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RESEARCH ARTICLE

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EXPLORING D-PHENYLALANINE AS A GASTRO-PROTECTIVE CHEMICAL DELIVERY SYSTEM FOR ACECLOFENAC

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ABSTRACT

Aceclofenac is a nonsteroidal anti-inflammatory drug used to relieve inflammation and associated pain in various forms of arthritis and has become popular due to its stimulatory effects on cartilage matrix synthesis even though it is known to induce erosion and ulcers in the gastro-intestinal tract. In the present work we synthesized a mutual prodrug of aceclofenac by exploring D-phenylalanine as a gastro-protective carrier system to lower the ulcerogenic potential and enhance effectiveness of aceclofenac as anti-arthritic agent. The study showed that D-phenylalanine enhanced the overall pharmacological and ulcerogenic profile of aceclofenac thus proving its utility as an effective gastro-sparing chemical delivery system in mutual prodrug design.

KEYWORDS

Aceclofenac, Anti-arthritic, D-phenylalanine, Gastro-protective, Mutual prodrug and Chemical delivery system.

INTRODUCTION

Arthritis involves inflammation of one or more joints in the body affecting the synovial or movable joints[1]. Destruction of connective tissue matrix components is the main cause of impairment of joint function[2]. The ordinary treatment of arthritis requires frequent intake of higher doses of nonsteroidal anti-inflammatory drugs (NSAIDs) over a longer duration which poses the risk of haemorrhage, gastro-intestinal tract (GIT) ulcers and significant renal, cardiovascular toxicities limiting their use[3].

Aceclofenac belongs to phenylacetic acid class of NSAIDs used widely in rheumatoid arthritis, osteoarthritis and ankylosing spondylitis[4]. It has been suggested that aceclofenac blocks prostaglandins production *via* cycloxygenase (COX-1) and COX-2 inhibition after intracellular metabolism to 4'-hydroxyaceclofenac and diclofenac in human rheumatoid synovial and other inflammatory cells[5]. In contrast to other NSAIDs, it has stimulatory effect on cartilage matrix synthesis[6]. The study by Cioli *et al.* suggests that direct tissue contact of the NSAID plays an important role in the production of GIT lesions[7]. The free carboxyl group in aceclofenac is responsible for local irritant effect on gastric mucosa. A structural modification to temporarily mask the –COOH group would help to lower this irritant effect. D-phenylalanine is reported to reduce chronic pain associated with arthritis, dental surgery, premenstrual cramps, lower back pain, migraine headaches, joint pains, whiplash, post-operative pain by stimulating nerve pathways in the brain that control pain[8]. We have reported anti-inflammatory and wound healing properties of D-phenylalanine[9]. Taking into consideration these findings, we thought of exploring this amino acid as a gastro-protective carrier to design a mutual prodrug of aceclofenac.

MATERIALS AND METHODS

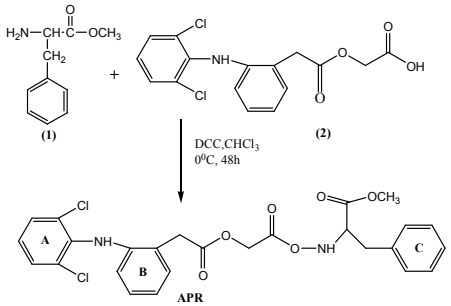
Aceclofenac was obtained as a gift sample from IPCA Mumbai, India and D-phenylalanine was purchased from Loba Chemicals, Mumbai. Reaction monitoring and purity check of synthesized prodrug was carried out by thin layer chromatography performed on silica gel 60 (Merck) coated plates using iodine vapours and UV light as detecting agents. Open capillary method was used to determine melting point and is uncorrected. The IR spectrum was recorded on JASCO, V-530 FTIR in potassium bromide (anhydrous I.R. grade) pellets while ^{1}H-NMR spectrum was recorded on ^{1}H- NMR Varian Mercury 300 MHz with super conducting magnet at University of Pune, Pune. All the experimental protocols used for pharmacological screening were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of Poona College of Pharmacy, Pune and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), Government of India. The animals used for the study were housed under standard environmental conditions of temperature 23±1°C and relative humidity of 50±5%. A 12 h light/dark cycle was followed. All animals had free access to water and standard pelleted laboratory animal diet. Statistical analysis was carried out by ANOVA followed by Dunnett's test to determine the significance of the difference between the control group and rats treated with the test compounds.

