ANTI-INFLAMMATORY, ANALGESIC, ULCEROGENIC ACTIVITIES OF SOME NEW NON-ACIDIC DICLOFENAC AND 1, 3, 4-OXADIAZOLE DERIVATIVES IN ANIMAL MODELS

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Summary

Diclofenac sodium is an important component of prescriptions of arthritis patients since last 25 years. But even this drug is not an exception to the limitations of gastrointestinal adverse effects associated with the traditional non-selective NSAIDs. The free –COOH group is reported to be the main culprit responsible for GI toxicity of these NSAIDs. Derivatives of 1, 3, 4-oxadiazole are also known to have a broad spectrum of biological activities. Schiff bases and 1, 3, 4-oxadiazole derivatives of diclofenac were tested in vivo for their anti-inflammatory activity. The compounds, which showed significant analgesic and anti-inflammatory activities comparable to the standard drug diclofenac, were screened for their ulcerogenic potential to make sure that designed and synthesized compounds lack ulcerogenecity associated with parent prototype Diclofenac Sodium from traditional non-selective NSAIDs. The study showed that compound 3k possessed most significant anti-inflammatory and analgesic activity compared to parent drug Diclofenac Sodium. The compound also showed non ulcerogenic action at 12 times the therapeutic dose in animal models. The ulcers in rats were analyzed by histopathological studies. Results showed that compound 3e, 3g, 3k, 4c, 4e and control group were unremarkable, and were also devoid of mucosal hemorrhages, mucosal congestion and ulceration compared to that of standard drug Diclofenac.

Key words: Diclofenac, 1, 3, 4-oxadiazole, anti-inflammatory, analgesic, ulcerogenicity.

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Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of pain, fever and inflammation, particularly arthritis [1, 2 and 3]. Among the most popular NSAIDs, diclofenac has been approved in 120 countries since its introduction 25 years ago and ranked 30th among the top 200 drugs with respect to new prescriptions [4].

The pharmacological activity of NSAIDs is related to the suppression of prostaglandin biosynthesis from arachidonic acid by inhibiting the enzyme cyclooxygenases (COXs) [5 and 6]. It was discovered that COX exists in two isoforms, COX-1 and COX-2, which are regulated differently [7, 8 and 9]. COX-1 provides cytoprotection in the gastrointestinal (GI) tract whereas inducible COX-2 mediates inflammation [10, 11 and 12]. Since most of the currently available NSAIDs in the market show greater selectivity for COX-1 than COX-2 [13], chronic use of NSAIDs, including Diclofenac may elicit appreciable GI irritation, bleeding and ulceration [14]. The incidences of clinically significant GI side effects due to long term use of NSAIDs is very high (30%) and causes some patients to abandon NSAID therapy [4]. GI damage from NSAID is generally attributed to two factors. Local irritation by the direct contact of carboxylic acid moiety of NSAID with GI mucosal cells (topical effect) and decreased tissue prostaglandin production, which undermines the physiological role of cytoprotective prostaglandins in maintaining GI health and homeostasis [5 and 15].

Synthetic approaches based upon NSAIDs chemical modification have been taken with the aim of improving NSAID safety profile. Several studies have described the derivatization of the carboxylate function [16, 17 and 18] of representative NSAID resulted in an increased anti-inflammatory activity with reduced ulcerogenic toxicity. Furthermore, it has been reported in literature that certain compounds bearing 1,3,4-oxadiazole nucleus possess significant anti-inflammatory activity [19-23]. In our attempt to discover new, safer and potent agents for treatment of inflammatory diseases, we have replaced the carboxylic acid group of diclofenac acid with additional heterocycle, 1, 3, 4-oxadiazole in order to accentuate potency and reduce GI toxicities associated with the parent Diclofenac due to its free –COOH group. The compounds designed so, were found to possess much significant analgesic-anti-inflammatory profile with significant reduction in potential for ulcerogenic toxicities.

