

PRELIMINARY STUDIES ON GASTRO-PROTECTIVE CHIMERIC DERIVATIVE OF BIPHENYL ACETIC ACID FOR RHEUMATOID ARTHRITIS

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ABSTRACT

Fenbufen, a bioprecursor prodrug owes its analgesic and anti-inflammatory activity to its active metabolite [1, 1'-biphenyl]-4-acetic acid (BPAA). It is used in the treatment of rheumatoid arthritis and osteoarthritis. Gastrointestinal complaints are the most frequently reported side effects of fenbufen and its major drawback is involvement of more than one metabolic pathway leading to inter-subject variations. BPAA is twice as active as fenbufen and has severe gastrointestinal side effects due to prostaglandin inhibition as well as local irritant effect of its carboxylic group. Therefore a gastro-protective carrier-linked chimeric derivative of BPAA (BPTR) was designed using L-tyrosine as the promoiety with an objective of potentiating its anti-inflammatory activity and minimizing local irritant effect. *In vitro* release of BPTR was studied in HCl buffer (pH 1.2) and phosphate buffer (pH 7.4). The prodrug was tested for anti-inflammatory activity by carrageenan- induced hind paw edema and Freund's adjuvant arthritis models while the analgesic and ulcerogenic activities were determined by hot plate method and Rainford's cold stress method respectively. *In vitro* release of BPTR in phosphate buffer (pH 7.4) furnished 16.14 % release of BPAA with a half-life of 1690.24 min, following first order kinetics while in HCl buffer it was found to be stable. BPTR showed better analgesic, anti-inflammatory and gastro-sparing activity than BPAA. The overall improved pharmacological profile of BPTR proves utility of L-tyrosine as effective carrier in latentiated chimeric design due to its synergistic contribution towards anti-inflammatory and wound healing effects.

Keywords: Fenbufen, Bioprecursor prodrug, Biphenylacetic acid, Arthritis, Gastro-sparing.

INTRODUCTION

Rheumatoid arthritis (RA) is a debilitating autoimmune disease which can lead to substantial loss of functioning and mobility. It is characterized by chronic inflammation and pain affecting many tissues and organs with primary involvement of joints producing an inflammatory synovitis, destruction of the articular cartilage and ankylosis. In RA, the balance between endogenous anti-inflammatory and pro-inflammatory mediators is disturbed with overwhelming production of pro-inflammatory cytokines - TNF, IL - 1 and others. The complex pathogenesis of RA is mediated by several mechanisms. Mobilization of leukocytes and their infiltration into the synovial membrane mark the early stages of RA followed by fibroblast proliferation and expansion of synovium responsible for characteristic erosions of cartilage and subchondral bone¹.

There is no known cure for RA, but the treatment can be focused on alleviating symptoms and / or modifying the disease process. The treatment options include use of nonsteroidal anti-inflammatory drugs (NSAIDs), slow-acting antirheumatic drugs (SAARDs) like antimalarials, sulphasalazine, methotrexate, gold, azathioprine, D-penicillamine, cyclophosphamide and chlorambucil either alone or in combination. The most common adverse reactions of NSAIDs reported to drug regulatory authorities are of gastrointestinal (GI) origin followed by renal damage, skin reactions, blood dyscrasias, headaches and nausea². COX-2 inhibitors decrease incidence of GIT adverse effects, but have significant renal and cardiovascular toxicities. The difficulties limiting use of SAARDs in the long-term treatment are those of side-effects and flares in disease activity despite initial control³.

NSAIDs is one of the most commonly prescribed group of drugs for RA world wide, with up to 20% of people admitted to hospitals due to severe GIT complaints^{4, 5}. Prostaglandins have an important role in maintaining normal GI physiology and controlling gastric acid secretion. NSAIDs inhibit prostaglandin synthesis causing abnormal GI function that eventually leads to gastric erosion, peptic ulcer formation, haemorrhage and inflammation in the small and large intestine⁵. Weakly acidic nature of NSAIDs helps in their preferential sequestration to inflamed joints, but also leads to accumulation in the kidney and the gut. The latter is responsible for the local irritant effect of NSAIDs on the gastric mucosa⁴.

Fenbufen (gamma-oxo[1,1'-biphenyl]-4-butanoic acid) from NSAID category is used in the treatment of rheumatic and other

musculoskeletal disorders. GI complaints are the most frequently reported side effects of fenbufen⁶. It is metabolized to four major metabolites: gamma-hydroxy [1, 1'-biphenyl]-4-butanoic acid (I), [1, 1'-biphenyl]-4-acetic acid (II), 4' hydroxy [1, 1'-biphenyl]-4-acetic acid (III), and gamma, 4'-dihydroxy [1,1'-biphenyl]-4-butanoic acid (IV). Fenbufen and metabolites I and II circulate to plasma and are pharmacologically active; metabolites III and IV are inactive and normally excreted in urine⁷. Involvement of more than one metabolic pathway leads to inter-subject variations. Mechanistic studies indicate that fenbufen has no intrinsic effect on cyclooxygenase activity, whereas its major metabolite, biphenylacetic acid (BPAA) which is twice as active as parent drug is a potent inhibitor of prostaglandin synthesis and therefore causes more GI injury on oral administration due to its direct local irritant effect caused by free carboxylic group⁸⁻¹⁰. Based on these facts, fenbufen can be considered as a bioprecursor prodrug of BPAA.