(i) Synthesis of aceclofenac prodrug with D-phenylalanine (APR) [10]

D-phenylalanine methyl ester hydrochloride was synthesized by Ronald's procedure.[11] It was dissolved in chloroform, triethylamine (0.1M) was added slowly and stirred for 2 h at 0^0C. The reaction mixture was then filtered and the chloroform layer was distilled off to get D-phenylalanine methyl ester **(1)**. DCC (0.1M) was then added to a solution of aceclofenac **(2)** (0.1M) in chloroform at 0^0C. Subsequently, D-phenylalanine methyl ester (0.1M) was added to the reaction mixture. It was stirred at 0^0C for 48 h and then filtered to remove the precipitated dicyclohexylurea and the filtrate was washed with 1M hydrochloride acid, 5% sodium bicarbonate and saturated solution of sodium chloride respectively. The organic layer was then dried over anhydrous sodium sulphate and concentrated under reduced pressure to give crude product. The product was recrystallized with ethyl acetate and was further purified by preparative TLC (chloroform: benzene: glacial acetic acid 2:1:0.1v/v) to give aceclofenac prodrug of D-phenylalanine (APR).

APR: 2- [2- {2- [2- (2, 6-Dichloro-phenylamino) phenyl] acetoxy} acetylamino]-3-phenyl propionic acid methyl ester; m.p. 174-176°C (uncorrected); yield: 70%; R_f: 0.64, ethyl acetate: methanol: glacial acetic acid (3:1:0.1v/v); aqueous solubility: 0.256 mg /ml; Log P_{oct}: -0.30; IR (KBr; cm^{-1}); 3327 (sec. amide NH stretching); 1748 (C=O stretching ester); 1669 (sec. amide C=O stretching); 1626 (NH bending amide); 1310 (C-N stretching aromatic amine); 1240 (C-O stretching ester); ^{1}H-NMR (DMSO-d_6; ppm): δ 8.53, [s, 1H] –NH; δ 7.30, [m, 5H] – aromatic CH Ring C; δ 7.00-7.28, [m, 3H] – aromatic CH of Ring A; δ 7.21, [m, 4H] – aromatic CH- Ring B; δ 4.97, [s, 2H] –CH_2; δ 4.55, [t, 1H] –CH; δ 3.72, [s, 2H] –CH_2.

Figure 1
Scheme of Synthesis



(ii) Anti-inflammatory activity by carrageenan-induced rat paw edema method[12]

Wistar rats of either sex (175-200 g) were used in this study. The animals were divided randomly in four groups with 6 rats per group. A freshly prepared solution of carrageenan (1% w/v, 0.1 ml) solution was injected into the planter region of right hind paw of each rat. One group was kept as a control and the animals of other group were pre-treated with the test drugs suspended in 1.0 % CMC given orally 1h before the carrageenan treatment. The paw volume was measured using UGO Basil Plethysmometer 7140 at 0 h, 1 h, 2 h, 3 h and 6h after carrageenan treatment. Percent inhibition was calculated according to the formula:

% Inhibition = {1- [(V_d – V_p)/ (V_c – V_p)]} × 100

where, (V_d – V_p) is the difference in the paw volume after carrageenan injection (V_d) and before carrageenan injection (V_p) in drug- treated group and (V_c – V_p) is the difference in paw volume after

carrageenan injection (Vc) and before carrageenan injection (V_p) in vehicle- treated group. Data was expressed as mean ± SEM. Results are shown in Table 1.

Table 1
Carrageenan –induced hind paw edema method

Group	Dose	Paw volumes #				Difference in paw volume #			% inhibition	
	mg/kg	0h	1h	3h	6h	1h	3h	6h	3h	6h
Arthritic Control	…	1.86 ± 0.11	2.17± 0.11	2.62± 0.22	3 ± 0.07	0.31± 0.11	0.76± 0.22	1.14 ± 0.07	…	…
Aceclo.	3.07	1.46 ± 0.23	1.72 ± 0.02	1.88 ± 0.02	1.84 ± 0.01	0.26 ± 0.02	0.30 ± 0.02	0.38 ± 0.01	60.52***	66.66***
Diclo.	0.714	1.46 ± 0.23	1.74 ± 0.02	1.79 ± 0.23	1.90 ± 0.23	0.26 ± 0.02	0.33 ± 0.01	0.44 ± 0.01	56.57	61.4
APR	4.4	1.64 ± 0.14	1.74 ± 0.06	1.76 ± 0.09	1.71 ± 0.08	0.31 ± 0.06	0.12 ± 0.09	0.07 ± 0.08	84.21***	93.85***
PA	1.41	1.59 ± 0.01	1.83 ± 0.01	2.11 ± 0.03	2.3 ± 0.005	0.28 ± 0.01	0.52 ± 0.03	0.68 ± 0.05	31.57	40.35

Aceclo: aceclofenac; Diclo: diclofenac; APR: prodrug of aceclofenac; PA: D-phenylalanine
*# Average of six readings; **P< 0.01; ***P< 0.001.*

(iii) Freund's complete adjuvant- induced arthritis model[13]

Anti-arthritic activity was evaluated using prophylactic and therapeutic protocols of Freund's adjuvant- induced arthritis method. Male rats (130-200 g) were used. On day 0, complete Freund's adjuvant (0.1 ml) was injected into the sub-plantar region of the left hind paw. Dosing with the test compounds or the standard was started depending on the protocol chosen. Paw volumes of both sides were recorded on the day of injection by using plethysmometer (7140-UGO Basil, Italy). On day 5, the volume of the injected paw was measured again. The severity of the non-injected paw (secondary lesions) was also recorded. The primary lesion was determined by measuring the paw volume on the 0, 5th, 13th, 18th and 21st day. % Inhibition of edema of both the injected left paw and non-injected right paw over vehicle control group was calculated by using the same formula as mentioned for carrageenan-induced rat paw edema method. Results are shown in Table 2.

Table 2
Anti-arthritic activity in Freund's adjuvant arthritis model

Group	Dose	Paw volume#					Difference in paw volume#		% inhibition	
	mg/kg	1d	5d	13d	18d	21d	18d	21d	18d	21d
Arthritic control	--	1.5 ± 0.012	2.4 ± 0.08	1.9 ± 0.04	2.0 ± 0.06	2.0 ± 0.07	0.5 ± 0.09	0.5 ± 0.11	--	--
Aceclo.	3.07	1.4 ± 0.08	2.4 ± 0.05	2.1 ± 0.08	1.7 ± 0.05	1.6 ± 0.05	0.2 ± 0.005	0.2 ± 0.01	55.6***	63.5***
Diclo.	0.714	1.6 ± 0.01	2.5 ± 0.02	2.34 ± 0.01	1.8 ± 0.01	1.78 ± 0.01	0.3 ± 0.02	0.21 ± 0.04	51.9***	59.6***
APR	4.4	1.55 ± 0.12	2.4 ± 0.09	2.25 ± 0.04	1.7 ± 0.13	1.7 ± 0.13	0.1 ± 0.01	0.14 ± 0.06	74.0**	73.0**
PA	1.41	1.6 ± 0.08	2.5 ± 0.06	2.3 ± 0.13	2.0 ± 0.006	2.0 ± 0.14	0.4 ± 0.06	0.41 ± 0.09	20.4	21.1

Aceclo: aceclofenac; Diclo: diclofenac; APR: prodrug of aceclofenac; PA: D-phenylalanine
*#Average of six readings; **P< 0.01; ***P< 0.001.*

(iv) Analgesic activity by acetic acid-induced writhing method[14]

Swiss mice of either sex (24 - 26 g) were used in the study. The animals were divided randomly in five groups with 6 mice per group. Mice were kept individually in the test cages, before acetic acid injection and habituated for 30 min. All compounds were dissolved in 1% CMC solution. The control group received p.o. administration of 1% CMC solution. After 1 h of drug administration 0.10 ml of 0.6% acetic acid was administered intra-peritoneally. After 5 min they were observed for a period of 10 min and the number of writhes was recorded for each animal. For scoring purpose, a writhe was indicated by stretching movements consisting of arching of the back, elongation of the body and extension of hind limbs. The formula for computing percent inhibition was:

% Analgesic activity = (n - n' / n) x 100

Where, n = mean number of writhes of control group, n' = mean number of writhes of test group.

Table 3
Analgesic activity by acetic acid- induced writhing model in mice

Group	Dose (mg/kg)	No. of writings#	% inhibition
Control	--	36 ± 2.16	--
Aceclo.	3.07	19.66 ± 1.15	47***
Diclo.	0.714	21 ±2	41.66**
APR	4.4	18.33 ± 0.57	50***
PA	1.41	31 ± 3.46	13.88

Aceclo:aceclofenac; Diclo: diclofenac; APR: prodrug of aceclofenac; PA: D-phenylalanine
*# Average of six readings; **P< 0.01;***P< 0.001.*

(v) Assessment of gastric ulcerogenic effect in rats[15]

The ulcerogenic activity was determined by the cold stress method. Groups of 6 male Wistar rats (175 – 200 g) were fasted for 24 h with free access to water. Test compounds were given orally to fasted animals at ten times higher dose than the normal equimolar dose of APR. After oral administration, animals were stressed by exposure to cold (-15°C for 1 h). The animals were kept in separate polypropylene cages. After 2 h of drug administration, the animals were sacrificed using chloroform. The stomach was opened along the greater curvature and washed with saline and the glandular portions were examined macroscopically for the number and size of mucosal lesions. The severity of the gastric damage was determined for each stomach examined and scored accordingly to the scale: 0- no lesion; 1- redness; 2- limited number of petechiae/diffused, pronounced lesions, 3- localized, severe lesions, more than 5 small ulcers and/or 1-2 large ulcers; 4 - extended, severe lesions; 5- perforation. The ulcerogenic index was calculated by using formula,

$UI = Un + Us + Up\ 10^{-1}$

where, UI- ulcer index, Un- average of number of ulcers per animal, Us- average of severity score, Up- percentage of animals with ulcers. Results are shown in Table 4 and Figure 1.

