Protective Effect of $A_{2B}$ Receptor Antagonist (TRP 1) on Acetic Acid Induced Ulcerative Colitis in Rats: in vitro, in vivo and in silico Methods

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ABSTRACT

Aim: Present study was elucidate the protective effect of pyridinone derivatives such as 7-amino-5-oxo-2-phenyl-5H, 8H-dihydro-[1, 2, 4] triazolo [1, 5-α] pyridine - 6-carbonitril (TRP 1) by in vitro, in vivo and in silico. Methods: Radioligand binding assay was performed on human adenosine receptors ($A_{2B}$) and assess $A_{2B}$ antagonist effect by adenylyl cyclase activity. In vitro study was carried out to determine the neutralize capacity against DPPH*, NO*, SO*, LPO* free radicals. TRP 1 at the doses 1 mg/kg bd.wt. and 10 mg/kg bd.wt p.o, was administered consecutively for 14 days in albino rats. Ulcerative colitis was induced with single dose of 2 ml of 3% acetic acid intrarectal on 14th day in treated rats. At the end of treatment, colonic tissue was collected and subjected for estimation of macroscopic score, glutathione, catalase, MPO and inflammatory parameters such as IL 1β, TNF α and IL 6. In silico study was carried out to evaluate the binding energy and IC$_{50}$ toward IL 1β, TNF α and IL 6. Results: TRP 1 was antagonized the $A_{2B}$ receptors at the concentration of 30000 nM. In vitro study was revealed that TRP1 (1 mg/ml) was significantly neutralizes the free radicals of DPPH*, SO*, NO* and LPO*. In vivo studies, intrarectal administration of acetic acid caused significantly ($**P<0.001$) increased macroscopic score, colon weight, colonic MPO, IL 6, IL 1β and TNF α. In silico study was carried out to evaluate the binding energy and IC$_{50}$ toward IL 1β, TNF α and IL 6. In vivo study was revealed that TRP1 (1 mg/ml) was significantly neutralizes the free radicals of DPPH*, SO*, NO* and LPO*. In vivo studies, intrarectal administration of acetic acid caused significantly ($**P<0.001$) increased macroscopic score, colon weight, colonic MPO, IL 6, IL 1β and TNF α. In vitro study was carried out to determine the neutralize capacity against DPPH*, NO*, SO*, LPO* free radicals. TRP 1 at the doses 1 mg/kg bd.wt. and 10 mg/kg bd.wt p.o, was administered consecutively for 14 days in albino rats. Ulcerative colitis was induced with single dose of 2 ml of 3% acetic acid intrarectal on 14th day in treated rats. At the end of treatment, colonic tissue was collected and subjected for estimation of macroscopic score, glutathione, catalase, MPO and inflammatory parameters such as IL 1β, TNF α and IL 6. Results: TRP 1 was antagonized the $A_{2B}$ receptors at the concentration of 30000 nM. In vitro study was revealed that TRP1 (1 mg/ml) was significantly neutralizes the free radicals of DPPH*, SO*, NO* and LPO*. In vivo studies, intrarectal administration of acetic acid caused significantly ($**P<0.001$) increased macroscopic score, colon weight, colonic MPO, IL 6, IL 1β and TNF α. In silico study was reported that the IC$_{50}$ of TPR 1 against IL 1β, IL 6 and TNF-α was 7.5 mM, 28.65 mM and 45.87 mM respectively. Conclusion: Our data demonstrated that the TRP 1 treatment improved clinical score in acetic acid induced colitis in rats. It also inhibited the proinflammatory cytokine IL-6, IL 1β and TNF α and improvements of antioxidant in colitis rats through $A_{2B}$ receptor antagonist property.

Key words: 7-amino-5-oxo-2-phenyl)-5H, 8H-dihydro-[1, 2, 4] triazolo [1, 5-α] pyridine - 6-carbonitril (TRP 1), Ulcerative colitis, Acetic acid, Myeloperoxidase (MPO), Glutathione (GSH), Catalase, TNF α, IL 1β and IL 6.