Materials and Methods

Experimental Animals:

Swiss albino mice of either sex weighing 20–25 g and wistar rats weighing in the range 100-120g were obtained from National Institute of Virology, Pune, India. All the animals were housed under standard environmental conditions of temperature (24±2°C) and relative humidity of 30-70 %. A 12:12 h light: dark cycle was maintained. All the animals were allowed to have free access to water and standard pelletized laboratory animal diet. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of College, Pune, constituted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), Government of India.

Chemicals

Carrageenan (Sigma-Aldrich, USA), acetic acid (Spectro. chem. Ltd., Mumbai), Anaesthetic Ether I.P. (TKM Pharma, Hyderabad) were procured. Diclofenac was obtained locally. All the chemicals were of analytical grade.

Anti-inflammatory activity: [27]

This activity was performed by the following procedure of Winter et al. on groups of six animals each. A freshly prepared suspension of carrageenan (1.0% w/v, 0.1 ml) was injected in the planter region of right hind paw of each rat. One group was kept as control and the animals of the other group were pretreated with the test drugs suspended in 1.0% Carboxy Methyl Cellulose (CMC) given orally 1 h before the carrageenan treatment. The volume was measured after 1h, 2 h and 3 h of carrageenan treatment with the help of digital plethysmometer (Panlab LE 7500 SI, Spain). The percent anti-inflammatory activity was calculated at 1 h, 2 h and 3 h according to the following formula:

$$\% \textit{ Anti-inflammatory activity} = (V_c - V_t / V_c) \times 100$$

Where V_t represents the mean increase in paw volume in rats treated with test compounds and V_c represents the mean increase in paw volume in control group of rats. Data are expressed as % anti-inflammatory activity ± S.E.M. and analyzed by one-way ANOVA followed by Dunnett's t test to determine the significance of the difference between the control group and rats treated with the test compounds. The difference in results were considered significant when $P < 0.01$. All statistical calculations were carried out using Graph Pad® Prism 3.0 (USA) statistical software.

Analgesic activity: [28]

Only compounds which exhibited good anti-inflammatory activity comparable to that of Diclofenac were screened for analgesic activity. The acetic acid induced writhing test was performed by injecting 0.1 ml of 1 % aqueous acetic acid solution intraperitoneally. Animals were divided in a group of 6 each. Mice were housed individually in the test cage, before acetic acid injection and allowed to acclimatize for 30 min. prior to dosing. Analgesic activity was screened at the dose of 10 mg/kg body weight. All the compounds were suspended in aqueous 1% CMC solution. The control group animals received only 1% CMC solution. Diclofenac was used as reference drug to validate the model on experimental animals. After 1 h of drug administration, 0.10 ml of 1% acetic acid solution was injected to mice intraperitoneally. Stretching movements consisting of arching of the back, elongation of body and extension of hind limbs were counted for 5–15 min after acetic acid injection. The analgesic activity was expressed in terms of % inhibition, and calculated as follows:

$$\% \textit{ Analgesic activity} = (nc - n_t/n_c) \times 100$$

Where,

nc = mean number of writhes of control group and

*n*t = mean number of writhes of test group.

Data are expressed as mean no. of writhes ± S.E.M., one way ANOVA followed by Dunnetts test was applied to determine the significance of the difference between the control group and mice treated with the test compounds. The difference in results were considered significant when $P < 0.01$. All statistical calculations were performed using Graph Pad® Prism 3.0 (USA) statistical software.

Acute ulcerogenecity studies: [29]

Albino rats were divided into different groups of six animals in each group. Potential for ulcerogenecity was evaluated after p.o. administration of test or standard compounds at 12 times the therapeutic doses. Control rats received 1% CMC as vehicle. Animals were fasted for 24 h before dosing, with water ad libitum. In order to induce prominent ulcers, after the drug treatment, the rats were exposed to cold stress at -20ºC for 4 h and then sacrificed by ether inhalation. The animals were sacrificed and dissected along the greater curvature of the stomach. And the stomach specimen were washed with distilled water and cleaned gently by dipping in saline. The mucosal damage was examined by means of a magnifying glass. For each stomach, the mucosal damage was assessed according to the following scoring system:

Score	Assignment
0.0	Normal (No injury, bleeding, and latent injury)
0.5	Latent injury or widespread bleeding.
1.0	Slight injury (2 to 3 dotted lines).
2.0	Severe injury (continuous lined injury or 5 to 6 dotted injuries).
3.0	Very severe injury (several continuous lined injuries).
4.0	Widespread lined injury or widened injury.

