) | 5.66 (5.41-5.92), \(df = 87\) |
| 7-Methylmarsanidine + BRL44408  | 4.14 (3.89-4.42), \(df = 37\) | NC      |
| 7-Methylmarsanidine + ARC239    | 4.5 (4.21-4.81), \(df = 37\) | NC      |
| 7-Methylmarsanidine + JP1302    | 5.65 (5.33-5.99), \(df = 37\) | NC      |
| 7-Methylmarsanidine + RX821002  | 18.11 (16.44-19.94), \(df = 47\) | 6.99 (6.81-7.17), \(df = 97\) |

Abbreviations: ARC239, 2-[2-4-(2-methoxyphenyl)piperazin-1-yl)methyl]-4,4-dimethyl-1,3-(2H,4H)-isouquinolindine dihydrochloride; BRL44408, 2-[4-(5-dihydro-1H-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1H-isooindole maleate; NC, not calculated; RX821002, 2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1H-imidazole hydrochloride.

\(^{a}\)Agonists (clonidine, marsanidine, 7-methylmarsanidine) were administered IV in increasingly cumulative doses at 5-minute intervals. Antagonists were administered IV 10 minutes before starting the administration of the series of agonist doses. ED\(_{50}\) values were calculated by nonlinear regression analysis with 95% confidence intervals and the number of \(df\).
In the present study, 2 newly synthesized imidazoline derivatives—marsanidine and 7-methylmarsanidine—as well as the reference drug: clonidine—produced dose-related mydriatic effects. The maximal pupillary dilations observed after administration of these agents to rats were similar but the effect of marsanidine was slightly greater than in the case of clonidine. It can be noted that the ED_{50} value for clonidine (8.34 µg/kg) is close to that previously reported by Koss and Koss.\textsuperscript{34}

Of the 2 new imidazolines, the relatively selective \( \alpha_2 \)-adrenergic receptor agonist marsanidine displayed about 5-fold lower potency than clonidine, while the potency of 7-methylmarsanidine (ED_{50} = 4.94 µg/kg), having \( \alpha_2 \)/imidazoline \( I_1 \) receptor agonistic properties, is comparable to clonidine. High central activity of these compounds could be connected with their physicochemical properties. The theoretically calculated by us with the use of ACD software\textsuperscript{39} lipophilicity parameter, Calculated LOGP, for 7-methylmarsanidine equals 1.70 and is greater than for the 2 remaining imidazolines: clonidine (1.41) and marsanidine (1.24). According to Sęczewski et al.,\textsuperscript{37} clonidine having pK\(_a\) value 8.2 is ionized at physiological pH (14\% of nonionized form at pH 7.4), whereas marsanidine and its 7-methyl analogue are characterized by lower basicity (pK\(_a\) values of these compounds are 6.32 and 6.53, respectively). Therefore, at physiological pH, it is very likely that marsanidine and 7-methylmarsanidine could exist primarily as nonionized bases (92\% and 82\%, respectively). That would explain their increased ability to permeate the blood–brain barrier. Boblewski et al\textsuperscript{38} proved that marsanidine and its 7-methyl derivative administered in the dose of 100 µg/kg to anesthetized rats induced marked decrease of blood pressure and heart rate, but the maximum hypotensive and negative chronotropic effects of the former compound (−30 mm Hg and −49 beats per minute) were less pronounced than that of the second one (−43 mm Hg and −122.9 beats per minute).

Yohimbine is an \( \alpha_2 \)-adrenergic antagonist commonly used in studies on the mydriatic activity of imidazoline compounds. In radioligand studies, it binds to all \( \alpha_2 \)-adrenoceptor subtypes, having the higher affinity to the \( \alpha_2A \) and \( \alpha_2C \) subtypes, lower to \( \alpha_2B \) one, and the lowest to \( \alpha_2D \)-adrenergic receptor. The corresponding pK\(_i\) values determined with the use of membranes of tissues containing only 1 subtype of \( \alpha_2 \)-adrenergic receptor—HT29 cells (\( \alpha_2A \)), rat neonatal lung (\( \alpha_2B \)), opossum kidney cells (\( \alpha_2C \)), and PC12 cells (\( \alpha_2D \))—are 8.72, 7.95, 8.94, and 7.27, respectively.