INTRODUCTION

IBD, including Crohn’s disease (CD) and ulcerative colitis (UC), is a lifelong disabling gastrointestinal disease. Although etiology of inflammatory bowel disease (IBD) is unknown it appears that an abnormal response of the mucosal innate immune system to luminal bacteria may trigger inflammation which is perpetual by disregulation of cellular immunity and imbalances between proinflammatory cytokines, such as...
TNF-α, IFN-γ, IL-1β, IL-6, and IL-12, and anti-inflammatory cytokines like IL-4, IL-10, IL-11. Therapeutic agents for IBD which include anti-inflammatory agents such as 5-aminosalicylates (5-ASA) and corticosteroids along with some immunomodulators like azathioprine, 6-mercaptopurine were used. However, treatments are associated with severe adverse events including diarrhea, cramps, abdominal pain accompanied by fever and high blood pressure. Thus, there is a need to develop new therapeutic options with low toxicity and minimal side effects. In the search for novel therapeutic options, increasing attention is being paid to the adenosine system and its involvement in the pathophysiology of IBDs. Extracellular adenosine binds to adenosine receptors (AR) 1, 2A, 2B and 3, all of which are expressed on the surface of immune cells. Low level expression of A2b R is demonstrated in small intestine. A2b R are highly expressed in the cecum and colon, esophagus, stomach, and jejunum but appears to be absent in the ileum. Inflammatory mediators like TNFα, IL6 are increased in the intestinal mucosa, serum and stools of patients with IBD through up regulation and over expression of A2b receptors. Past scientific studies are supported that fused pyridinone ring derivatives have been found to versatile pharmacophore with wide range of useful biological activities due to antagonize adenosine receptors and ameliorated the inflammation. Hence A2b Rare great deal of interest, its primary molecular target and its mechanism of action remain to be clarified. In the present study was evaluated the protective effect of pyridinone derivatives like 7-amino-5-oxo-2-phenyl-5H,8H-dihydro-[1,2,4] triazolo[1,5-α] pyridine - 6- carbonitril on colitis rats. Acetic acid induced colitis in rats is one of the common models in IBD research and resembles human ulcerative colitis in histology. To test our hypothesis, the present study was undertaken to determine the possible mechanism of action of TRP 1 on the acetic acid induced ulcerative colitis in wistar rats.

MATERIALS AND METHODS

Materials

Adult male Wistar rats (200 - 220 g) were purchased from Mahaveer enterprises, Hyderabad. The animal room was maintained at 22°C –24°C and a lighting regimen of 12 hr light/12 hr dark. Rats were fed with standard house chow and water ad libitum. All animal experiments were performed after getting prior approval from the Institutional Animal Ethics Committee. TRP 1 procured from Chemistry department (Shri Vishnu college of Pharmacy), acetic acid (Loba Chemie), NBT- (Loba Chemie), reduced glutathione (Otto Chemie), trichloro acetic acid (Loba Chemie). ethylenediamino tetra acetic acid (Loba Chemie). O-Dianisidine (Loba Chemie). 2,2 Diphenyl picryl hydrazyl (Siaco Research laboratory Pvt.Ltd), 5, 5 DithioBis 2 Nitro benzoic acid -Siaco Research laboratory Pvt.Ltd, TNF α, IL-1β and IL-6 (Ray Biotech inc.). [3H]CCPA ([3H]2-chloro-N6-cyclopentyladenosine) was obtained from NEN Life Sciences (48.6 Ci/mmoll), [3H]MSX-2([3H]3-(3-hydroxypropyl)-7-methyl-8-((m-methoxystyryl)-1-propargyloxanthine) from Amersham (85 Ci/mmoll), [3H]PSB-603 (8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propargyloxanthine) from GE Healthcare (73 Ci/mmoll), and [3H]PSB-11 ([3H]-8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2,1-i]-purin-5-one) from Quotient Biosciences (53 Ci/mmoll). All other chemicals used were of analytical grade.

