Dr. H.B. Singh  
Scientist F & Head  
Raw Materials Herbarium & Museum  
Phone: 011-25841143  
E-mail: hbs@nic-sair.res.in; hbsbhati@yahoo.com

Dear Mr. Nagar,

Kindly refer to your letter No. nil dated 29.10.2009 regarding identification of four crude drug samples. The samples have been identified as given below:

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Sample Received as</th>
<th>Part</th>
<th>Sample identified as</th>
<th>Remarks</th>
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<tr>
<td>01</td>
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<td>Achillea millefolium Linn.</td>
<td>O.K.</td>
</tr>
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<td>Roots</td>
<td>Rubia cordifolia Linn.</td>
<td>O.K.</td>
</tr>
<tr>
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<td>Saussurea lappa</td>
<td>Roots</td>
<td>Saussurea lappa C. B. Clarke</td>
<td>O.K.</td>
</tr>
</tbody>
</table>

With regards,

Yours sincerely,

(\[Signature\])

Mr. Ashish Nagar  
Ph. D. Scholar  
Delhi Institute of Pharmaceutical Sciences & Research (DIPSAR), Pusp Vihar, sector-III  
M.B. Road, Opp. Sainik Farms  
New Delhi-110017
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<tr>
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<th>Query / Remark: click on the Q link to go to the location in the proof</th>
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<td>Q2</td>
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<td>Q4</td>
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<td>Q5</td>
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<td>Please provide a conflict of interest statement. If there is no conflict of interest, please state that.</td>
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<td>Q7</td>
<td>Please provide volume for reference [24].</td>
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Thank you for your assistance.
Spasmolytic effect of novel antagonists of the adenosine A<sub>2</sub> receptor in isolated guinea pig tracheal smooth muscle

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Abstract

We have investigated the relaxation effect of nine novel adenosine A<sub>2B</sub> antagonists on precontracted guinea pig tracheas by carbachol (10<sup>−6</sup> M). Isometric contractions of isolated guinea pig tracheae were recorded at 2 g resting tension, and carbachol dose-response curves were performed. EC<sub>50</sub> and IC<sub>50</sub> values were identified by plotting cumulative concentration-response curve and pD<sub>2</sub> values were calculated. Theophylline was able to relax carbachol precontracted tracheas on 10<sup>−5</sup> M conc. The relaxant effect of 1 × 10<sup>−4</sup> M compound 5 (ARR-1) was found highly significant as compared to theophylline (P<0.001), whereas compound 6 (ARR-2) showed equipotent effect in a concentration dependent manner. However, other compounds were not able to attenuate contractions induced by carbachol significantly as compared to theophylline. Compound 5 (ARR-1) may be an important new compound in testing the current hypotheses proposing a therapeutic role for adenosine antagonist in asthma. This effect might be attributed to inhibition of cAMP through adenyl cyclase enzyme. The results exclude an important contribution of adenosine A<sub>2B</sub> receptors.

Keywords: Asthma, Adenosine, PD<sub>2</sub>, Tracheal smooth muscle.
potassium dihydrogen phosphate, and glucose were purchased from Merck, India; DMSO were purchased from CDH, Delhi and test compounds: ARR/BC-1; ARR/BC-2; ARR/BC-3; ARR/RK-2; ARR-1; ARR-2; H₂ATP; M₂ATP; P₂ATP (compounds 1–9, respectively) were procured from Dr. A. Raghuram Rao, Department of Pharmaceutical Chemistry, Punjab University, India.

2.1.3. Isolation of trachea

Animals were euthanized by overdose of diethyl ether followed by incision on ventral skin region of neck without damaging the tracheal smooth muscles. The exposed portion of the trachea was isolated by giving a transverse cut just below the thyroid cartilage and above the carinal portion of the trachea. The piece of trachea obtained was quickly transferred to a petridish containing freshly prepared modified Krebs Henseleit solution (M-KHS) (composition per litre: sodium chloride, 6.9 g; potassium chloride, 0.35 g; calcium chloride, 0.28 g; magnesium sulphate, 0.28 g; potassium dihydrogen phosphate, 0.16 g; sodium bicarbonate, 0.21 g; glucose, 1.5 g), which was aerated with 95% O₂ in CO₂ (5%) at pH 7.4 ± 0.05 and temperature of 37 °C.

2.1.3.1. Preparation of tissue. This was performed according to the method described by Costantin et al. [16–18]. The tracheal piece was cleaned of extraneous tissue, cut spirally into two to three strips of tissues depending on the length of trachea, one end to other in spiral fashion such that two or three segments of cartilage separate each turn. The single channel physiography with PolyVIEW software (version 16) (RADNOTI, Monrovia, CA, USA) was calibrated as per the standard operating procedures [19]. Each strip was mounted in a tissue bath containing 60 mL of pre-warmed M-KHS at 37 °C aerated with 5% CO₂ balanced in oxygen with the help of thread between tissue hook and grooved shaft of research grade isometric force transducer of instrument, connected to an analogical/digital converter to record the signals.

