ANTI-INFLAMMATORY AND ANALGESIC ACTIVITY OF 4'-METHYLBIPHENYL-2- (SUBSTITUTED PHENYL) CARBOXAMIDE ANALOGS IN ANIMAL MODELS OF INFLAMMATION

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Summary

A new class of anti-inflammatory compounds containing 4'-methylbiphenyl-2- (substituted phenyl) carboxamide derivatives evaluated *in vivo* for anti-inflammatory, analgesic and its ulcerogenic potential in animal models of inflammation. A series of biphenyl analogue was investigated for acute anti-inflammatory model by carrageenan induced rat paw edema. Among the most active compounds 4a, (4'-methylbiphenyl-2-(4-nitro phenyl) carboxamide was selected for detail investigation on various phases of inflammation. Pretreatment of rats with 4a (100 mg/kg) reduced carrageenan induced rat paw edema at 3 h compared to control group. Dose dependent percent inhibition of granuloma formation, was observed in 4a (25, 50 and 100 mg/kg) and celecoxib (5 mg/kg) treated groups in cotton pellet granuloma in rats. C – reactive proteins were absent in 4a treated group. 4a inhibited acetic acid induced writhing dose dependently (10, 20 and 30 mg/kg). 4a was inactive to pain produced by hot plate method. Gastric toxicity screening experiments showed that compound 4a, both after single and repeated oral administration is devoid of any gastric irritation in rats. The LD_{50} was found to be more than 2000 mg/kg.

Keywords: 4'-Methylbiphenyl-2- (substituted phenyl) carboxamide, Analgesic, Anti-inflammatory**,** Ulcerogenicity

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are extensively used throughout the world in the treatment of a number of pathological conditions, but several adverse effects limit their clinical usefulness. Among these the most frequent complication is gastrointestinal lesions, and these are often responsible for discontinuation of therapy in patients taking NSAIDs (1, 2).
The pharmaceutical industry has endeavored to discover, develop and market an efficacious NSAID with little or no gastrointestinal liabilities. This has lead to the arrival of the Cyclooxygenase-2 inhibitors, a new class of NSAID that is prominently represented by Vioxx (Rofecoxib, Merck), Celebrax (Celecoxib, Pfizer) and Bextra (Valdecoxib, Pfizer). Although, these drugs demonstrate analgesic and anti-inflammatory activity comparable with traditional NSAIDs, they cause markedly less gastrodudenal irritation than aspirin and the traditional NSAIDs (3)**.** However, Merck voluntarily withdrew Vioxx from the market in 2004 because it was demonstrated to increase the risk of heart attacks and stroke in some individual participating in a colon polypstrial, after 18 months of therapy. The medical needs of patients suffering from chronic inflammatory disorders remain unmet and no current therapy constitutes a cure for this. A great amount of concerted efforts are being made world wide to find a safe and acceptable anti-inflammatory drug.
In recent years, the computer-aided quantitative structure-activity relationship (QSAR) and molecular modeling approaches have been extensively applied for finding new molecular entities as therapeutic agents (4-6). The close examination of chemical structure of COX enzyme inhibitors reveal that the drugs possess diaryls substituted differently around the 5- or 6-membered heterocyclic nucleus. In order to deviate from such a traditional strategy, a series of compounds containing biphenyl nucleus substituted variously with carboxamide linkage at position-2 was designed on the basis of physico-chemical properties and molecular similarity. These were then synthesized and tested preliminarily for anti-inflammatory activity (7). In the present work, out of nine compounds screened for anti-inflammatory activity, most active compound 4a (4'-methylbiphenyl-2-(4-nitro phenyl) carboxamide) was investigated in detail for their effect on sub acute model of inflammation, analgesic action and ulcerogenic effect on the mucosa of the stomach.

Materials and Methods

Animals

Swiss albino mice of either sex weighing 20–25 g and wistar rats of 175-200 g were obtained from National Toxicological Centre, Pune, India who is approved breeder of laboratory animals. They were housed under standard environmental conditions of temperature (24±1°C) and relative humidity of 30-70 %. A 12:12 h light dark cycle was followed. All animals had free access to water and standard pelleted 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 (Pure Chem. Ltd, Pune), methanol (Qualigens, Mumbai) were purchased. Celecoxib (CAS number: 169590-42-5), pentazocin (CAS number: 359-83-1) and aspirin (CAS number: 50-78-2) were obtained locally. All the chemicals were of analytical grade.