Radioligand binding studies

The binding studies were conducted at Human A2b following the reported procedure. In brief, membranes for radioligand binding were prepared from CHO cells stably transfected with human Adenosine receptors subtypes in a two-step procedure. In a first low-speed step (1000 g), cell fragments and were removed. The crude membrane fraction was sedimented from the supernatant at 100000 g. The membrane pellet was resuspended in the buffer used for the respective binding experiments, frozen in liquid nitrogen and stored at -80°C. For the measurement of adenylyl cyclase activity, only one high speed centrifugation of the homogenate was used. The resulting crude membrane pellet was resuspended in 50 mM Tris/ HCl, pH 7.4 and immediately used for the cyclase assay.

Adenylyl Cyclase Activity

The potency of antagonists at the A2b R was determined in adenylyl cyclase experiments. The procedure was carried out as described previously with minor modifications. Membranes were incubated with about 150000 cpm of ATP for 20 min in the incubation mixture as described without EGTA and NaCl. For agonists, the EC50 values for the stimulation of adenylyl cyclase were calculated with the Hill equation. Hill coefficients in all experiments were near unity. IC50 value for concentration-dependent inhibition of NECA-stimulated adenylyl cyclase caused by antagonists was calculated accordingly. Dissociation constants (ki) for antagonists were calculated with the Cheng and Prusoff equation.

Antioxidant activity by in vitro
Antioxidant activity was tested by scavenging of DPPH* assay,\textsuperscript{25} NO* assay,\textsuperscript{26} SO* assay,\textsuperscript{27} \( \text{Fe}^{2+} \) ascorbate induced lipid peroxidation assay.\textsuperscript{28}

**Anti-inflammatory activity by human RBC (HRBC) method\textsuperscript{29}**

Blood was collected from the healthy volunteers and mixed with equal volume of sterilized Alsevers solution (composition Glucose 20.5 g, Sodium chloride 4.2, Tri-sodium citrate 8.0 g, and citric acid 0.55 g, distilled water 1000 mL). Blood solution was centrifuged at 3000 rpm and the packed cells were separated, then washed with isosalone (0.85%; pH 7.2) solution and a 10% v/v suspension was made with isosaline. This HRBC suspension was used for the estimation of anti-inflammatory property.

Different concentrations of TRP1 (100 ng/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml and 1000 µg/ml), standard Sulfasalazine and control were separately mixed with 1ml of phosphate buffer (0.15 M, pH 7.4), 2mL of hyposaline (0.36%) and 0.5mL of HRBC suspension. All the assay mixtures were incubated at 37°C for 30 min and centrifuged at 3000 rpm. The supernatant liquid was decanted and the hemoglobin content was estimated by a spectrophotometer at 560 nm. The percentage hemoysis was estimated by assuring the hemoysis produced in the control as 100%. Instead of hyposaline 2 mL of distilled water was employed as control. The anti-inflammatory potency was estimated by measuring % of inhibition of hemoysis

Percentage inhibition of Hemolysis = \( \left( 1 - \frac{\text{ABS}_{\text{sample}}}{\text{ABS}_{\text{control}}} \right) \times 100 \).

**Acute toxicity**

The acute oral toxicity was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD), revised draft guidelines 423, received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

**Experimental procedure for the study the protective effect of TRP 1 on acetic acid induced ulcerative colitis**

Animals were divided into five groups (n=6). In this study Sulfasalazine used as standard compound due to their potential anti-inflammatory activity against ulcerative colitis in rats and also used clinically.\textsuperscript{30,31}

**Group I:** Serve as sham control

**Group II:** Animals were pretreated with 80% DMSO for 14\textsuperscript{th} days and 2 ml of 3.0% acetic acid administered intra rectally on 14\textsuperscript{th} day

**Group III:** Animals were pretreated with standard Sulfasalazine (360 mg/kg bd.wt) for 14 days + 2 ml of 3.0% acetic acid administered intra rectally on 14\textsuperscript{th} day.