The mean score of each treated group minus the mean score of control group was regarded as severity index of gastric mucosal damage. Data are expressed as mean ± S.E.M., data analyzed by one way ANOVA followed by Dunnett's test to determine the significance of the difference between the standard group and rats treated with the test compounds. The differences in results were considered significant when P is < 0.01.

Histopathology studies: [30,31]

For the histopathological study, rats were sacrificed 4h after the cold stress and their stomach were removed and put into 10% formalin solution. A longitudinal section of stomach along the greater curvature, which included the ulcer based and both sides of the ulcer margin, was taken and fixed in 4% formalin for 24 h at 4ºC and embedded in paraffin. Morphological examination was performed with haematoxylin and eosin staining for histological changes and examined under light microscope.

Results

The analgesic and anti-inflammatory activities were evaluated using equimolar doses compared to the standard, Diclofenac at 0.0003378 molar concentrations, i.e. 10 mg/kg body weight for Albino mice as well as Wistar rats.

Effect of diclofenac derivatives on caraageenan induced rat paw edema.

Subplanter injection of carrageenan produced increase in paw volume (inflammation) of all the animals of various groups. The onset of action was evident from one hour in all the groups. The onset of reduction at 1 h. of rat paw volume was shown by only few compounds viz. 3g, 3k, 4c and 4e. The significant reduction of rat paw edema was observed by all the test compounds at 3 h compared to vehicle treated group. (Table 1)

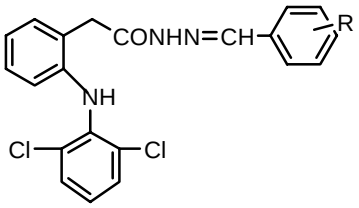
Results of Analgesic activity for synthesized compounds by acetic acid induced writhing model in Swiss Albino mice

Acetic acid (0.1 ml, 0.6%) produced 27.83 number of writhing in control group, the number of writhings after administration of acetic acid in various test groups are given in table 2. The number of writhes in Diclofenac (10 mg/kg) treated group were significantly reduced to 9.83 than that of control group. Dose dependent percentage inhibition of acetic acid induced writhing was observed in group, which were statistically significant compared to the control group , 27.83 ± 0.477 respectively. The results are reported in Table 2.

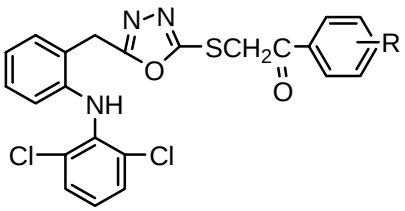
Gastric Ulcerogenic studies after single oral administration of the compounds under investigation.

Close inspection of the results obtained by ulcerogenecity studies indicate that Ulcerogenic effect of 3e, 3g, 3k , 4c, 4e at doses 10, 30, 50 mg/kg on stomach was negligible compared to parent drug Diclofenac Sodium at the same dose levels. (Table-3). Hence it can be said that gastro intestinal tolerance to these compounds is better than that of parent molecule, Diclofenac Sodium.. The results of potential for ulcerogenecity studies by the synthesized compounds are tabulated in the Table 3.

Table 1: Chemical structure of diclofenac derivatives and there anti-inflammatory activity against carrageenan induced rat paw edema.



