European Journal of Pharmacology 678 (2012) 48–54

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and Inflammation

Anti-inflammatory potential of thienopyridines as possible alternative to NSAIDs

Kuncha Madhusudana [a], Boyapati Shireesha [b], Vegi Ganga Modi Naidu [c], Sistla Ramakrishna [a,*,1], Banda Narsaiah [d], Akkinepally Raghuram Rao [e], Prakash V. Diwan [f,**,1]

[a] Pharmacology Division, Indian Institute of Chemical Technology (IICT), Tarnaka, Hyderabad, Andhra Pradesh-500607, India
[b] University College of Pharmaceutical Sciences, Kakatiya University, Warangal, Andhra Pradesh-506009, India
[c] Department of Anatomy, YLL School of Medicine, National University of Singapore, Singapore
[d] Fluoroorganics Division, Indian Institute of Chemical Technology (IICT), Tarnaka, Hyderabad, Andhra Pradesh, 500607, India
[e] University Institute of Pharmaceutical Sciences, Punjab University, Chandigarh, India
[f] Lalitha College of pharmacy, Ghatkesar, Hyderabad, Andhra Pradesh, India

ARTICLE INFO

Article history:
Received 28 June 2011
Received in revised form 7 December 2011
Accepted 9 December 2011
Available online 24 December 2011

Keywords:
Thienopyridine analog
Anti-inflammatory
Carrageenan
Dextran
Arachidonic acid
Freund's complete adjuvant induced arthritis

ABSTRACT

The present study was designed to evaluate the anti-inflammatory and antiarthritic activity of the new synthetic thienopyridine analogs. The anti-inflammatory activity of thienopyridines was assayed by using carrageenan; dextran and arachidonic acid induced paw edema models (acute), cotton pellet granuloma model (Sub acute) and Freund's complete adjuvant induced arthritis (chronic) in experimental rats. The compounds BN-4, BN-14 and BN-16 have shown significant inhibition of edema in carrageenan and arachidonic acid induced paw edema model at a dose of 100 mg/kg compared to the dextran induced paw edema model and also showed significant inhibition in granuloma tissue formation and Freund's complete adjuvant induced arthritis in experimental rats. These thienopyridine analogs also inhibited the proinflammatory mediators such as Tumor necrosis factor (TNF)-α, Interleukin (IL)-1β and Nitric Oxide (NO) in Lipopolysaccharide (LPS) challenged murine macrophages. Ulcerogenecity study results revealed less ulcerogenic potential of BN-4, BN-14 and BN-16 compared to nonsteroidal anti-inflammatory drug (NSAID) indomethacin in rats. In conclusion, the new thienopyridine analogs were promising for the potential use as anti-inflammatory agents for both acute and chronic inflammatory disorders with low toxic effects.

1. Introduction

During recent years there has been a large investigation on development of different classes of thienopyridine compounds, many of which were found to possess an extensive spectrum of pharmacological activities. The general skeletal structure for thienopyridine was depicted in Fig. 1. Thienopyridines have been reported for treatment of cancer, stroke, osteoporosis, rheumatoid arthritis (Harris et al., 2004), anti-inflammatory (Vasuma et al., 2010), antiplatelet agents (Boneu and Destelle, 1996), acetyl cholinesterase inhibitory activity Ikuo et al., 1992), antimicrobial activity (Bakhite et al., 2002) and anti-asthmatic agents Yamaguchi et al., 1995). As part of our ongoing program in the development of novel lead molecules with anti-inflammatory activity, a series of thiophene analogs of tacrine were synthesized.

Inflammation involves complex array of enzyme activation, release of mediators, and extravasation of fluids, cell migration, tissue breakdown and repair (Vane and Bolting, 1995). During the inflammation process, macrophages play a central role in managing many different immunopathological phenomena including the overproduction of pro-inflammatory cytokines and inflammatory mediators such as interleukin (IL)-1β, IL-6, Nitric Oxide (NO), and tumor necrosis factor (TNF)-α. Hence LPS (Bacterial Lipopolysaccharide) stimulated macrophage cells (Raw 264.7) has been selected as *in vitro* anti-inflammatory cellular model. Based on the cardinal signs of inflammation (*i.e.* edema, exudation) the *in vivo* models were chosen to study the anti-inflammatory effect of thienopyridine derivatives on acute inflammation (carrageenan, dextran and arachidonic acid models), sub acute inflammation (Cotton pellet granuloma model) and chronic inflammatory conditions (Freund's complete adjuvant induced arthritis model). The present study was therefore, aimed to evaluate the newly synthesized thienopyridine derivatives, for their possible anti-inflammatory, antiarthritic potential in various *in vitro* and *in vivo* models and also evaluate the efficacy and minimizing the toxicity of the molecules.