2.1.4. Measurement of tension due to standard drugs and compounds

A pretension of 2 g was applied to the tracheal strips in all the experiments conducted followed by a stabilisation period of 30 to 40 minutes to obtain a constant baseline [20]. Tissues were replenished with the fresh M-KHS two to three times during stabilisation period. The relaxation due to theophylline and test compounds (1–9) was measured on the tracheal muscles, precontracted with carbachol (10 μM). The dose of 10 μM of carbachol was optimised following a cumulative concentration-response curve (CCRC) between the ranges (10⁻⁶ M to 3 × 10⁻³ M). Guinea pigs were allocated randomly to the groups: group 1: M₂Mol dose of carbachol served as negative control; group 2: CCRC of theophylline, served as standard; group 3–12: CCRC of test compounds; group 13–20: CCRC of theophylline served as positive controls. The cumulative increase in muscle relaxation was recorded over a concentration range. Theophylline exposed rings served as positive controls. The cumulative increase in muscle relaxation was recorded over a concentration range of 10⁻⁹ to 10⁻³ M, whereas for the test compounds CCRCs were obtained in the concentrations ranging from 10⁻⁶ to 3 × 10⁻³ M except for compound ARR-1 (1 × 10⁻⁹ to 3 × 10⁻³ M). Adenosine at the dose of 10⁻⁶ M was used in the presence of carbachol (10⁻³ M) to elucidate the mechanism of ARR-1. Percentage contraction and percentage relaxation were obtained from the plots obtained by CCRC of respective drugs. EC₅₀ values were obtained graphically followed by calculation of pD² values as per Patel et al. [22].

2.2. Statistical analysis

Analysis of each data set was performed by one way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test (sigma plot 11, USA). The data was considered to be statistically significant at P < 0.05.

3. Results

CCRC of contraction and relaxation were expressed as a percentage of the maximal response for each substance. Theophylline and tested compounds produce relaxation in a dose dependent manner in carbachol (10 μM) induced precontracted strips [Figs 1 and 2]. Carbachol produced 100% contraction at the dose of 30 μM with an EC₅₀ 75.974 μM and pD² 4.128. A dose of 10 μM carbachol was selected to attain submaximal response and all the relaxant drugs including the standard theophylline were tested for the relaxation produced, their EC₅₀ value and pD² values are tabulated in Table 1. Our results revealed that 10⁻³ M theophylline was able to completely restore the spasm induced by submaximal concentration (10 μM) of carbachol via non-selective inhibition of phosphodiesterases [23]. Among the all test compounds, the most potent spasmolytic compound was ARR-1. The compound 6 was equipotent to theophylline. Compounds 3, 4 and 7 (ARR/BC-3, ARR/RK-2 and H₂ATP) were not significant when compared to theophylline. Out of these, compound 4 and compound 7 equipotent and less potent when compared to compound 3. The compounds 1, 2, 8 and 9 (ARR/BC-1; ARR/BC-2; M₂ATP; P₂ATP) did not show any marked relaxation in carbachol precontracted tracheal strips.

4. Discussion

Adenosine modulates neuronal excitability via interaction with different cell surface receptor subtypes, heterogeneously distributed in the mammalian central nervous system as well as in many peripheral tissues. Four different adenosine receptor subtypes have been cloned and characterized (A₁, A₂A, A₂B, and A₃ receptors) [24]. It is generally accepted that A₂A and A₂B receptors are coupled to Gs proteins. They are known to activate adenylyl cyclase in virtually every cell in which they are expressed. Although activation of adenylyl cyclase is arguably an important signaling mechanism

<table>
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<tr>
<td><strong>EC₅₀ and pD² values of theophylline and test compounds [1–9].</strong></td>
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<tr>
<td><strong>Compound</strong></td>
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</tr>
<tr>
<td>Theophylline</td>
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<tr>
<td>Compound 1 (ARR/BC-1)</td>
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<td>Compound 2 (ARR/BC-2)</td>
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<td>Compound 3 (ARR/BC-3)</td>
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<td>Compound 4 (ARR/RK-2)</td>
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<td>Compound 5 (ARR-1)</td>
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<td>Compound 6 (ARR-2)</td>
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<tr>
<td>Compound 7 (H₂ATP)</td>
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<tr>
<td>Compound 8 (M₂ATP)</td>
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<td>Compound 9 (P₂ATP)</td>
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Values are expressed as mean ± SEM; n: number of tissues; pD²: negative log of EC₅₀; EC₅₀: concentration of antagonist inducing 50% of the maximum effect achievable. *** P < 0.001 as compared with carbachol.