For these reasons several alternative drug delivery systems such as mutual ester prodrugs with phytophenols/alcohol as promoieties¹¹, hydrazide derivatives of probenecid and diclofenac with BPAA¹², synergistic α -mercapto prodrugs of BPAA¹³, macromolecular prodrugs of BPAA with cyclodextrins for colon specific delivery¹⁴ and changes in formulation such as topical preparation of BPAA¹⁵, suppositories, enteric coated tablets and sustained release preparations have been investigated with the objective of developing safer NSAIDs devoid of ulcerogenic side effects. Utility of mutual prodrug concept through derivatization of free carboxylic group of NSAIDs with amino acids into amides to reduce their ulcerogenic potential has been reported by Dhaneshwar et al.¹⁶⁻¹⁸

The present work aims at exploring a novel approach of designing a latentiated chimeric derivative of BPAA with L-tyrosine that has been reported to possess anti-inflammatory activity and wound healing properties with the objectives of potentiating anti-inflammatory activity and minimizing local irritant effect of BPAA.

MATERIALS AND METHODS

BPAA was purchased from Sigma-Aldrich Chemie GmbH, Germany and L-tyrosine was purchased from Loba Chemie Pvt. Ltd., Mumbai, India. Fenbufen was purchased from Shanghai AoBo Biopharmaceutical Technology Co., Ltd., Shanghai, China. All other chemicals used in the synthesis were of A.R. grade and those of synthetic grade were purified prior to use. ¹HNMR spectrum of the

BPTR was recorded in DMSO-d₆ using ¹H NMR Varian Mercury 300 MHz with super conducting magnet using TMS as internal standard at Department of Chemistry, University of Pune, Pune. Chemical shift values are reported in ppm downfield on δ scale. The IR spectrum of the synthesized compound was recorded on JASCO, V-530 FTIR in potassium bromide (anhydrous IR grade). The absorbance maxima (λ_{max}) of synthesized compound were determined on JASCO V530, UV-Visible double-beam spectrophotometer. Partition coefficient was determined in n-octanol/ phosphate buffer (pH 7.4) whereas the aqueous solubility was determined in distilled water at room temperature (25 ± 1°C).

The reactions were monitored by TLC on precoated silica gel plates- 60 F₂₆₄ (Merck).

Synthesis of amide conjugate of BPAA with L-tyrosine (BPTR)

L-tyrosine (**a**) was esterified to L-tyrosine methyl ester. HCl (**b**) by a reported procedure (Dhaneshwar & Chaturvedi, 1994). It was treated with triethylamine to release free L-tyrosine methyl ester (**c**) which was then coupled with BPAA (**d**) using DCC at 0°C for 48 h. The crude product was recrystallized with ethyl acetate and dried under vacuum. For further purification preparative TLC technique was used. The scheme of synthesis is illustrated in figure 1.