The another 3 \( \alpha_2 \)-adrenergic receptor antagonists, applied in this work, are characterized by a selectivity toward particular subtypes of this receptor. BRL44408 is classified as selective \( \alpha_2A \)-adrenoceptor antagonist having a good affinity for this subtype but significantly lower for all other subtypes of \( \alpha_2 \)-adrenergic receptor. In radioligand binding studies on Chinese hamster ovary (CHO) cells, transfected with human \( \alpha_2A \), \( \alpha_2B \), and \( \alpha_2C \) receptors, K\(_i\) values were 109, 1800, and 700 nM, respectively.\textsuperscript{20}

ARC239 exerted a slight preference for \( \alpha_2A \)-adrenoceptors and showed significant \( \alpha_2B \)-adrenoceptor selectivity displaying a 100-fold \( \alpha_2B/\alpha_2A \) selectivity ratio in cell line experiments. Its binding affinity values, pK\(_i\), in CHO cell lines expressing human \( \alpha_2A \), \( \alpha_2B \), and \( \alpha_2C \) adrenergic receptors were 6.65, 8.03, and 7.78, respectively.\textsuperscript{40}

JP-1302 is a novel highly specific \( \alpha_2C \)-adrenergic receptor ligand. In in vitro competition binding assays with \(^{3}H\)-rauwolscine, on membranes from S115 cells transfected with 1 of the 3 human \( \alpha_2 \) receptor subtypes (\( \alpha_2A \), \( \alpha_2B \), \( \alpha_2C \)), the agent displayed an affinity of 28 nM for the \( \alpha_2C \) subtype. The same K\(_i\) values obtained for the \( \alpha_2A \) and \( \alpha_2B \)-adrenergic receptors are 3150 and 1470 nM, respectively.\textsuperscript{41} JP-1302 displayed strong antagonistic potency, characterized by K\(_B\) value of 16 nM, at the human \( \alpha_2C \)-adrenoceptor subtype. In comparison, the K\(_B\) for human \( \alpha_2A \) and \( \alpha_2B \) subtypes equals 1500 and 2200 nM, respectively. All these data were established with membranes from CHO cells, stably expressing the human \( \alpha_2A \), \( \alpha_2B \), and \( \alpha_2C \) adrenergic receptor subtypes, by antagonizing the adrenaline-induced stimulation of \(^{[35]}S\)-GTP\(_\gamma\) binding.\textsuperscript{41}

According to Sallinen et al., JP-1302 did not antagonize dexmedetomidine-evoked mydriatic effect in rats, but this effect was antagonized by atipamezole known as a nonselective antagonist of \( \alpha_2 \)-adrenoceptor subtypes.\textsuperscript{41}

RX821002 is relatively selective for both \( \alpha_2A \) and \( \alpha_2C \) versus \( \alpha_2B \) adrenoceptor subtypes. Its binding activity, pK\(_i\), at 3 human \( \alpha_2 \)-adrenergic receptor subtypes expressed in CHO cells, is 9.73 (\( \alpha_2A \)), 8.77 (\( \alpha_2B \)), and 9.52 (\( \alpha_2C \)), respectively.\textsuperscript{42} At the same time, in experiments on brain cortex slices, this compound is an antagonist with high power to distinguish \( \alpha_2A \) from \( \alpha_2D \)-adrenoceptors while having markedly higher affinity for guinea pig \( \alpha_2D \) (pK\(_d\) = 9.7) than rabbit \( \alpha_2A \) (pK\(_d\) = 8.2) subtypes.\textsuperscript{43}

Dose–pupillary dilation curves, obtained not only for clonidine but also for marsanidine and 7-methylmarsanidine, were parallelly shifted to the right by yohimbine, which supports the participation of brain \( \alpha_2 \)-adrenergic receptors in mydriatic action of a model compound and 2 new imidazoline derivatives. Analysis of variance (\( P = .02 \)) and Tukey analysis of the results of our further experiments with the use of RX821002 showed that in the case of clonidine the subtype \( \alpha_2D \) seems to be predominantly engaged in pupillary response evoked by the imidazolines studied. The results of our further experiments with the use of BRL44408, ARC239, JP-1302, and RX821002 showed that the subtype \( \alpha_2D \) is predominantly engaged in pupillary response evoked by imidazolines studied. It was demonstrated by marked changes of pA\(_2\) values for clonidine, marsanidine, and 7-methylmarsanidine pretreated with RX821002, as compared to corresponding pA\(_2\) values calculated for these agents studied at the presence of yohimbine. Whereas in the case when selective antagonists of \( \alpha_2A \), \( \alpha_2B \), \( \alpha_2C \) subtypes of \( \alpha_2 \)-adrenoceptor were administered in single doses prior to clonidine, marsanidine, and 7-methylmarsanidine, no changes in the mutual position of the corresponding dose–response curves were noted.

Previously Heal et al\textsuperscript{44} provided the data from experiments in vitro on rat brain cortex preparation using a series of ligands
having different affinity to particular α2-adrenergic receptor subtypes (α2A-α2D). Displacement of [1H]RX821002 from cortical membranes with these compounds yielded pKᵣ values correlated very well with the same values for the α2D receptors in rat submaxillary gland reported earlier by Michel et al. At the same time, no significant correlations were obtained with literature pKᵣ data characterizing the binding of the agents to α2A, α2B, and α2C subtypes localized in rabbit spleen (α2A), rat kidney (α2B), and OK cells (α2C). Heal et al. determined also in conscious rats the potencies of various α2-adrenoceptor antagonists to inhibit clonidine-evoked mydriasis and found good relationships between −log ID₅₀ values and pKᵣ values for α2D-adrenoceptor binding, whereas poor correlations with the Kᵣ for the remaining subtypes of these receptors were noted. Based on the presented data, the conclusion has been drawn by these authors that postsynaptic α2-adrenergic receptors localized in both brain cortex and the Edinger-Westphal nucleus of the rat could be mainly of the α2D subtype.

However, lack of highly selective antagonists of particular subtypes of α2-adrenoceptors was for a long time an obstacle to demonstrate directly which subtype is engaged in mydriatic activity of imidazoline agents in rats. Especially, it concerns the following receptors: α2A, α2B, and α2C. Nowadays, some of these antagonists are available, for example, BRL4408 (α2A), ARC239 (α2B), JP-1302 (α2C), but according to existing literature, they were not yet applied (except of JP-1302) in functional studies on rat eye mydriasis. Only some antagonists, such as RX821002, MK 912, and benoxathian, are known to unambiguously differentiate α2A from α2D-adrenoceptors. Therefore, in this work, we undertook an attempt to evaluate directly the involvement of α2D-adrenoceptors in pupillary dilation produced by imidazoline drugs in anesthetized rats. Our results seem to confirm the earlier observations by Heal et al. Moreover, results support observations by Yu and Koss that clonidine-evoked mydriasis is triggered by postsynaptic α2-adrenergic stimulation of sciatric nerve, which produced mydriasis by the reduction of parasympathetic neural tone to the iris. Presented results (especially as regards to marsanidine and 7-methylmarsanidine) indicated that rat eye mydriasis model seems to be a valuable tool not only for detailed studies on the mechanism of imidazolines action on brain α2-adrenoceptors but also for identification of potential cardiovascular and other (eg, antinociceptive, antidepressant, anesthetic) drug “candidates” among newly synthesized imidazoline derivatives.

**Conclusion**

New imidazoline compounds, marsanidine and 7-methylmarsanidine show strong mydriatic effects in rats as compared to clonidine. Experiments performed in the presence of α2-adrenoceptor subtype(s) antagonists seem to confirm that α2-adrenoceptor 2D subtype has been engaged in mydriatic effects of clonidine, but results for marsanidine and 7-methylmarsanidine are ambiguous.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was supported by National Science Centre of Poland, Grant No. 2012/05/N/NZ7/03544.

**References**

1. Khan ZP, Ferguson CN, Jones RM. Alpha-2 and imidazoline receptor agonists. Their pharmacology and therapeutic role. *Anaesthesia. 1999;54(2):146-165. doi:10.1046/j.1365-2044.1999.00659.x.*

2. Nikolic K, Filipic S, Agbaba D. QSAR study of imidazoline antihypertensive drugs. *Bioorg Med Chem. 2008;16(15):7134-7140. doi:10.1016/j.bmc.2008.06.051.*

3. Koss MC. Pupillary dilation as an index of central nervous system alpha 2-adrenoceptor activation. *J Pharmacol Methods. 1986;15(1):1-19. doi:10.1016/0160-5402(86)90002-1.*

4. Raczk-Gutknecht J, Frąckowiak T, Nasal A, Kaliszanz R. Mydriasis model in rats as a simple system to evaluate alpha2-adrenergic activity of the imidazoline compounds. *Pharmacol Rep. 2013;65(2):305-312.*

5. Hey JA, Gherzghizerh T, Koss MC. Studies on the mechanism of clonidine-induced mydriasis in the rat. *Naunyn Schmiedebergs Arch Pharmacol. 1985;328(3):258-263. doi:10.1007/BF00515551.*

6. Koss MC. Rilmenidine produces mydriasis in cats by stimulation of CNS alpha 2-adrenoceptors. *Auton Autacoid Pharmacol. 2003; 23(1):51-56. doi:10.1046/j.1365-2044.2003.00276.x.*

7. Christensen HD, Mutzig M, Koss MC. CNS alpha 2-adrenoceptor induced mydriasis in conscious rats. *J Ocul Pharmacol. 1990; (2):123-129.*

8. Heal DJ, Prow MR, Butler SA, Buckett WR. Mediation of mydriasis in conscious rats by central postsynaptic alpha 2-adrenoceptors. *Pharmacol Biochem Behav. 1995;50(2):219-224. doi:10.1016/0091-3057(94)00299-X.*

9. Civantos Calzada B, Alexandre de Artiñano A. Alpha-adrenoceptor subtypes. *Pharmacol Res. 2001;44(3):195-208. doi:10.1006/phrs.2001.0857.*

10. Bylund DB. Subtypes of alpha 1- and alpha 2-adrenergic receptors. *Faseb J. 1992;6(3):832-839. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids¼1346768.*

11. Bylund DB, Ray-Prenger C, Murphy TJ. Alpha-2A and alpha-2B adrenergic receptor subtypes: antagonist binding in tissues and cell lines containing only one subtype. *J Pharmacol Exp Ther. 1988;245(2):600-607. http://www.ncbi.nlm.nih.gov/pubmed/2835476.*

12. Michel AD, Loury DN, Whiting RL. Differences between the α2-adrenoceptor in rat submaxillary gland and the α2A- and α2B-
13. Latifpour J, Jones SB, Bylund DB. Characterization of [3H]yohimbine binding to putative alpha-2 adrenergic receptors in neonatal rat lung. *J Pharmacol Exp Ther.* 1982;223(3):606-611.

14. Murphy TJ, Bylund DB. Characterization of alpha-2 adrenergic receptors in the OK cell, an opossum kidney cell line. *J Pharmacol Exp Ther.* 1988;244(2):571-578.

15. Simonneaux V, Ebadi M, Bylund DB. Identification and characterization of alpha 2D adrenergic receptors in bovine pineal gland. *Mol Pharmacol.* 1991;40(2):235-241. http://molpharm.aspetjournals.org/content/40/2/Abstract.%2Cmolpharm.aspetjournals.org/content/40/2/235.full.pdf.