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Fig. 1. Cumulative concentration-response curves fitted by non-linear iterative regression analysis of standard drugs and test compounds (1–9). The preparations were precontracted by carbachol. Responses are expressed as % relaxation of the tension produced by the spasmoden. Vertical bars show S.E. Values, n = 6 for all C/R-curves.

Fig. 2. Cumulative relaxant dose-response curve of ARR-1 on carbachol precontracted guinea pig tracheal strips.

for A2A receptors, this is not necessarily the case for A2B receptors, as other intracellular signaling pathways have been found to be functionally coupled to A2B receptors in addition to adenyl cyclase [25]. Many extracellular signal proteins increase cAMP content via stimulating adenyl cyclase rather than decreasing the activity of phosphodiesterase [26].

Increase in cAMP concentration through activation of adenyl cyclase via stimulation of A2 receptors leads to increased intracellular concentration of Ca²⁺ ions, which eventually leads to breakdown of Ca²⁺-calmodulin (CAM) complex. Ca²⁺-CAM complex is a key mediator for the activation of MLCK (myosin light chain kinase), an enzyme responsible for phosphorylation of myosin light chain thereby causing smooth muscle contraction [27]. Furthermore, adenosine binds to A2B and stimulates phospholipase C and thereby increases the production of IP₃ and DAG. IP₃ binds to receptors on sarcoplasmic reticulum and increases the release of calcium from smooth endoplasmic reticulum thereby causing smooth muscle contraction. Therefore, an A2B receptor antagonism is solicited for bronchial smooth muscle relaxation to relieve bronchospasm [28].

In the present study, we have evaluated the potential of nine newly synthesized novel antagonists on tracheal chain preparations. Compounds were procured from Punjab University, Chandigarh – (courtesy A. Raghu Ram Rao), these compounds were found to have A2B receptor antagonist property on the basis of molecular modelling (unpublished results). All the molecules (compounds 1–9; ARR/BC-1, ARR/BC-2, ARR/BC-3, ARR/RK-2, ARR-1, ARR-2, H₂ATP, M₂ATP, P₂ATP, respectively) were synthesized for the first time and were evaluated for their potential against spasmyotic activity on tracheal smooth muscle of guinea pigs. Tracheal strips were contracted with carbachol (Fig. 3) and a dose-response curve was obtained to standardise the submaximal concentration to evaluate relaxant effect of standard and test compounds. Among the nine test compounds, relaxation was achieved with ARR/BC-3, ARR/RK-2, ARR-1, ARR-2 and H₂ATP (Table 1, Fig. 1), wherein ARR-2 was found to be equipotent to standard theophylline and ARR-1 was found to be more potent as compared to theophylline. Further studies were therefore carried out only on ARR-1 to elucidate the mechanism of the compound.

The relaxation achieved by ARR-1 in carbachol precontracted tracheal strips was markedly reduced in the presence of adenosine, indicative of the adenosine antagonistic nature of the test compound. However, a minor relaxation obtained by ARR-1 in presence of adenosine at higher concentrations is indicative of involvement of some other mechanism apart from adenosine antagonistic property. The ARR-1 was further subjected to radio-ligand-binding assay (results unpublished) and it was found to have selective A₂
binding properties. However, the compound was synthesised with aim to achieve selective A2B binding. Our results revealed a non-selective binding of the compound at A2B receptors. Furthermore, ARR-1 exhibits a higher potency on tracheal strips compared to theophylline, thereby a possible drug lead for the treatment of bronchial asthma. Since ARR-1 fails to relax carbachol induced contractions completely (Fig. 4) in presence of adenosine therefore involvement of other possible mechanisms i.e. Ca2+ channels and cAMP related pathways could play a significant role. Although there are several other pathways which are known to be involved in the relaxation of the tracheal strips including H2 antagonism [29], β2 agonism [30], NO induced relaxation [31] and prostanoids induced relaxation [32] which were ruled out with our earlier studies (results unpublished). The involvement of A2B gives an insight into tracheal smooth relaxation and could be the novel target for the development of new chemical entities in bronchospasm therapy.

5. Conclusion

Our results showed that compound 5 (ARR-1) was more potent and compound 6 (ARR-2) was equipotent than standard drug (theophylline) but our studies does not reveal specific mechanism of action for their muscle relaxing activity. Therefore, we suggest further ligand-binding assays to establish the ability of these compounds to bind A2A and A2B receptors specifically or equipotent for both the receptors or higher specificity for any particular receptor.

Disclosure of interest

The authors have not supplied their declaration of conflict of interest.

Acknowledgments

We are thankful to Department of Training and Technical Education, Government of NCT Delhi for the funding assistance.

References