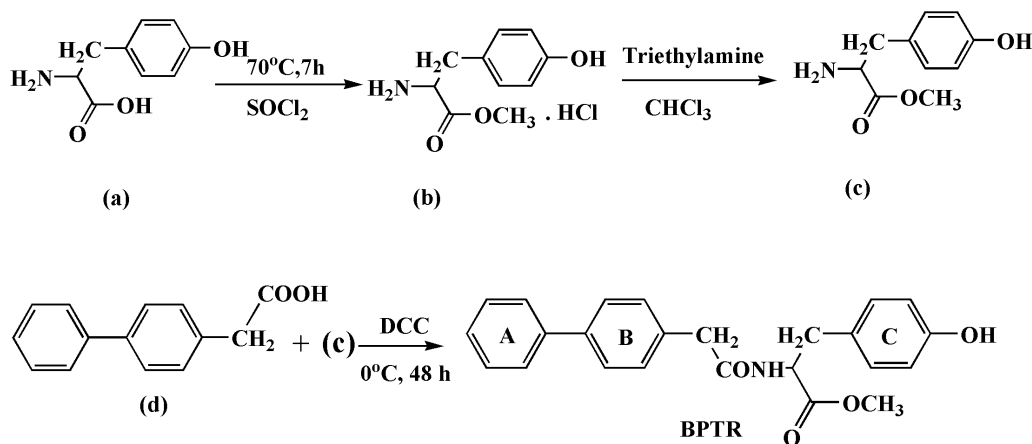


Fig. 1: It shows scheme of synthesis

Synthesis of L-tyrosine methyl ester hydrochloride (**b**)¹⁶

Freshly distilled thionyl chloride (0.05 mol + 30% excess; 5 ml) was slowly added to methanol (100 ml) with cooling and **(a)** (0.1 mol, 16.518g) was added to it. The mixture was refluxed for 7 h at 60-70°C with continuous stirring on a magnetic stirrer. Excess of thionyl chloride and solvent was removed under reduced pressure giving crude **(b)** which was triturated with 20 ml portions of cold ether at 0°C, until excess dimethyl sulphite was removed. The resulting solid product was collected and dried under high vacuum to give dried **(b)**. It was recrystallized from hot methanol by slow addition of 15-20 ml of ether, followed by cooling at 0°C. Crystals were collected on the next day and washed twice with ether: methanol mixture (5:1v/v) followed by pure ether and dried under vacuum to give pure **(b)**.

Melting point: 220-223°C, R_f = 0.76 in ethyl acetate: methanol: glacial acetic acid (3:1:0.1v/v). IR (KBr; cm⁻¹): 3404, 3320 cm⁻¹ Phenolic OH and primary amine -NH stretching, 3010 cm⁻¹ aromatic C - H stretching, 1740 cm⁻¹ C=O stretching ester, 1470 cm⁻¹ C-H bending methylene, 1250 cm⁻¹ C-O stretching ester.

Synthesis of 2-(2-biphenyl-4-yl-acetyl-amino)-3-(4-hydroxy-phenyl)-propionic acid methyl ester (BPTR)

To a solution of **(b)** (0.01M, 2.32g) dissolved in chloroform, triethylamine (0.01M, 1.4 ml) was added slowly and stirred for 2 h at 0°C. The reaction mixture was then filtered and the chloroform was distilled off to get **(c)** which was dried under reduced pressure. DCC (0.01M, 2.06g) was then added to a solution of **(d)** (0.01M, 2.12g) in chloroform at 0°C. Subsequently, **(c)** (0.01M, 1.95g) was added to the reaction mixture. It was stirred at 0°C for 48 h. The reaction mixture was filtered to remove the precipitated dicyclohexylurea and the filtrate was washed with 1M hydrochloric acid, 5% sodium bicarbonate and saturated solution of sodium chloride respectively. The organic layer was then dried under anhydrous sodium sulphate and concentrated under reduced pressure to give crude product. The product was recrystallized in ethyl acetate and it was further purified by preparative TLC (benzene: ethyl acetate: glacial acetic acid 2:1:0.1v/v) to give white crystals of BPTR.

BPTR: m.p. 176°C (d), (uncorrected), R_f 0.62; benzene: ethyl acetate: glacial acetic acid (2:1:0.1), yield 50%, aqueous solubility 0.109 mg/ml, Log P 1.05, λ_{max} 254 nm; HCl buffer (pH 1.2), 255 nm; phosphate buffer (pH 7.4), IR (KBr; cm⁻¹) 3449 (broad) (phenolic OH stretching), 3360 (secondary amide NH stretching), 1759 (C=O stretching ester), 1628 (secondary amide C=O stretching), 1574 (NH bending amide), 1273 (C-O stretching ester).

¹H NMR (DMSO-d₆) δ 7.12-7.8 [m, 9H]; aromatic protons ring A and B, δ 6.68 [d, 2H] and 6.95 [d, 2H]; aromatic protons ring C, δ 8.5 [d, 1H]; NH amide, δ 3.38 [s, 3 H]; methoxy, δ 5.6 [d, 1H]; CH methine, δ 2.94 [d, 2 H]; CH₂ methylene, δ 4 [bs, 1H]; phenolic OH.

In vitro Release Studies

pH- dependent activation and stability of BPTR was studied in aqueous buffers (pH 1.2 and pH 7.4) to simulate the pH with stomach and small intestine. The studies were carried out in triplicate. The release of BPAA from BPTR was monitored by HPLC and the methods developed were validated as per ICH guidelines. The LC system consisted of a Pump (Jasco PU 2080); with sampler programmed at 20 μl capacity per injection. The detector consisted of a UV/VIS (Jasco UV 2075) model operated at a wavelength of 254 nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system. The column used was Thermo Scientific ODS Hypersil (250mm × 4.6mm, 5.0 μm) from MA, USA. The dissolution studies were performed using USP 27 Type II dissolution test apparatus (TDT-067, Electrolab, India) and for the calculation of release kinetics, software 'PCP Disso' developed by Department of Pharmaceutics, Poona College of Pharmacy, Pune, India was used.

In vitro release in HCl buffer (pH 1.2)

BPTR (7.5 mg) was introduced in 500 ml of HCl buffer taken in a basket at 37 ± 1°C. The solution was occasionally stirred and 2 ml aliquot portions were withdrawn at various time intervals over a period of 3 h. The aliquots were estimated on HPLC using methanol: acetic acid (5%) 75:25 as the mobile phase at a flow rate of 1 ml/min with the retention time of 4.4 min for BPTR and 5.19 min for BPAA. Chromatogram at 3 h is illustrated in figure 2.

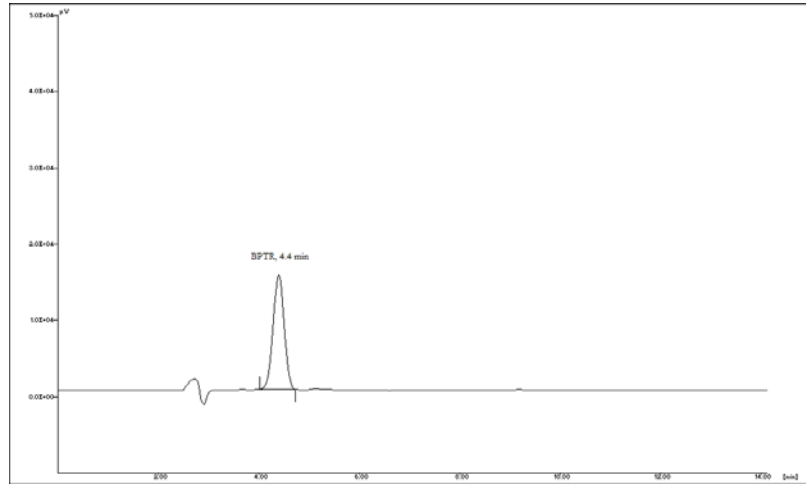


Fig. 2: It shows chromatogram indicating stability of BPTR at 3h in HCL buffer (pH 1.2)

In vitro release in phosphate buffer (pH 7.4)

Same procedure as described above was followed. HCL buffer was replaced by phosphate buffer and release of BPAA was studied over

a period of 8 h. The aliquots were estimated on HPLC using methanol: water 70:30 as the mobile phase at a flow rate of 1 ml/min with the retention time of 2.5 min for BPAA and 5.9 min for BPTR. Chromatogram at 8 h is illustrated in figure 3.

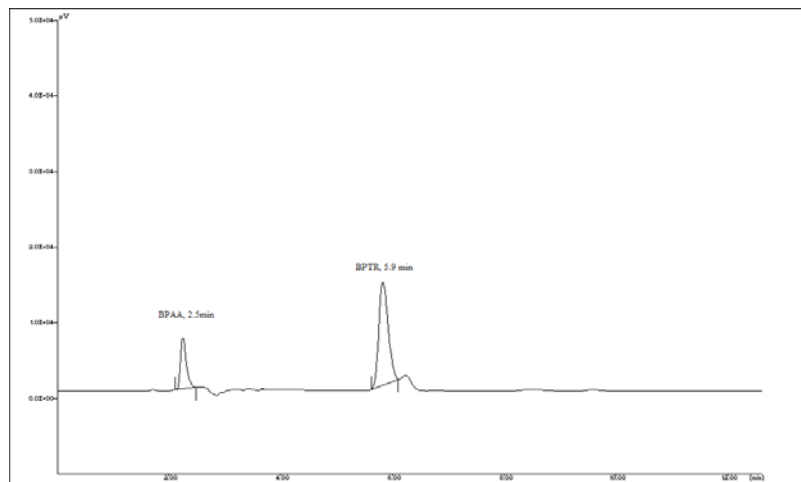


Fig. 3: It shows chromatogram indicating release of BPAA from BPTR in phosphate buffer (pH 7.4)

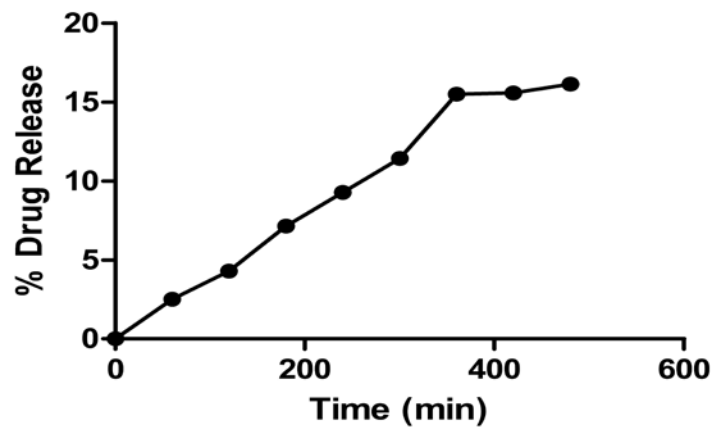


Fig. 4: It shows percent release of BPPAA from BPTR in phosphate buffer (pH 7.4)