16. Ruuskanen JO, Xhaard H, Marjamaki A, et al. Identification of duplicated fourth alpha2-adrenergic receptor subtype by cloning and mapping of five receptor genes in zebrafish. *Mol Biol Evol.* 2004;21(1):14-28. doi:10.1093/molbev/msg224.

17. Bylund DB. Alpha-2 adrenergic receptor subtypes: are more better? *Br J Pharmacol Rep.* 2005;144(2):159-160. doi:10.1038/sj.bjp.0706060.

18. Wróblewska M, Kasprzyk J, Sączewski F, et al. Marsanidine and 7-Me-marsanidine, the new hypotensive imidazolines augment sodium and urine excretion in rats. *Pharmacol Rep.* 2013;65(4):1025-1032.

19. Lanier SM, Downing S, Duzic E, Homcy CJ. Isolation of rat genomic clones encoding subtypes of the alpha 2-adrenergic receptor. Identification of a unique receptor subtype. *J Biol Chem.* 1991;266(16):10470-10478.

20. Beeley LJ, Berge JM, Chapman H, et al. Synthesis of a selective alpha(2A) adrenoceptor antagonist, BRL 48962, and its characterization at cloned human alpha-adrenoceptors. *Bioorg Med Chem.* 1995;3(12):1693-1698.

21. Nakamura M, Suk K, Lee MG, Jang IS. α(2A) adrenoceptor-mediated presynaptic inhibition of GABAAergic transmission in rat tuberomammillary nucleus neurons. *J Neurochem.* 2013;125(6):832-842. doi:10.1111/jn.12259.

22. Romero TRL, de Castro Perez A, de Francischi JN, Gama Duarte ID. Probable involvement of alpha(2C)-adrenoceptor subtype and endogenous opioid peptides in the peripheral antinociceptive effect induced by xylazine. *Eur J Pharmacol.* 2009;608(1-3):23-27. doi:10.1016/j.ejphar.2009.02.019.

23. Neubig RR, Spedding M, Kenakin T, Christophoules A; International Union of Pharmacology Committee on Rceptor Nomenclature and Drug Classification. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol Rev.* 2003;55(4):597-606. doi:10.1124/pr.55.4.4.

24. Ośmialowski K, Halkiewicz J, Radecki A, Kaliszan R. Quantum chemical parameters in correlation analysis of gas-liquid chromatographic retention indices of amines. *J Chromatogr A.* 1986;361:63-69. doi:10.1016/S0021-9673(01)86894-1.

25. MacMillan LB, Hein L, Smith MS, Piascik MT, Limbird LE. Central hypotensive effects of the alpha2a-adrenergic receptor subtype. *Science.* 1996;273(5276):801-803. http://www.ncbi.nlm.nih.gov/pubmed/8670421.

26. Altman JD, Trendelenburg a U, MacMillan L, et al. Abnormal regulation of the sympathetic nervous system in alpha2A-adrenergic receptor knockout mice. *Mol Pharmacol.* 1999;56(1):154-161.

27. Hunter JC, Fontana DJ, Hedley LR, et al. Assessment of the role of alpha2-adrenoceptor subtypes in the antinociceptive, sedative and hypothermic action of dexmedetomidine in transgenic mice. *Br J Pharmacol.* 1997;122(7):1339-1344. doi:10.1038/sj.bjp.0701520.

28. Lakhiani PP, MacMillan LB, Guo TZ, et al. Substitution of a mutant alpha2a-adrenergic receptor via “hit and run” gene targeting reveals the role of this subtype in sedative, analgesic, and anesthetic-sparing responses in vivo. *Proc Natl Acad Sci U S A.* 1997;94(18):9950-9955. doi:10.1073/pnas.94.18.9950.

29. Hein L, Altman JD, Kobilka BK. Two functionally distinct alpha2-adrenergic receptors regulate sympathetic neurotransmission. *Nature.* 1999;402(6758):181-184. doi:10.1038/46040.

30. Link RE, Desai K, Hein L, et al. Cardiovascular regulation in mice lacking alpha2-adrenergic receptor subtypes b and c. *Science.* 1996;273(5276):803-805.

31. Gavin KT, Colgan MP, Moore D, Shanik G, Docherty JR. Alpha 2C-adrenoceptors mediate contractile responses to noradrenaline in the human saphenous vein. *Naunyn Schmiedebers Arch Pharmacol.* 1997;355(3):406-411.

32. Kable JW, Murrin LC, Bylund DB. In vivo gene modification elucidates subtype-specific functions of alpha(2)-adrenergic receptors. *J Pharmacol Exp Ther.* 2000;293(1):1-7.

33. Heal DJ, Prow MR, Buckett WR. Clonidine produces mydriasis in conscious mice by activating central alpha 2-adrenoceptors. *Eur J Pharmacol.* 1989;170(1-2):11-18. doi:10.1016/0014-2999(89)90127-1.

34. Yu Y, Koss MC. Rat clonidine mydriasis model: imidazoline receptors are not involved. *Auton Neurosci Basic Clin.* 2005;117(1):17-24. doi:10.1016/j.autneu.2004.10.001.

35. Raczk-Gutknecht J, Frąckowiak T, Nasal A, et al. Effect of the reference imidazoline drugs, clonidine and rilmenidine, on rat eye pupil size confirms the decisive role of α2-adrenoceptors on mydriasis. *Int J Pharmacol.* 2014;10(8):470-478. doi:10.3923/ijp.2014.470.478.

36. Sączewski F, Kornicka A, Hudson AL, et al. 3-[(imidazolidin-2-yl)imino]indazole ligands with selectivity for the α(2)-adrenoceptor compared to the imidazoline I(1) receptor. *Bioorg Med Chem.* 2011;19(1):321-329. doi:10.1016/j.bmc.2010.11.020.

37. Słączewski F, Korzicka A, Hudson AL, et al. 1-[(imidazolidin-2-yl)imino]indazole. Highly alpha(2)/I(1) selective agonist: synthesis, X-ray structure, and biological activity. *J Med Chem.* 2008;51(12):3599-3608. doi:10.1021/jm800112s.

38. Boblewski K, Lehmann A, Sączewski F, Kornicka A, Rybczynska A. Vagotomy reveals the importance of the imidazoline receptors in the cardiovascular effects of marsanidine and 7-ME-marsanidine in rats. *Pharmacol Rep.* 2014;66(5):874-879. doi:10.1016/j.pharep.2014.05.009.
40. Gentili F, Pigini M, Piergentili A, Giannella M. Agonists and antagonists targeting the different alpha2-adrenoceptor subtypes. *Curr Top Med Chem*. 2007;7(2):163-186.

41. Sallinen J, Hoglund I, Engstrom M, et al. Pharmacological characterization and CNS effects of a novel highly selective alpha2C-adrenoceptor antagonist JP-1302. *Br J Pharmacol*. 2007;150(4):391-402. doi:10.1038/sj.bjp.0707005.

42. Audinot V, Fabry N, Nicolas JP, et al. Ligand modulation of [35S]GTPgammaS binding at human alpha(2A), alpha(2B) and alpha(2C) adrenoceptors. *Cell Signal*. 2002;14(10):829-837.

43. Trendelenburg AU, Wahl CA, Starke K. Antagonists that differentiate between alpha 2A-and alpha 2D-adrenoceptors. *Naunyn Schmiedebersgs Arch Pharmacol*. 1996;353(3):245-249.

44. Heal DJ, Cheetham SC, Butler SA, Gosden J, Prow MR, Buckett WR. Receptor binding and functional evidence suggest that postsynaptic α2-adrenoceptors in rat brain are of the α2D subtype. *Eur J Pharmacol*. 1995;277(2-3):215-221. doi:10.1016/0014-2999(95)00078-Y.

45. Clarke RW, Harris J. RX 821002 as a tool for physiological investigation of alpha(2)-adrenoceptors. *CNS Drug Rev*. 2002;8(2):177-192.