Table 4
Results of ulcerogenic activity

Compound	Dose (mg/kg)#	Ulcer Index ± S.D.*
Healthy control	--	3.52 ± 0.21
Aceclo.	30.7	14.01 ± 0.90
Diclo.	7.14	28.91 ± 2.12
APR	44	5.14 ± 0.42***
PA	14.1	2.56 ± 0.26

Aceclo: aceclofenac; Diclo: diclofenac; APR: prodrug of aceclofenac; PA: D-phenylalanine
*# Ten times equimolar dose; *Average of six readings; **P < 0.01; ***P< 0.001.*

Isolated stomach pictures of rat showing normal and ulcerated mucosa.

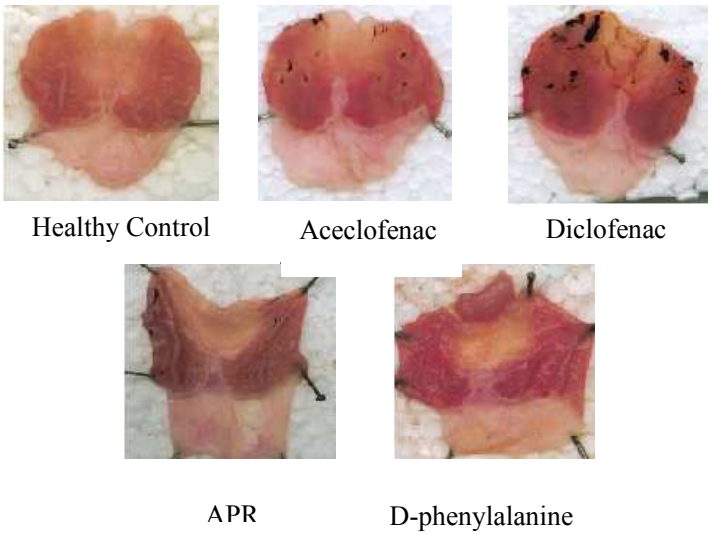


Figure 2
Photographs of stomach of rat

DISCUSSION

The purity of prodrug was checked by TLC and single spot was obtained for the same. The structure was assigned on the basis of IR and ^{1}H NMR. The log P value of APR was found to be -0.30. D-phenylalanine enhanced hydrophilicity of aceclofenac (log P 0.22). The anti-inflammatory activity of APR by carrageenan- induced rat paw edema showed 84.21% and 93.85 % inhibition of edema after 3 h and 6 h respectively which was much higher compared to aceclofenac (60.52 % and 66.66 % respectively) indicating positive contribution of D-phenylalanine. The anti-arthritic activity of APR was assessed by Freund's complete adjuvant induced arthritis model. Few hours after the induction of adjuvant arthritis by sub planter injection of 0.1 ml *Mycobacterium butyricum* to rats, the animals showed a local inflammatory reaction in the injected paw (primary response) with an increase in planter volume of about 60% over the baseline value. In addition, a disseminated arthritic reaction (secondary response) developed from day 1 after Freund's complete adjuvant (FCA) injection. Thus, swelling could be observed both in the injected paw and the non-injected contra-lateral paw. The effect of APR on the development of adjuvant arthritis is shown in Table 2. The reduction of the planter volume was statistically significant for both hind legs and the reduction of edema was more pronounced in the injected paw than in the non-injected paw. For peripheral analgesic activity APR was tested by acetic acid- induced writhing in mice. The analgesic activity of APR in this test proved to be a little more (50%) than that of aceclofenac (47%). The compound was further screened for ulcerogenic activity. The extent of gastric damage caused by APR and reference drugs after single oral administration to 24 h fasted male rats is shown in the Table 4. APR when administered orally, showed a lowest ulcer index (5.14 ± 0.42), as compared to plain aceclofenac and diclofenac which showed a higher ulcer index (14.01 ± 0.90) and (28.91±2.12) respectively at ten time higher doses. Ulcerogenic effect of APR on the stomach was nonexistent at the dose at which it showed anti-inflammatory effect. Hence gastric tolerance to APR was better than the reference drugs. These results are promising enough to justify and prove the correctness of mutual prodrug hypothesis.

CONCLUSION

In order to lower the ulcerogenic potential and enhance effectiveness of aceclofenac as anti-arthritic agent, a mutual prodrug was successfully synthesized with D-phenylalanine. It was interesting to note that conjugation of aceclofenac with D-phenylalanine not only lowered its ulcerogenic potential but also enhanced its analgesic, anti-inflammatory and anti-arthritic activities noticeably thus proving its utility as an effective gastro-protective carrier.

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