Biological evaluation

The biological screening of BPTR was carried out in the Department of Pharmacology, Poona College of Pharmacy and its animal facility is approved by Committee for Purpose, Control and Supervision of Experimental Animals, (Reg. No.100/1999/CPCSEA). The study protocols were approved by Institutional Animal Ethics Committee. The standard and the test compounds were administered orally as a suspension in distilled water using 1% sodium CMC. Albino Wistar rats of either sex (200-300 g) were used for the screening of anti-inflammatory and ulcerogenic activity whereas for Freund's adjuvant arthritis model, Wistar rats of either sex (150-200 g) were used. Analgesic activity was carried out using mice of either sex (20-25 g). The animals were distributed into control, standard and test groups, having six animals each. The dose of BPTR was calculated on an equimolar basis to fenbufen.

Statistical analysis

Average of six readings was calculated and data was expressed as mean \pm SEM. Statistical differences between the groups were calculated by two-way ANOVA followed by Bonferroni post- hoc

test for carrageenan induced rat paw edema and one-way ANOVA followed by Dunnett's post- hoc test for Freund's adjuvant arthritis model, analgesic activity and ulcerogenic activity.

Anti-inflammatory activity

Carrageenan- induced rat paw edema method¹⁹ was used to evaluate the anti-inflammatory activity of BPTR on the acute inflammation. Wistar rats of either sex (200-300 g) were distributed into control, standard and test groups, having six animals each. At 0 h the standard and test compounds were administered orally in the form of suspension. One hour after this treatment, edema was induced by injecting 0.1ml of 1% w/v suspension of carrageenan (C-3889 Type IV, Sigma Chemicals, St. Louis, MO) in subplantar region of the hind paw. The control group received 1% carboxymethylcellulose (CMC). Paw volumes were measured before and after the administration of carrageenan using a UGO-Basile Plethysmometer. The percent inhibition was calculated using formula $(1 - V_t / V_c) \times 100$, where V_t and V_c are the mean relative changes in the paw volume in test and control respectively (Winter et al, 1962). The results are summarized in figure 5.

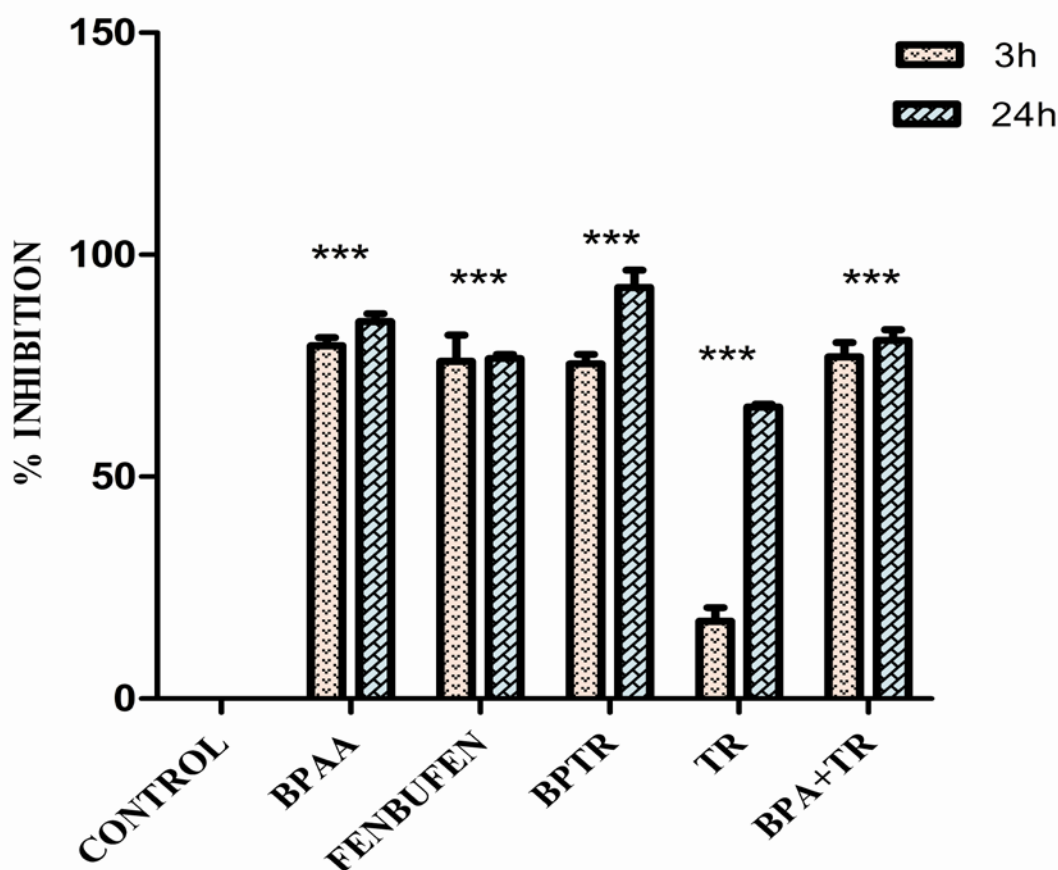


Fig. 5: It shows percent inhibition of edema in carrageenan-induced rat paw edema model