* Correspondence to: S. Ramakrishna, IICT, Hyderabad, Andhra Pradesh, India. Tel./fax: +91 40 27193753.
** Correspondence to: P.V. Diwan, Tel./fax: +91 8415 255311.
E-mail addresses: kuncha_m@yahoo.com (K. Madhusudana), sirimedchem@yahoo.co.in (B. Shireesha), vgmnaidu@gmail.com (V.G.M. Naidu), sistla@iict.res.in (S. Ramakrishna), narsaiah_1984@yahoo.co.in (B. Narsaiah), raghumed@gmail.com (A.R. Rao), diwanpv@gmail.com (P.V. Diwan).
[1] Authors who have equally contributed.

0014-2999/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.ejphar.2011.12.019

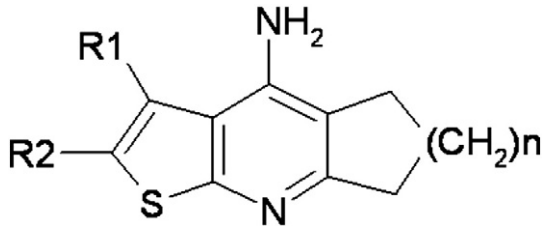

Fig. 1. General skeletal structure of thienopyridine.

2. Materials and methods

2.1. Chemicals

Carrageenan, Indomethacin, Prednisolone, Griess Reagent, Dextran, Dulbeccos Modified Eagle's Medium, Minimum essential medium, Sodium bicarbonate, Antibiotic and Antimycotic mixture, Trypsin solution, and MTT were purchased from Sigma, USA. Glutamine (Fluka), Fetal bovine serum (Gibco) and TNF-α, IL-1β kits were purchased from R&D Biosystems, USA. Freund's complete adjuvant was purchased from Difco, USA.

2.2. Test animals

Male Albino Wistar rats weighing between 130 and 150 g were used for the experiments. They were kept in polypropylene cages under standard laboratory conditions (12:12 h light/dark cycle) at 24 °C. Rats were provided with commercial rat diet (NIN, Hyderabad) and water *adlibitum*. The experiments were conducted after obtaining approval from Institutional Animal Ethical Committee of IICT (97/1999/CPCSEA, dated 28.04.1999). Animals were quarantined and acclimatized to laboratory conditions for 7 days prior to study initiation. Animals were observed for general health and suitability for testing during this period.

2.3. Synthesis

Poly substituted 2-aminothiophenes with an electron-withdrawing cyano group in third position and alkyl or cycloalkyl groups in the fourth and fifth positions are prepared via Gewald reaction (Haung et al., 2005). Fifteen new derivatives of amino thienopyridines were synthesized in the second step using zinc chloride as catalyst without using any solvent and has got more than seventy five percent yield (Shireesha et al., 2010).The procedure involves condensation of thiophene aminonitrile and cyclic ketones in 1:2 ratio in presence of zinc chloride as Lewis acid catalyst by refluxing at their boiling points. The reaction of thiophene aminonitriles with various cyclic ketones in presence of zinc chloride results in nucleophilic attack of amino nitrogen on partially positive carbonyl carbon to form Schiff's base along with progressive formation of the intra molecular metal chelate which also helps the reaction to drive towards cyclization. The acceleration of the cyclization is observed in the presence of the Zinc salts. Complexation of the Lewis acidic metal salt to the nitrile at third position activated the nitrile triple bond towards nucleophilic addition. The attack of active methylene carbon adjacent to imine linkage on cyanide carbon led to cyclization *in situ* to form tricyclic ring. Immediately after cyclization has occurred, rapid tautomerization of the initially formed imine would result in aromatization of the central ring to give the quinoline/acridine analogs. After total conversion of the starting material into amino thienopyridines, the reaction mixture was added to 40% sodium hydroxide solution to release the product from zinc chloride complex. The separated compounds were collected by vacuum filtration and purified using column chromatographic technique. All the compounds were characterized using physical and spectral data and reported elsewhere (Shireesha et al., 2010).