**Groups IV:** Animals were pretreated with TRP 1 (1mg/kg bd.wt dissolved in 80% DMSO) for 14 days + 2 ml of 3.0% acetic acid administered intrarectally on 14\textsuperscript{th} day.

**Groups V:** Animals were pretreated with TRP 1 (10 mg/kg bd.wt in 80% DMSO) for 14 day + 2 ml of 3.0% acetic acid administered intra rectally on 14\textsuperscript{th} day.

**Assessments of colitis**

Animals were scarified at the end of treatment, the distal 10 cm portions of the colon were removed and cut longitudinally, cleaned with physiological saline to remove fecal residues.

Macroscopic inflammation scores are assigned based on the clinical features of the colon using an arbitrary scale ranging from 0 to 10 as follows:

0 = No damage,
1 = Focal hyperemia (water oozes out),
2 = Ulcerization without hyperemia or bowel wall thickness,
3 = Ulcerization with inflammation at one site,
4 = Ulcerization with inflammation at two sites,
5 = Major sites of inflammation >1 cm along the organ with redness,
6 = Major sites of inflammation >2 cm along the organ with redness,
7 = Major sites of inflammation >3 cm along the organ with redness,
8 = Major sites of inflammation > 4 cm along the organ with redness,
9 = Major sites of inflammation >5 cm along the organ with redness and bleeding, and
10 = Major sites of inflammation >6 cm along the organ with redness, swelling, and bleeding.\textsuperscript{32}

**Biochemical assays**

The colorectal tissue was collected, homogenized in 10 mM Tris-HCl buffer (pH 7.1). The homogenate was used for the measurement of antioxidant enzyme levels such as catalase,\textsuperscript{33} glutathione,\textsuperscript{34} colonic MPO activity,\textsuperscript{35} inflammatory cytokines such as TNF-\( \alpha \), IL-1\( \beta \) and IL-6 (Ray Biotech Inc., US) using standard sandwich enzyme-linked immune sorbent assay (ELISA) kit specific for rat cytokines according to the manufacturer’s instruction.

**Histolopathological study**
A portion (2 cm) of the colonic specimen from each rat (n= 6) was fixed in 10% formalin, cut into 5 μm thickness, stained using heamatoxylin–eosin and histopathological observations were made. The stained sections of colon were examined for any inflammatory changes like infiltration of the cells, necrotic foci and damage to tissue structures like payers patches, damage to nucleus.

**In silico method**

To evaluate the compound TRP 1 binding capacity by using AUTODOCK 4.2 version and the images are rendered using Accelry’s Discovery studio visualizer v4.0 interface.

**Statistical analysis**

All data values are expressed as mean ± SD. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by Dunnett’s test, using (Graph pad version 5.0) *P<0.05, **P<0.01, ***P<0.001 were considered as statistically significant.

**RESULTS**

**Effect on human adenosine receptors**

Compound TRP 1 exhibited inhibitory concentration toward A<sub>2b</sub> receptors is 30,000 nM (Table 1).

**Effect on hemolysis**

At the dose of 1000 μg/ml of TRP 1 exhibited 60.09±3.2% protection of HRBC in hypotonic solution (Table 2, Figure 1).

**Effect on free radical scavenging activity**

At the dose of 1 mg/ml of TRP 1 neutralize free radicals of DPPH (28.07±0.1%), SO (35.3±0.03%), NO (44.03±0.05%) and LPO (58±0.8%) all the results compared with Sulfasalazine (Table 3, Figure 2).

**Acute toxicity**

TRP 1 treated rats were safe upto the dose level 2000 mg/kg bd.wt. As per OECD guidance 423. At the dose level of 300 mg/kg bd.wt. And 2000 mg/kg bd.wt. Treated rats were exhibited drowsy.
Effect on colon parameters in acetic acid induced colitis rats

At the end of the treatment, acetic acid administered rats exhibited severe macroscopic edematous inflammation in the colon. The inflammation score and weight of colon was significantly (**P<0.001) increased in colitis rats 8.3±0.7, 2.9±0.3 respectively, also increase in content of MPO (43±1.28, ***P<0.001), decrease colonic catalase (19±0.31, ***P<0.001) and glutathione (2.9 ± 0.16) when compared to normal rats. The pretreatment of TRP 1 (10 mg/kg bd.wt.) significantly reduces inflammation score (4.83 ±0.3; **P<0.01), colon weight (1.25± 0.03; **P<0.01), colonic MPO activity (19.6±1.5; *P<0.05) and increases catalase activity (16.8 ± 0.38; *P<0.05), colonic GSH (6.9 ± 0.20; **P<0.01), the alteration in these biochemicals parameters when compared to colitis rats (Figure 3 and 4; Table 5).

Effect on cytokines TNF-α, IL-1β and IL-6 levels

Pro inflammatory cytokines are in acetic acid induced colitis rats showed significantly increased TNF-α (3800 ± 54.9; **P<0.01), IL 1β (5200 ± 73.1; **P<0.01) and IL-6 (700 ± 90.4; **P<0.01) compared with those in the sham control group. TRP1 at the doses 1 mg/kg bd.wt. and 10 mg/kg bd. Wt., decreases the level of TNF-α from 3800 ± 54.9 pg/g tissue to 3200±22.8 pg/g, 3800 ± 54.9 pg/g tissue to 2900±22.6 pg/g (**P<0.05), respectively; correspondingly decrease of IL-1β from 5200±73.1 pg/g tissue to 4300±28.8 pg/g, 5200±73.1 pg/g tissue pg/mg to 3900±33.9 pg/mg tissue (**P<0.01), respectively. Correspondingly decrease of IL-6 from 700±90.4 pg/g tissue to 650±13.1 pg/g, 700±90.4 pg/g tissue pg/mg to 400±18.7 pg/mg tissue (**P<0.01), respectively (Table 5, Figure 5).

Binding energy and inhibition of IL1-beta, IL6 and TNF-α by in silico

Docking is widely used in modern drug discovery process and effective tool for quickly and accurately predicting biomolecular conformation with binding energy of protein ligand complex. Compound TRP 1 exhibited potent inhibition on IL-1β (-6.7 Kcal/mol, 7.5 mM), TNF α (-5.92 Kcal/mol, 45.87 mM) and IL 6 (-6.2 Kcal/mol, 28.65 mM) (Table 4).

<table>
<thead>
<tr>
<th>S. No</th>
<th>Drug targeting Protein</th>
<th>Binding Energy in Kcal/mol</th>
<th>IC₅₀ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IL 1β</td>
<td>-6.7</td>
<td>7.5</td>
</tr>
<tr>
<td>2</td>
<td>IL 6</td>
<td>-6.2</td>
<td>28.65</td>
</tr>
<tr>
<td>3</td>
<td>TNF α</td>
<td>-5.92</td>
<td>45.87</td>
</tr>
</tbody>
</table>

Table 4: Binding energy and inhibition of IL1-beta, IL6 and TNF-α by in silico
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**Table 5: Effect on colon parameters in acetic acid induced colitis rats**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Treatment group</th>
<th>Inflammation score</th>
<th>Weight of colon (gm)</th>
<th>Colonic MPO levels (U/ml)</th>
<th>Colonic Catalase levels (U/ml)</th>
<th>Colonic GSH levels (µg/ml)</th>
<th>TNF-α (pg/g tissue)</th>
<th>IL-1β (pg/g tissue)</th>
<th>IL-6 (pg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sham control</td>
<td>0±0</td>
<td>1.6±0.11</td>
<td>2.9±0.13</td>
<td>9±1.25</td>
<td>29±1.40</td>
<td>2900 ± 54.9</td>
<td>2300 ± 43.3</td>
<td>190 ± 11.9</td>
</tr>
<tr>
<td>2</td>
<td>Diseased control</td>
<td>8.3±0.70***a</td>
<td>2.9±0.13***a</td>
<td>43±1.25***a</td>
<td>22±0.88***a</td>
<td>16±0.30</td>
<td>3800 ± 54.9</td>
<td>5200 ± 73.1***a</td>
<td>700 ± 80.4</td>
</tr>
<tr>
<td>3</td>
<td>Sulfasalazine (360 mg/kg bd. wt.)</td>
<td>3.9±0.4***b</td>
<td>1.4±0.05***b</td>
<td>22±0.88***b</td>
<td>24±0.53***b</td>
<td>16±0.30</td>
<td>2900 ± 22.7</td>
<td>3200 ± 22.7</td>
<td>3900 ± 33.9**b</td>
</tr>
<tr>
<td>4</td>
<td>TRP 1 (1 mg/kg)</td>
<td>7.3±0.33</td>
<td>1.4±0.05***b</td>
<td>22±0.88***b</td>
<td>24±0.53***b</td>
<td>16±0.30</td>
<td>2900 ± 22.7</td>
<td>3200 ± 22.7</td>
<td>3900 ± 33.9**b</td>
</tr>
<tr>
<td>5</td>
<td>TRP 1 (10 mg/kg)</td>
<td>4.8±0.30*</td>
<td>1.2±0.03*</td>
<td>16±0.80*</td>
<td>19±1.25*</td>
<td>16±0.80*</td>
<td>2900 ± 22.7</td>
<td>3200 ± 22.7</td>
<td>3900 ± 33.9**b</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SD, from six groups of rats and analyzed by one way ANOVA followed by Dennett’s test.

* P<0.05, ** P<0.01; compared with sham control, b compared with disease control.

**Histopathological changes in colon of experimental rats**

Acetic acid induced colitis showed massive necrotic destruction of epithelium, submucosal edema, areas of haemorrhages and inflammatory cellular infiltration. TRP 1 at low dose level showed minimal damage of the mucosa with slight submucosal edema and mild inflammatory cell infiltration. 10 mg/kg bd.wt. TRP 1 showed remarkable recovery of colonic mucosa from acetic acid induced colitis damage (Figure 6).

**DISCUSSION**

Compound TRP 1 has been demonstrated to have protective effect against acetic acid induced ulcerative colitis. Adenosine plays prominent roles in maintaining tissue integrity by modulation of immune functions, down-
The authors declare no conflict of interest.

CONCLUSION

TRP 1 exhibited antagonist on A_{2B} receptors, possess anti-inflammatory activity and reduces inflammatory mediator’s levels, enhanced antioxidant activity in acetic acid induced colitis in rats. All the above scientific evidence suggested that the selected compounds improve clinical score in acetic acid induced colitis rats.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATION USED

TRP: 1-(7-aminomethyl-5-oxo-2-phenyl)-5H,8H-dihydro-[1,2,4] triazolo[1,5-α] pyridine - 6- carbonitril), A 2B; Adenosine 2B; DPPH: 2,2-diphenyl-1-picrylhydrazyl; IBD: Inflammatory bowel disease; TNF: Tumor necrosis factor alpha; IL: Interleukins.
REFERENCES


Ulcerative colitis is an inflammatory bowel disease that causes long lasting inflammation and sores (ulcers) in the innermost lining of large intestine (colon) and rectum. Activation of $A_{2B}$ receptors and depletion of antioxidant defensive system are contributing factors in development of ulcerative colitis. TRP 1 antagonize the $A_{2B}$ receptors, proved in radioligand binding assay and also exhibited anti-inflammatory activity, antioxidant activity reported in $in vitro$ models. Pretreatment with TRP 1 significantly minimizes the proinflammatory mediators release as well as enhance antioxidant defensive system in acetic acid induced ulcerative colitis rats. Eventually TRP 1 evoked a significant protective effect against colonic damage induced by intrarectal injection of acetic acid.

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