Antiarthritic activity

Antiarthritic activity was evaluated using Freund's adjuvant induced arthritis model²⁰. Wistar rats of either sex (150-200 g) were distributed into control, standard and test groups, having six animals each. On day one, 0.1 ml of complete Freund's adjuvant (F-5881, Sigma Aldrich Co., USA) was injected into the subplantar region of the hind paw. The animals were housed in cages to allow the development of full arthritis up to 13 days. The paw volumes

were measured on 5th and 13th day using UGO BASILE Plethysmometer 7140, Italy. On 13th day the drug administration was started and continued up to 21st day. The arthritic control group received 1% CMC solution. Finally, paw volume was again measured on 21st day. The average foot swelling in standard and test groups was compared with the arthritic control group and % inhibition of edema was determined by the using the same formula which was used to evaluate anti-inflammatory activity. The results of antiarthritic activity are summarized in figure 6.

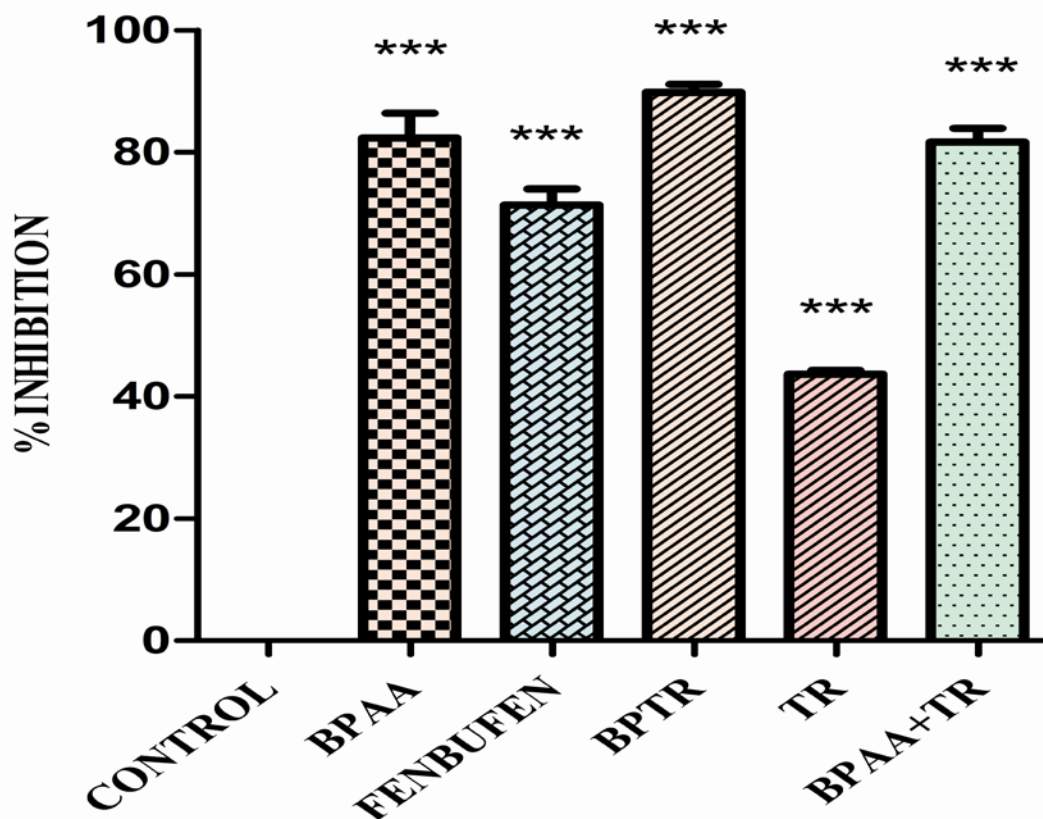


Fig. 6: It shows percent inhibition of edema in Freund's adjuvant- induced arthritis model

Analgesic activity

The analgesic activity was carried out using hot plate method²¹. Mice of either sex (20-25 g) were distributed into control, standard and test groups, having six animals each. The paws of mice being sensitive to heat are exposed to a temperature not damaging to the skin and the response in the form of jumping, withdrawal of the paws or the licking of the paws is observed. The mice were placed on

Eddy's hot plate (equipped with a thermostat) kept at a temperature of 55 ± 0.5 °C. A cut off period of 15 s, was observed to avoid damage to the paw. Reaction time and the type of response were noted using a stopwatch. At 0 min the standard and test compounds were administered orally in the form of suspension. The latency period was recorded at 20, 90, 120 and 180 min following oral administration of the compounds. Average reaction times were then calculated and the results are summarized in figure 7.