Acute oral toxicity studies (8)

Healthy adult albino mice of either sex, starved overnight were subjected to acute toxicity studies as per guidelines (AOT No. 425) suggested by Organization for Economic Co-operation and Development (OECD) 2001. The mice were observed continuously for 2h for behavioral, neurological and autonomic profiles for any lethality or death for next 48 hrs. Based on the results obtained from this study, the doses for further pharmacological studies were fixed at 25, 50 and 100 mg/kg, p.o.

Anti-inflammatory activity against carrageenan-induced rat paw edema (9)

Wistar rats of either sex weighing 175 to 200 g were used in this study. The animals were divided randomly in different groups with 6 rats per group. Group 1 was control group (1 % CMC, 0.5 ml p.o.); test compounds and standard drug were administered orally at a dose of 100 mg/kg and 10 mg/kg to respective groups of animals. The pretreatment time was 1 hour after which 0.1 ml of 1% carrageenan solution was injected into the right hind paw of the rat. The paw volume was recorded at 1 h, 2 h and 3 h by using plethysmometer (UGO Basile 7140). Percentage inhibition of edema was calculated at different hours (1 h, 2 h and 3 h).

Anti-inflammatory activity against cotton pellet granuloma (10)

Subcutaneous implantation of pellets of compressed cotton provokes foreign body granuloma. Cotton rolls were cut and made into pellets weighing 20 mg each and sterilized in an autoclave at 100°C for 30 min. Four individual pellets were inserted in each ether anesthetized rats by making small subcutaneous incisions in both axillae and groin regions. The incisions were sutured by sterile catgut. After recovery from anesthesia; the animals were treated orally for 7 days. On eighth day animals were sacrificed, and the granulomas were freed from extraneous tissue, dried and weighed to obtain constant weight. The average weight of the pellets of the control group as well as of the test group was calculated. The percent change of granuloma weight relative to vehicle control group was determined.

C - reactive protein (CRP) measurement during systemic inflammation (11)

Wistar rats were anesthetized with anesthetic ether. The blood was removed by retro orbital puncture method and collected into the bottles without anticoagulant solution. CRP was detected by using Plasmatec CRP latex test kit. Four individual cotton pellets were inserted in each ether-anesthetized animal by making small subcutaneous incisions in both axillae and groin regions. The incisions were sutured by sterile catgut. The animals were treated orally for 7 days. On eighth day, the blood samples were again collected and CRP was detected.

Analgesic activity against acetic acid induced writhing (12)

Pain was induced by injection of irritant (acetic acid) into the peritoneal cavity of mice. The animals react with a characteristic stretching behavior called writhing i.e. contractions of abdomen, turning of trunk and extension of hind limb, which was observed in various groups of animals. Vehicle (1 % w/v CMC, 0.5 ml p.o.) was given to control group (n=6 per group) of mice. A mouse in the test groups (n = 6 per group) received 4a at 10, 20 and 30 mg/kg p.o. Aspirin (100 mg/kg) was used as the reference analgesic drug. One hour following the administration of test drugs, 0.1ml/100gm of 0.6% v/v acetic acid solution was injected intra-peritoneally to each of the test mice. The number of writhes that occurred within the next 10 min following acetic acid administration was counted and recorded. Results were expressed as percentage inhibition of writhing.

Analgesic activity by Randall & Selitto model (13)

Male wistar rats were used. Assessment of pain consisted of measurement of the threshold stimulus for reaction (escape or paw withdrawal) using a weight (maximum limit of 500 g) applied to the pads of hind paws of animals. The threshold for pain sensation was measured before (basal) and 120 min after the intra plantar injection of 1% carrageenan (0.2 ml). 4a or aspirin or vehicle (1 % w/v CMC, 0.5 ml p.o.) was administered orally 15 min prior to carrageenan injection. The treatment groups were vehicle (1 % CMC, 0.5 ml p.o), test compound-4a (10, 20 and 30 mg/kg, p.o.) or aspirin (100 mg/kg, p.o.)

Analgesic activity by hot plate method (14)

In hot plate method various responses (jumping and licking) were recorded. The control group of mice (n= 6) received vehicle (1 % w/v CMC, 0.5 ml p.o.). The test group mice received 4a at 10, 20 and 30-mg/kg p.o. and pentazocin (4 mg/kg i.p.), respectively. One hour following the test compound or pentazocin administration, the mice were individually placed on Eddy's hot plate (Ugo Basile 7250) maintained at 55 ± 0.2 ºC. The latency period of 20 sec. was defined as complete analgesia. To avoid injury, the measurement was terminated if latency period exceeded 20 sec.

Assessment of gastric ulcerogenic effects in the rat (15)
Ulcerogenic effect on acute administration of drugs

Groups of 6 male wistar rats weighing 175 – 200 g were fasted for 24 h with free access to water. Test compounds were given orally to fasted animals, which were killed 5 h later by ether inhalation. At this time gastric lesions were fully developed. The stomach was dissected out, sectioned along the greater curvature, washed with saline, and the glandular portion examined macroscopically for the number and size of mucosal lesions. The severity of the gastric damage was determined for each stomach examined and the ulcer index for each treatment was calculated.

Ulcerogenic effect on repeated administration of drugs

Male Wistar rats weighing 150-175 g were housed in groups of 6 and allowed free assess to normal food and water except for three hour before and after treatment. Animals were given test compound orally at 10 am for 5 consecutive days. 6 h after the last administration the rats were killed by ether inhalation and the stomach of each animal was removed, examined, scored as described previously.

Statistical analysis

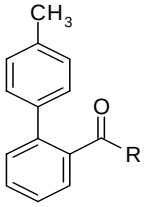
Data obtained for each set of anti-inflammatory models was expressed as mean ± S.D and analyzed by one-way ANOVA followed by Dunnett's test. Data from acetic acid induced writhing model was expressed as mean ± SEM and analyzed by Kruskel-Wallis ANOVA followed by Mann-Whitney "U" test. Level of significance was set at $P < 0.05$. All statistical manipulations were carried out using Graph Pad® Prism 3.0 (USA) statistical software.

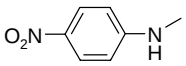
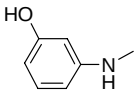
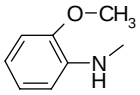
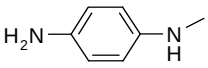
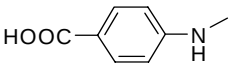
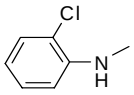
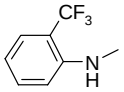
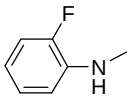
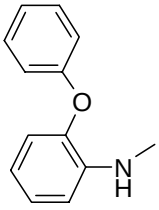
Results

Effect of biphenyl derivatives on carrageenan-induced rat paw edema

Sub planter injection of carrageenan produced increase in paw volume (inflammation) of all the animals of various groups. The onset of action was evident after one hour in various test groups. The onset of reduction of rat paw volume occurred by compound 4a, 4e and 4g at 1 h. The other compound 4b, 4c, 4d, 4f and 4i did not show any significant anti-inflammatory activity. The significant ($p<0.01$) reduction of rat paw edema was observed by test compound 4a, 4e, 4g and 4i at 3 h compared to vehicle treated group. (Table-1)

Table 1: Chemical structure of biphenyl analogue and there anti-inflammatory activity against carrageenan induced rat paw edema



Group	-R	% Inhibition at hour		
		1 h	2 h	3 h
Vehicle	-	-	-	-
Celecoxib	-	19.40	30.89	45.24**
4a		15.42	27.83	40.48*
4b		5.47	18.04	25.24
4c		4.48	14.98	19.52
4d		2.49	18.04	23.81
4e		13.43	28.44	41.43**
4f		12.50	18.14	26.12
4g		14.42	26.96	39.55*
4h		7.69	24.02	33.21
4i		15.92	19.67	25.75

Data analyzed by ANOVA followed by Dunnett's test, *P< 0.05, ** P< 0.01, n=6,
Dose: 100 mg/kg p.o.

Effect of 4a on cotton pellet granuloma

Implantation of cotton pellets produced granuloma formation, fluid infiltration and undifferentiated connective tissue was measured by weighing the dried pellets after 7 days of implantation and treatment. Dose dependent percent inhibition of granuloma formation was observed in 4a (25, 50 and 100 mg/kg) and celecoxib (5 mg/kg) treated groups on cotton pellet granuloma in rats. (Table-2)

Table 2: Effect of test and reference drugs on cotton pellet granuloma in rat

Drug	Daily oral dose mg/kg	Dry granuloma weight	Inhibition (%)
Control	-	44.62±2.4	-
	25	34.12±2.26**	23.53
4a	50	29.66±3.45**	33.53
	100	28.55±1.4**	36.02
Celecoxib	5	25.22±1.5**	43.48

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

Effect of 4a on C reactive protein

CRP was found to be absent in all the animals of all the groups before administration of the test drugs. After implantation of cotton pellets, CRP was detected (positive) only in vehicle treated (control) group on 7th day. The compound 4a (100 mg/kg) and celecoxib (5 mg/kg) pretreated groups showed absence of CRP (Table-3).

Table 3: CRP detection in 4a and celecoxib treated rats

Group	Dose (mg/kg)	C – Reactive Protein	
		Before treatment	After 7 days treatment
Vehicle	-	Positive	Positive
4a	100	Positive	Negative
Celecoxib	5	Positive	Negative

Effect of 4a on acetic acid induced writhing test in mice

Acetic acid (0.1 ml, 0.6%) produced 29.16 number of writhing in control group, the number of writhings after administration of acetic acid was 18.6, 17.0, 16.78 in 4a (10, 20 and 30 mg/kg) pretreated group respectively. The number of writhes in aspirin (100 mg/kg) treated group was 13.88. Dose dependent percentage inhibition of acetic acid induced writhing was observed in test compound treated group at various doses (10, 20 and 30 mg/kg), which were statistically significant compared to the control group respectively. (Table-4)

Table 4: Effect of 4a on acetic acid induced writhing in mice

Group	Treatment	Dose (mg/kg)	No. of writhing. (Mean ± SE).	% Inhibition.
I	Control	-	29.16 ± 0.66	-
		10	18.6± 1.11**	37.22
II	4a	20	17.0±1.27**	41.70
		30	16.78±1.23**	42.75
III	Aspirin	100	13.88± 1.54 **	52.40

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

Effect of 4a on Randall & Selitto model

In control rats receiving a sub planter injection of carrageenan, the mean threshold of pain in the inflamed foot 3 h after irritant increased (82 as compared to 66) with the normal non-injected foot. Test compound (4a) showed moderate analgesic effect in the doses tested 10, 20 and 30 mg/kg (Table-5).

Table 5: Effect on % increase in pain threshold by Randall Selitto test in rat

Group	Dose Mg/kg	Pressure (gm) ± Mean S.D.	% Increase in pain threshold
Control	-	66±7.76	-
	10	82±8.89*	24.24
4a	20	84±7.18*	27.31
	30	86±8.91**	30.30
Aspirin	100	90±7.86**	36.36

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

Effect of 4a on hot plate method in mice

In the hot plate method, 4a was found to be inactive to inhibit pain produced by thermal means at various doses (10, 20 and 30 mg/kg).

Gastric ulcerogenic effects after single and repeated oral administration

Ulcerogenic effect of 4a (100 mg/kg) on stomach was nonexistent (Table-6). Hence gastric tolerance to 4a was better than that of celecoxib. After repeated oral administration for 7 consecutive days to partially fasted (3 h) rats, 4a showed better gastric tolerability as compared with celecoxib (Table- 7).

Table 6: Gastric ulcerogenic effects of 4a and reference drugs after single oral administration in rats

Drug	**Dose (mg/kg, p.o.)**	**Average of no. of ulcers per animal**	**Mean Score of Lesion intensity**	**No. of animals with ulcer (%)**	**Ulcer index (UI)**
Control	-	-	-	-	-
4a	25	-	-	-	-
	50	-	-	-	-
	100	-	-	-	-
Celecoxib	10	3	1.3	1 (16.16)	2.09

** P<0.01, one way ANOVA followed Dunnett's test, n = 6

Table 7: Gastric ulcerogenic effects of 4a and reference drugs after repeated oral administration for 7 consecutive days to the male rats

Drug	**Dose (mg/kg, p.o.)**	**Average of no. of ulcers per animal**	**Mean Score of Lesion intensity**	**No. of animals with ulcer (%)**	**Ulcer Index (UI)**
Control	-	-	-	-	-
4a	25	-	-	-	-
	50	-	-	-	-
	100	-	-	-	-
Celecoxib	10	2	1.6	2 (33.33)	3.69

** P<0.01, one way ANOVA followed Dunnett's test, n= 6

Toxicity study

Acute oral toxicity studies revealed that the test compound was safe up to a dose level of 2000 mg/kg of body weight. No lethality or any toxic reactions were found up to the end of the study.

DISCUSSION

The emergence of COX-2 inhibitors offered hope and optimism to patients and physicians but the surprising and untoward liabilities associated with these drugs have triggered significant consternation and frustration in the medical community and the FDA. A resolution and refocused perspective to this sensitive issue would bring clinical relief, peace of mind and hope to the patients suffering from inflammatory disorders like rheumatoid arthritis.

In the present study biphenyl analogues were synthesized by reacting 4'- methylbiphenyl-2-carboxylchloride with substituted anilines to obtain desired compounds (Table-1). The compound 4a has structural resemblance with some known non-steroidal anti-inflammatory drugs such as diclofenac, flurbiprofen and diflunisal. The rationale for the synthesis of these analogues was to develop the anti-inflammatory drug without gastrointestinal irritation.

The pharmacological experiments of this study showed that some of the compounds tested possess promising anti-inflammatory activities; celecoxib was used as a standard drug for comparing the anti-inflammatory activity and also as an agent with less gastrointestinal tract irritant activity. Compound 4a was one of the most active compound in the series of compounds tested against carrageenan induced rat paw edema model. Carrageenan induced paw edema is believed to be biphasic, of which the first phase is mediated by the release of histamine and 5-hydroxytryptamine in the early stage followed by kinin release and then prostaglandin in the later phase (16). It has been reported that the second phase (3 h) of edema is sensitive to most clinically effective anti-inflammatory agents. Anti-inflammatory effects of 4a in 3h of edema suggest involvement of inhibition of prostaglandin in the action of 4a.

The cotton pellet granuloma method is commonly used to evaluate the proliferative aspects of the chronic tissue injury (inflammation). Subcutaneous implantation of pellets of compressed cotton provokes foreign body granuloma. Dose dependent percent inhibition of granuloma formation was observed with 4a indicating suppression of the proliferative phase of inflammation by the test compound. C - reactive protein (CRP) is one of the acute phase protein that is increased during systemic inflammation and used as a marker of inflammation. The physiological function of CRP is to induce non-specific mechanisms against infection and to help macrophages to scavenge altered lipoproteins. After the 4a treatment there was absence of CRP indicating additional contributing mechanisms of anti-inflammatory effects.

Writhing test is based on the principle that tissue injury increases the sensitivity to pain and this sensitivity is susceptible to modification by analgesics. In this model of chemical induced tissue injury, 4a showed non-steroidal anti-inflammatory drug like peripheral analgesic activity which was also confirmed by Randell-Selitto test. Absence of analgesic effect against hot plate induced pain supports absence of central component in mechanism of 4a analgesia. The acute toxicity of 4a in mice was found to be greater than 2000 mg/kg. Thus, tested compound (4a) was found to be safe in the anti-inflammatory dose range. In conclusion, the compound 4a showed good acute and sub-acute anti-inflammatory with peripheral analgesic activity. Our gastric toxicity screening experiments showed that compound 4a is devoid of any gastric irritation, both after single and repeated oral administration in rats.

ACKNOWLEDGEMENT

The authors are grateful to Dr. S. S. Kadam, Principal and Dr. K. R. Mahadik, Vice-Principal, Poona College of Pharmacy for keen interest and encouragement in the project.

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