(3)



(4)

Compound	-R	% inhibition at different time intervals.		
		1 hr	2hr	3hr
Vehicle	-	-	-	-
Diclofenac Sodium		**28.69 ± 3.94****	**48.99 ± 0.75****	**71.3 ± 1.91****
3e	**-3-NO_2**	**20.87 ± 4.74****	**47.02 ± 2.31****	**69.79 ± 2.15****
3g	**-4-OH**	**16.28 ± 4.24***	**38.14 ± 3.10****	**70.94 ± 2.57****
3j	**-4-$N(CH_3)_2$**	**28.20 ± 2.54****	**48.75 ± 3.10****	**69.36 ± 4.17****
3k	**-4-Br**	**30.53 ± 3.43****	**51.72 ± 2.57****	**80.07 ± 3.30****
4c	**-3,4-$(OCH_3)_2$**	**23.43 ± 3.28****	**48.63 ± 0.89****	**72.63 ± 3.44****
4d	**-3,4-$(Cl)_2$**	**30.46 ± 4.27****	**52.26 ± 3.00****	**60.39 ± 10.91****
4e	**-4-OH**	**31.55 ± 4.82****	**48.91 ± 3.06****	**71.99 ± 3.63****
4f	**-3-NO_2**	**28.34 ± 5.21****	**49.47 ± 2.73****	**60.32 ± 11.17****

Data analyzed by one way ANOVA followed by Dunnett's test, (n=6), *P<0.05, **P<0.01.

Table no 2: Analgesic Effect of compound syntjesized in series 3 and 4 Acetic acid induced writhing model

Compound No.	Dose (mg/kg, p.o)	No of Writhes in 25 min. after treatment (Mean ± S.E)	% Inhibition
Control	-	27.83 ± 0.477**	-
Std.	10	9.83 ± 0.60**	64.65**
3e	10	15.16 ± 0.83**	45.27**
3g	10	11.0 ± 0.57**	60.46**
3j	10	12.83 ± 1.13**	53.86**
3k	10	8.66 ± 0.55**	68.66**
4c	10	9.16 ± 0.70**	66.89**
4d	10	13.5 ± 0.99**	51.36**
4e	10	8.83 ± 0.47**	68.22**
4f	10	12.5 ± 0.76**	55.00**

Data analyzed by one way ANOVA followed by Dunnett's test, (n=6), *P<0.05, **P<0.01.

Table No 3: Ulcerogenic effects of compounds in 3 and 4 series in comparison with Diclofenac Sodium.

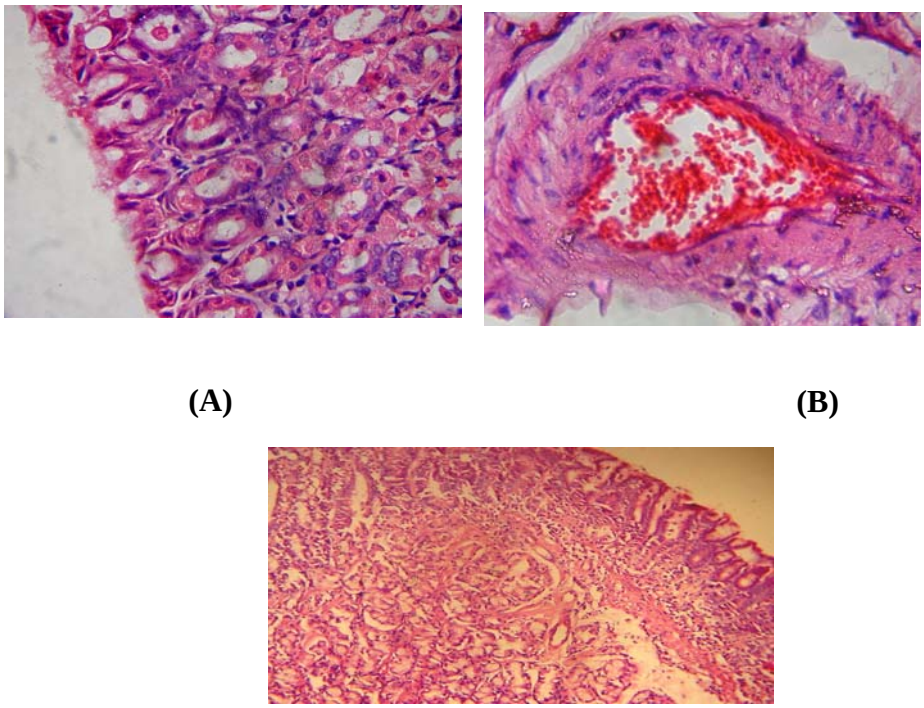
Compound	Dose (mg/kg, p.o)	Ratio of ulcerated animals	Ulcer index (mean±S.E)
Diclofenac	10	4/6	1.8±0.1
	30	6/6	2.1±0.2
	50	Not tested	N.A.
3e	10	Nil	Nil
	30	Nil	Nil
	50	Nil	Nil
3g	10	Nil	Nil
	30	Nil	Nil

	50	Nil	Nil
3k	10	Nil	Nil
	20	Nil	Nil
	50	Nil	Nil
4c	10	Nil	Nil
	30	Nil	Nil
	50	Nil	Nil
4e	10	Nil	Nil
	30	Nil	Nil
	50	Nil	Nil

Data analyzed by one way ANOVA followed by Dunnett's test, (n=6), *P<0.05, **P<0.01.

Histopathology studies:

Histopathological analysis showed characteristic features of ulceration in standard drug Diclofenac. After inducing ulcers the tissue samples, isolated from the control group rat stomach, consisted of fibroblasts, macrophages and proliferating endothelial cells forming micro vessels. The sample tissue of Diclofenac treated rat stomach showed some epithelial cells proliferated in the ulcer margin and these epithelial cells were found to be migrated over and into the ulcer crater, which was strongly infiltrated by inflammatory cells, fibroblasts and endothelial cells. The typical representative examples of ulcerated tissues by Diclofenac Sodium and nonulcerated tissue samples by control and drug treated tissue samples are shown in Fig. 1.



(A) **(B)**

(C)

Figure 1 : Haematoxylin and eosin Immunohistochemical staining of gastric ulcers after ulcer induction in rats. Intact Mucous membrane in control treated rat (A) showing granulation tissue composed of macrophages, fibroblasts and and endothelial cells forming microvessels. Congestion of mucosal blood vessels in Diclofenac (B). No damage was seen to mucosa of rat treated with 3k drug (C).Original magnification x 200.

Discussion

The compounds were tested at equimolar doses equivalent to 10 mg/kg oral dose of Diclofenac the parent drug and the results of test compounds were compared with the standard drug diclofenac.The tested compound showed anti-inflammatory activity ranging from 60.32 to 80.07% at 3 h. (Table 1), where as the standard drug Diclofenac Sodium showed 71.3% edema inhibition at 3 h after drug treatment. The compound 3k, 4c, 4e showed the most significant (>70%)

activity while compound 4d, 4f showed significant (60 to 70 %) activity. The maximum activity (80.07%) was shown by 3k derivative having bromo group at 4th position. When this group was replaced by 3, 4-dimethoxy oxadiazole derivative the activity was found to be decreased but was found to be equipotent to the standard drug. Effect of diclofenac and test compounds on percent inhibition of paw edema in rats at various time intervals (1 h and 3 h) is shown below (Fig 2).

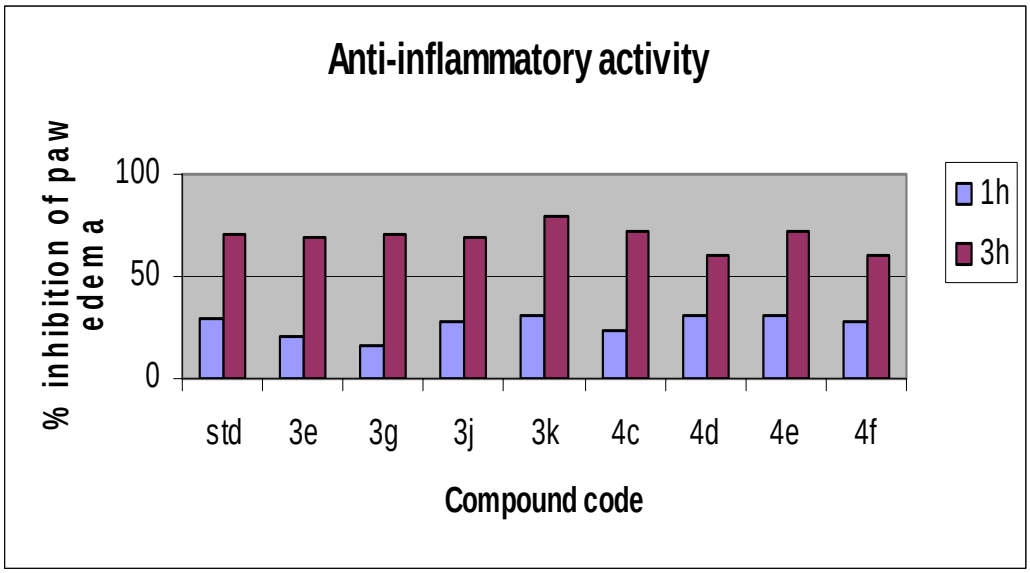


Fig.2 Combined effect of drug at 1h. and 3h.

All the synthesized compounds showed analgesic activity ranging from 45.27% to 68.66%. The compound 3k (68.66%), 4c (66.89%), 4e (68.22%) showed better analgesic activity than the standard drug diclofenac (64.65%).

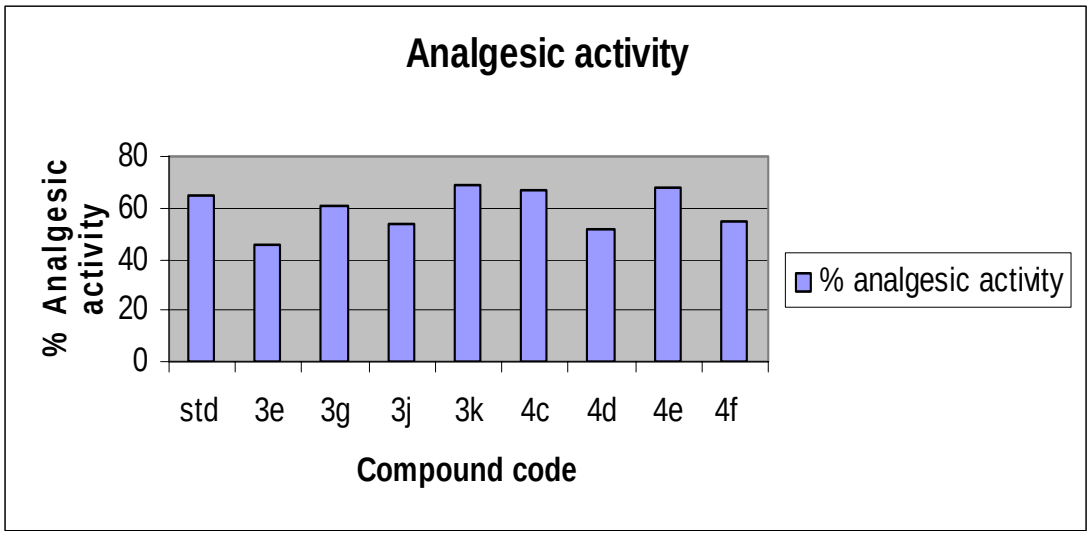


Fig.3. comparison of % analgesic activity of test compounds with diclofenac.

The compound which showed anti-inflammatory activity comparable to that of standard drug diclofenac and also showed high analgesic activity were screened for potential for ulcerogenecity.

Histological analysis showed no ulcerative features in rats treated with 3e, 3g, 3k, 4c, 4e group, the sections were also devoid of mucosal hemorrhages, and mucosal congestion and ulceration .In the tissue of control group the mucous membrane was intact without any damage to the adjacent cells. Same effect was seen in the group treated with the 3e, 3g, 3k, 4c, 4e 3k treated groups. While the group treated with standard drug Diclofenac showed proliferation and migration of epithelial cells in the region of ulcer crater, which was strongly infiltrated by inflammatory cells. It also showed marked evidence of congestion of mucosal blood vessels, mucosal ulceration, and mucosal hemorrhages

In all it can be concluded that compound 3e, 3g, 3k, 4c, 4e are devoid of ulcerogenic activity at 10, 30, 50 mg/kg dose while retaining their anti-inflammatory properties in animal models. The most potent and safer and less acidic diclofenac derivatives can be further subjected to acute, toxic toxicity studies and to clinical studies if found to be nontoxic.

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