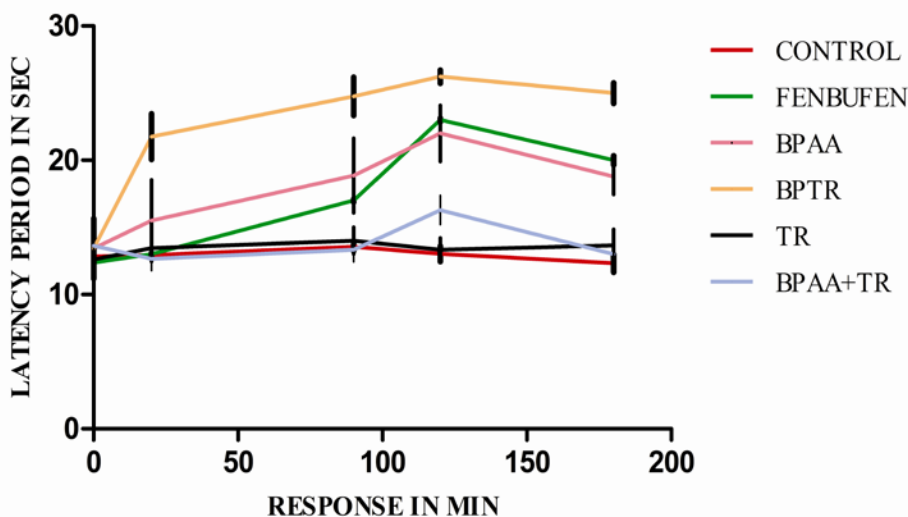


Fig. 7: It shows analgesic activity

Ulcerogenic activity

The ulcerogenic activity was determined by the cold stress method²². Wistar rats of either sex (200-300 g) were distributed into control, standard and test groups, having six animals each. Standard and test compounds were administered at a dose of 10 times that of usual dose, orally. After 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 along with duodenal

part was opened along the greater curvature and washed thoroughly with normal saline and scanned using UMAX ASTRA 5600 Crystal Clear Display (CCD) Scanner, Novex Technologies Inc., China.

The images were analyzed using Adobe Photoshop and Image J software. All ulcers larger than 0.5 mm were counted and recorded as average number of ulcers per animal and were scored according to the method reported by Cioli et al. (1979)²³ and the ulcer index was determined. The results are summarized in figure 8 and the gastric ulcers are photographically represented in Fig 9.