2.4. Carrageenan induced rat paw edema

Carrageenan induced paw edema was produced according to the method of Winter et al. (1963). Overnight fasted animals were divided into different groups with six animals in each group. The test compounds were administered by oral route as gum acacia suspension (2% w/v) at the dose of 100 mg/kg whereas animals in the standard group received indomethacin at the dose of 10 mg/kg, p.o. Rats in the control group received the vehicle alone. One hour after test drugs administration, rats in all the groups were challenged with 0.1 ml of (1%w/v) carrageenan in PBS into the sub plantar region of right hind paw. Paw volumes were measured before and after 3 h following the carrageenan administration using digital Plethysmometer (Ugo Basile, Italy). The percent inhibition of paw volume for treated groups was calculated by comparing with that of mean paw volume of control group.

2.5. Dextran induced rat paw edema

Wistar rats were divided into different groups containing six rats in each group. Acute inflammation was induced according to the method (Murkherjee et al., 1997). A volume of 0.1 ml of 1% w/v of dextran in normal saline was injected to the sub plantar region of right hind paw. The test compounds were suspended in gum acacia and administered to the rats 1 h before dextran injection. The paw volumes were measured by using Plethysmometer at 0 h and 3 h for all the rats following the dextran injection. The percent inhibition of paw volume for treated groups was calculated by comparing with mean paw volume of the control group.

2.6. Arachidonic acid induced paw edema

Paw edema was produced in rats by arachidonic acid following the reported methods (DiMatrino et al., 1987; Seguraa et al., 1998). Rats were divided into different groups of six animals in each. The test and standard compounds were administered as gum acacia suspension by oral route. After 2 h a volume of 0.1 ml of 0.5% arachidonic acid in 0.2 M carbonate buffer was injected in to the sub plantar region of the right hind paw of the rat. Paw volumes were measured using plethysmometer at 0 h and 1 h after arachidonic acid injection for all the rats.

2.7. Cotton pellet granuloma

In this experiment, the effect of thienopyridine analogs and indomethacin on priliferative phase of inflammation was investigated employing cotton pellet granuloma method (Swingle and Shideman, 1972). Wistar rats weighing between 180 and 220 g were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) by intra peritoneal injection. Rats were divided into 5 groups of six animals in each. The test compounds (BN-4, BN-14 and BN-16) and indomethacin (Standard) were administered as gum acacia suspension. Then cotton pellets, weighing 10 ± 1 mg each, were implanted on both sides in scapular region under sterile condition. The same doses of test compounds and standard were administered daily for seven consecutive days. On the 8th day the rats in all groups were sacrificed with high dose of anesthesia. The pellets surrounded by granuloma tissues were dissected out and kept overnight for incubation at 37 °C. Pellets were then dried at 60 °C until they reached constant weight. The wet weight of pellets was also recorded after sacrificing. The average weights of pellets of control group and other treated groups were calculated. The percent change of granuloma weight was calculated for all the test groups by comparing with that of control group.

2.8. Acute oral toxicity study

The acute oral toxicity study was conducted as per OECD guidelines by fixed dose method adopted by OECD (420). In the sighting study, the effect of various doses was investigated in single animal of each sex. Dosing was sequential allowing at least 24 h before dosing the next animal. All animals were carefully observed for signs and symptoms of toxicity continuously up to 24 h and later up to 7 days. The sighting study was conducted with sequential doses of 5, 50, 500 and 2000 mg/kg of the test article. If the initial dose chosen did not produce severe toxicity, the next higher dose was selected. In this sighting study, the dose that produced evident toxicity but not death was identified. Dose escalation was continued up to 2000 mg/kg.

In main study at least 10 animals (5 males and 5 females) for each species were used for the dose level. