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Docking study of the Rohitukine for the prevention of peptic ulcer- A New Target

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Abstract

Context: In our previous study we have suggested that Rohitukine attenuates gastric mucosal injury; however its exact mechanism has not yet been established. **Objective:** The aim of present study was to evaluate the gastro protective mechanism of Rohitukine. **Materials and methods:** Sprague dawley rats and guinea pigs weighing 180–200 g were used. Dudenol ulcer was observed through Histamine induced gastric ulcer in guinea pigs. **Result and Discussion:** The present study was considered to evaluate the anti-ulcerogenic properties of an alkaloid chromane, Rohitukine from *Dysoxylum binectariferum*. Moreover, we studied the role of Rohitukine on the cytosolic concentration of cAMP and Histamine level in parietal cell-enriched cell suspension in order to ascertain its mechanism of action. In addition, Rohitukine failed to show protection in histamine induced gastric ulcer. **Conclusion:** Our docking study revealed that Rohitukine moderately bind with CCK2 receptor with binding energy as compare with standard drug benzotript. Furthermore, anti-secretory mechanism of Rohitukine mediated apparently through bind with CCK2 and inhibited the acid secretion, is novel to our finding.

Keywords: Peptic ulcer, CCK2 receptor, Docking study, Rohitukine, *Dysoxylum binectariferrum*.

Introduction

The gastrointestinal system comprises the body organs that perform digestion, absorption and excretion. These organs include the mouth, pharynx, oesophagus, stomach, small intestine, large intestine and anus as well as major role of the some important glands like salivary glands, liver and pancreas. Gastric acid play an important role in the digestion of food. After researcher discovers the gastric acid and pepsin in the stomach, the queries arises regarding "Why does the stomach not digest itself?" and "how does the stomach protect itself against great offensive factors like acid, pepsin and *Helicobacter pylori*. After decades it was found that the offensive factors was neutralized by defensive factors and if any disturbance was generated it causes gastric ulcer. Gastric ulcer is a very common global problem today. Which occur due to an imbalance between offensive (acid, pepsin and *Helicobacter pylori*) and defensive (mucin, prostaglandin, bicarbonate, growth factors and nitric oxide) factors? Consequently reduction of gastric acid production as well as reinforcement of gastric mucosal protection has been the major therapeutic approaches of peptic ulcer disease.[1]

In this study, we want to confirm the anti-ulcer effect of Rohitukine that by which pathway it protects the gastric mucosa. In our literature survey, we found very little information available regarding its pharmacological pathophysiology on the gastrointestinal system. We carried out in vitro studies of the effect of Rohitukine on intracellular cAMP and Histamine level in parietal cell. In order to verify the role of Rohitukine may act as a CCK2 antagonist, we also performed the docking study in order to ascertain its mechanism of action.

Materials and Methods

Plant material

The stem bark of *Dysoxylum binectariferum* was collected from the Andaman Coast of India and identified by the Botany Division of the Central Drug Research Institute. The voucher specimen (No. 8091) has been kept in the herbarium of the Institute.

Extraction/Fractionation Procedure

The extraction and fractionation from 1.0 kg of plant material was carried out adopting the procedure described previously.[2] Briefly, air-dried powdered plant material was extracted with distilled ethanol, concentrated under reduced pressure and fractionated into four fractions (n-hexane, chloroform, soluble n-butanol and insoluble n-butanol fraction). From chloroform fraction, a known alkaloid Rohitukine [5,7-dihydroxy-2-methyl-8-4-(3-hydroxy-1-methyl)-piperidinyl-4H-1-benzopyran-4-one] was isolated by repeated column chromatography over silica gel and further purification by HPLC on LC-20-AD using methanol-water (55:45 v/v, flow rate 1.0ml/min.) as a solvent The characterization of compound was done using IR, NMR, mass, derivatization and comparison with available literatures. The yield of Rohitukine was 1% and its purity was 99.6%.[3] It was also compared with authentic samples as co-tlc.

Experimental Animals

Guinea pigs of either sex, weighing 300–350 g were used for histamine- induced ulcer model, were housed three to four per cage, in a room with temperature regulated at 22 ± 2ºC, with a 12h/12h light/dark cycle (lights on 07:00 h, lights off 19:00 h). Adult Sprague Dawley rats, weighing 130-180 g procured from National Laboratory Animal Centre, CDRI, were used in the study. Experimental protocols were approved by the Institutional Ethical and Usage Committee of Central Drug Research Institute, Lucknow, following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Standard chow pellets and water were given *ad libitum*, except during the period when food deprivation was applied.

Treatment schedule

Rohitukine, standard anti-ulcer drug omeprazole (Omz) (Sigma Chemicals, USA) were prepared in 1% sodium carboxy methyl cellulose (CMC) suspension and administered orally, 45 min prior to exposure to ulcerogens to the animals. All animals were deprived of food for 16 h before ulcerogens exposure and were divided into two groups each group containing six animals (n=6).

Group: 1. Control group of animals were treated with vehicle 1% CMC.

Group: 2. Rohitukine (20 mg/kg, p.o.) were tested against histamine induced gastric ulcer model to identify the mechanism of action.

Group: 3. Standard anti-ulcer drugs such as omeprazole (Omz) (10 mg/kg, p.o.) were tested against histamine induced gastric ulcer model.

Drugs and reagents

Omeprazole and other chemicals were obtained from M/s. Sigma Chemicals, St Louis, MO, USA.

Docking Simulations

Preparation of ligand structures

Ligand files Rohitukine and benzotript (as control drug) were downloaded in mol. format from Chem Spider Chemical Database (http://www.chemspider.com/). These files could not be directly used by Auto Dock 4.0 [4] thus we have to generate 3D pdm format using CORINA online tool. Further the ligands were submitted for minimization in Chimera (Version 1.5.3)[5] using Genetic Algorithm: Steps 2000 and 0.5 grid units Optimized[6].

Preparation of protein structures

3D structure of CCK2 receptor (PDB ID: 1L4T) receptor was obtained from Protein Data Bank

(http://www.rcsb.org/). Published structure was edited to remove HETATM using Discovery Studio Visualizer (Version 2.5.5). Chimera was used for energy minimization, removal of steric collision with the steepest descent steps 1000, steepest descent size 0.02 A, Conjugated gradient steps 1000 and the conjugate gradient step size 0.02 A for the conjugate gradient minimization.[7, 8]

Docking Studies

Docking studies were performed by Auto Dock Tools (Version 4.0) suit.[4, 9] Water molecules were removed from the protein structures before docking and hydrogen atoms were added to all target proteins. Kollman united charges and salvation parameters were added to the proteins. Gasteiger charge was added to the ligands. Grid box was set to cover the maximum part of proteins and ligand. The values were set to 60×60×60 Å in X, Y and Z axis of grid point. The default grid points spacing was 0.375 Å. Lamarckian Genetic Algorithm (LGA) [10] was used for proteins ligands flexible docking calculations. The LGA parameters like population size (ga_pop_size), energy evaluations (ga_num_generation), mutation rate, crossover rate and step size were set to 150, 2500000, 27000, 0.02, 0.8 and 0.2 Å, respectively. The LGA runs were set to 10 runs. Molecular docking methods followed the search of the best conformation of receptor and ligand on the basis of binding energy. All obtained 10 conformations of proteins and ligand complex were analysed for their interactions in terms of binding energy of the docked structure using Discovery Studio Visualizer (version 2.5.5).

Anti-ulcer studies

Histamine induced duodenal ulcer in guinea pigs (HA)

Duodenal ulcers were induced in guinea pigs by intramuscular application of histamine acid phosphate at a dose of 0.25 mg/kg at every 30 mins interval for 4 hours and the animals were sacrificed after 30 mins of the last dose.[11] Animals were treated with Rohitukine (20 mg/kg, p.o.) and Omz 45 mins prior to histamine administration. Promethazine hydrochloride at a dose of 2.5 mg/kg of body weight was injected intraperitoneally to each animal 15 min. prior to administration of histamine, in order to protect the animals from histamine toxicity. Stomach was cut along the lesser curvature down to the duodenum to observe the formation of ulcer on the anterior and posterior wall of duodenum.

Measurement of ulcer index

Ulcers formed in stomach of Histamine induced duodenal ulcer in guinea pigs were scored according to the arbitrary scoring system. In HA model the length of the lesions were measured using Biovis image analyzer software (Expert Vision Lab Private Ltd., Mumbai, India) and summated to give a total lesion score.

Preparation of isolated parietal cells from rat stomach

Gastric cell isolation was performed as described[12] with some modifications. Media of the following compositions were used:

Medium A (mmol./l) NaH2PO4 (0.5), Na2HPO4 (1.0), NaHCO3 (20.0), NaCl (70.0), KCl (5.0), glucose (11.0), EDTA (2.0), HEPES (50.0), and BSA (2%).

Medium B was of same composition as A but was EDTA -free and contained CaCl2 (1.0), MgCl2 (1.5), and BSA (1%).

Medium C differed from B in having 0.1% BSA.

Hanks' balanced salt solution (HBSS) with 10 mM glucose was used for final suspension of the isolated cells.

After anaesthetization, the rats were given in situ perfusion through the heart with normal saline. Stomach was removed and washed with normal saline, mucosa was minced. The minced mucosa was incubated in 5 ml of buffer A and was digested with 1 mg/ml pronase with continuous stirring at 37°C. After 30 min, the supernatant was collected into a fresh tube and the undigested mucosa was digested with fresh enzyme solution (0.5 mg/ml) and incubated for 15 min. It was then centrifuged at 600 g for 5 min and the pellet was re-suspended in 5 ml buffer B along with 0.5 mg/ml collagenase. Another digestion for 30 min with continuous stirring was given and later it was centrifuged at 600 g for 10 min and the supernatant was collected. The pellet was re-suspended in buffer C and was washed twice with buffer C at 600 g for 10 min. The parietal cells were then separated from the final cell suspension by density-gradient centrifugation through linear Opt prep gradient.

Cyclic adenosine monophospahate (cAMP) Enzyme immunoassay

The concentration of cAMP in gastric parietal cell was determined using a cAMP enzyme immunoassay kit. The assay is based on the competition between free cAMP and a acetylcholinesterase (AChE) conjugate for a limited number of cAMP specific rabbit antibody binding sites. The concentration of the conjugate is held constant while the concentration of cAMP varies. Therefore, the amount of conjugate that binds to the rabbit antibody will be inversely proportional to the concentration of cAMP in the well.

Statistical analysis

Results are expressed as the mean ± S.E.M. from six rats per group. Statistical analysis was performed with Prism version 3.0 software using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Probabilities of less than 5% ($P < 0.05$) were considered significant.

Results

Experiment 1

(i) Rohitukine alkaloid expressed the binding energy values of -4.39 kcal/mol and standarad drug Benzotript exhibiting-6.39 kcal/mol with CCK2 receptor respectively.

(ii) For Docking Analysis (Table 1)

Experiment 2

As represented in Figure 1, Rohitukine pre-treatment was not effective in histamine induced ulcer model 12.63% protection in comparison to control which was comparable to protection afforded by omeprazole (70.80%).

Experiment 3

To understand the mechanism of anti-ulcerogenic effect of Rohitukine, we have examined the alterations in the following parameters.

(i) The involvement of Rohitukine on the cAMP level.

We examined cAMP level in isolated parietal cell after forskolin stimulation. As evident forskolin increases the level of cAMP, we also found that it increases the cAMP. Rohitukine has not altered the level of cAMP, after the stimulation of forskolin (Figure 2). In our previus study we have shown the protective effect of Rohitukine on the entire gastric ulcer model.

(ii) Rohitukine also has not changed the histamine level (Figure 3). It was same as control group but in the case of ranitidine (50 mg/kg) it decreases the level of histamine.

Table1: Docking analysis results in terms of binding energy (Kcal/mol), H-bonding residues and residues creating hydrophobic region.

Receptor	Ligands	Residues involved in H- Bond	Distance of Hydrogen bond (Å)	Residues creating hydrophobic region	Binding energy (Kcal/Mol)
1L4T	Rohitukine	ARG15	2.113	ALA2,ASN3,THR4,TRP5,ARG6,ASP9, GLY10,GLY12,ARG15,ALA16,ALA20 ,PRO21,ILE22,ILE25	-4.39
		ARG15	1.751		
		ALA20	2.1		
1L4T	Benzotript	ASP9	2.015	TRP5,ARG6,ASP9,GLY10,PRO11,GL Y12,ARG15,ALA16	-6.31
		GLY10	1.8		
		ARG15	1.901		
		ARG15	1.997		

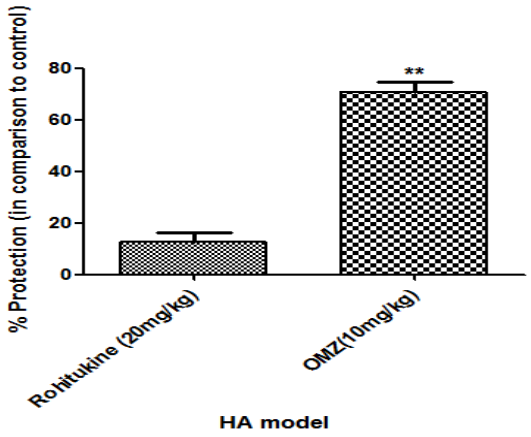


Figure 1: Effect of Rohitukine and standard drugs (Omz) on percentage protection of ulcer against histamine induced dudenol ulcer models in guinea pigs. Data expressed as mean % protection ± S.E.M. Statistical analysis was done by One Way ANOVA followed by Dunnett's Multiple Comparison Test. *Statistically significant at P<0.05 and **P< 0.01, in comparison to control. n = 6 in each group.

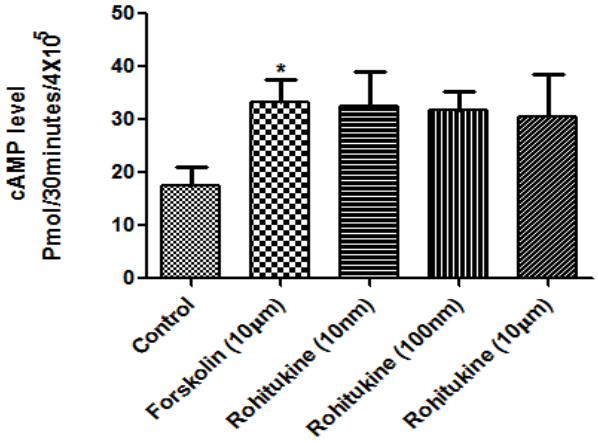


Figure 2: Effect of Rohitukine on cAMP level in isolated parietal cell. Data expressed as mean % protection ± S.E.M. Statistical analysis was done by One Way ANOVA followed by Dunnett's Multiple Comparison Test. *Statistically significant at P<0.05 and **P< 0.01, in comparison to control. n = 6 in each group.

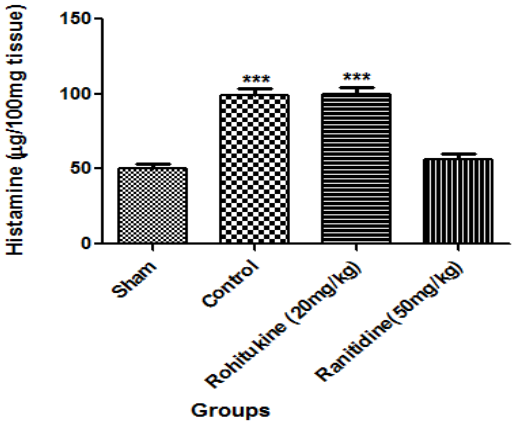
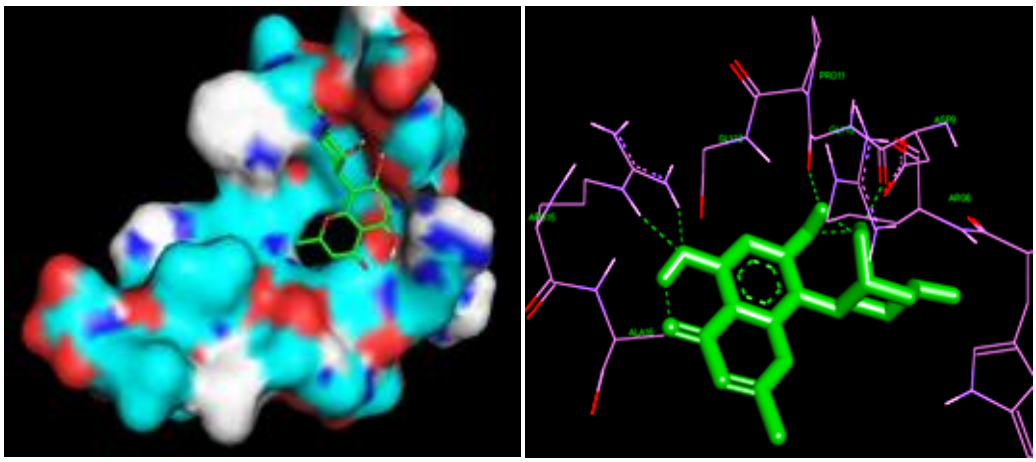
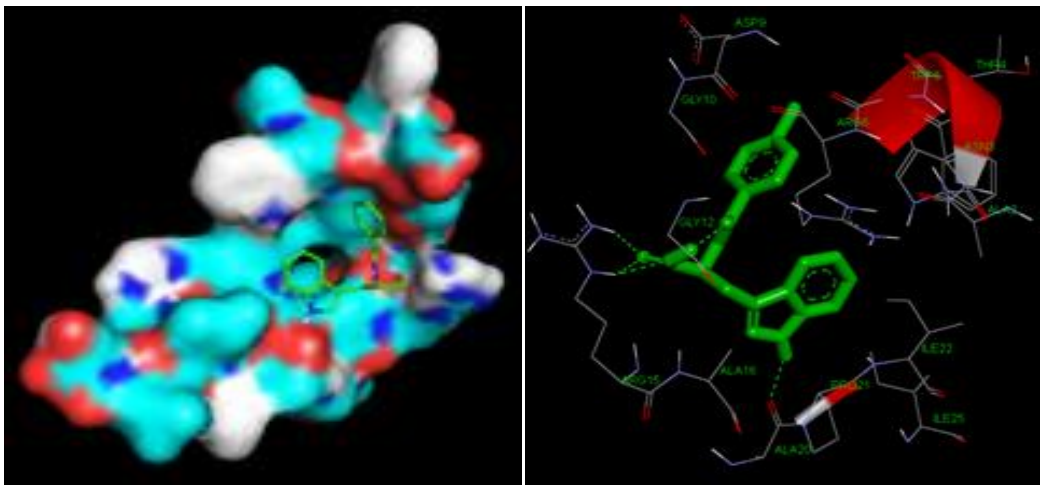


Figure 3: Effect of Rohitukine on histamine level in gastric tissue. Data expressed as mean % protection ± S.E.M. Statistical analysis was done by One Way ANOVA followed by Dunnett's Multiple Comparison Test. *Statistically significant at P<0.05 and **P< 0.01, in comparison to control. n = 6 in each group.



A



B

Figure 4: Rohitukine (A) and Benzotript (B) docked into CCK2 receptor as visualized iAccelrys Discovery StudioVisualizer

Discussion

From the docking studies of Rohitukine and benzotript with CCK2 receptor was that; though the binding energy values of Rohitukine was slightly less than benzotript, but at the molecular level, the binding pockets used by these compounds were significantly same. Even the surrounding residues in both the docked complexes around the ligand molecules (Table1) involved the same TRP5, ARG6, ASP9, GLY10, PRO11, GLY12 and ARG15 amino acid residues. Both the compounds Rohitukine and benzotript were able to form similar two hydrogen bonds with same ARG15 amino acid residues (Figure 4). The docked structure obtained in the process further revealed Rohitukine and benzotript having the same orientation and position in CCK2 receptor and even being docked into the same binding cavity available in the middle of the CCK2 receptor pocket, in the upper third of the lipid bilayer within the helical confluence as a potential site of docking of these compounds. The promising results obtained above, further supports our data at the molecular level, as Rohitukine an efficient gastrin antagonist.

In concurrence to previous reports we observed that incubation of parietal cell with forskolin it significantly elevated the intracellular cAMP level over basal values. This forskolin elicited amplification of cAMP level was not affected by Rohitukine. Comparing the effect of Rohitukine on intracellular cAMP level, with reference to cAMP level incresed by forskolin.

In our present study Rohitukine has not change the cAMP and histamine level. This finding has confirmed the in vivo anti-gastrinic activity of Rohitukine. The results suggested that Rohitukine exhibited anti-ulcerogenic potential possibly due to its act as a CCK2 receptor antagonist.

Rohitukine has not altered the level of cAMP and histamine level, as well as its antagonistic effect on the functional responses of gastrin on acid secretions from parietal cells.

Rohitukine may represent a promising agent as it possess several medicinal properties and offers advantage over existing anti-secretory drugs, in that it act as a anti-gastrinic agent with a cytoprotective potential, and thus would call for more detailed investigation in the different stages of drug development.

Conclusion

In conclusion, our results demonstrated that Rohitukine has not altered the cAMP and histamine levels; therefore it is not following the cAMP dependent pathway. It has an antagonistic effect on the gastrin, acid secretions from parietal cells. So it is following the Ca^{2+} dependent pathway and good target for future because all the marketed PPI have a lot of side effects, therefore we are targeting the CCK2 and not the proton pump which may be to yield a better therapy for future.

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