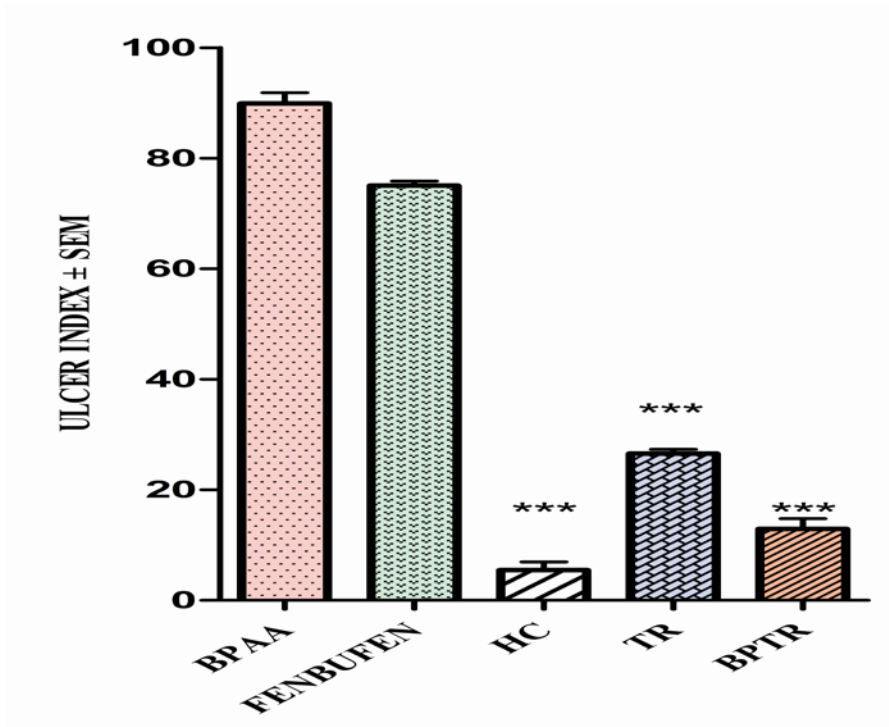


Fig. 8: It shows ulcerogenic activity

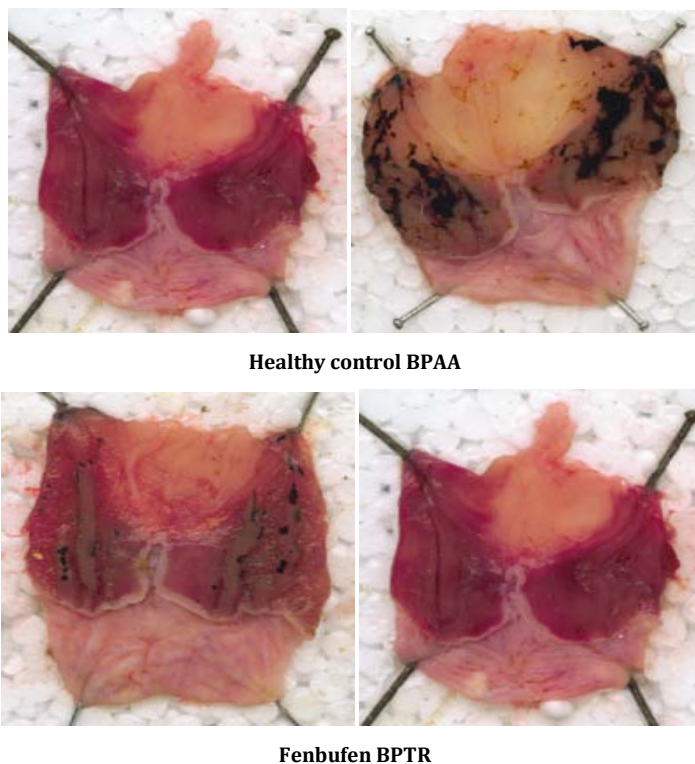


Fig. 9: It shows photographic representation of gastric ulcers in Wistar rats

RESULTS AND DISCUSSION

The purity of BPTR was checked by TLC and single spot was obtained for the same. Conjugation of BPAA with L-tyrosine enhanced its lipophilicity as evident from higher log P (1.05) as compared to BPAA (0.106). IR spectrum showed a broad peak at 3449 of phenolic OH stretching typical of L-tyrosine, secondary amide NH stretching at 3360 and secondary amide C=O stretching at 1628 cm^{-1} confirming formation of an amide that was further justified by ^1H NMR spectrum showing chemical shift at δ 8.5 ppm for proton of NH amide. Thus the spectral analysis of the prodrug confirmed its anticipated structure. The prodrug was found to be stable in HCl buffer (pH 1.2) which suggests that free BPAA responsible for gastric irritation would not be available in stomach. Slow rate of release of BPAA (16.14%, $t_{1/2}$ =1690 min, first order kinetics) from BPTR is in accordance with the fact that amides are resistant to pH dependent hydrolysis. The release profile of BPAA from BPTR is illustrated in figure 8. *In vivo* release studies in blood, urine and feces are currently underway to ensure enzymatic (amidases) hydrolysis of BPTR into faster and complete release of BPAA that would help to justify the biological actions of this prodrug. In carrageenan- induced hind paw edema model, after 3h; BPTR showed 70.2% inhibition of inflammation which was comparable to BPAA (72%) but at 24h, % inhibition of inflammation achieved by BPTR was slightly more (92%) than BPAA (87.5%). In Freund's adjuvant induced arthritis model, BPTR showed significantly more protection against chronic inflammation (85%) on 21st day than BPAA (71.3%). The effect of physical mixture of BPAA with L-tyrosine was also evaluated in the above acute and chronic models of inflammation to prove the anti-inflammatory effect of L-tyrosine. As predicted, the physical mixture provided better protection against inflammation in both the models (77% at 3h / 81% at 24h in carrageenan induced edema model and 80.5% on 21st day in Freund's adjuvant induced arthritis model). Maximum analgesic effect was observed for BPTR at 120 min which was maintained till 180 min where as BPAA could not maintain the analgesic effect after 120 min. A seven fold decrease in ulcerogenic potential of BPAA was achieved by temporary masking of its free carboxylic group with L-tyrosine. The overall improvement in the pharmacological profile of BPTR could be ascribed to certain prominent attributes of BPTR namely enhanced lipophilicity resulting in better absorption, longer duration of action owing to slow rate of hydrolysis and synergistic contribution of L-tyrosine towards anti-inflammatory activity with gastro-protective effect.

CONCLUSION

A latentiated carrier-linked chimeric derivative of BPAA with better analgesic, anti-inflammatory and gastro-sparing activities was successfully designed and developed. *In vivo* comparison of the pharmacokinetic behavior of BPTR with fenbufen is required to prove its utility in the safer management of rheumatoid arthritis.

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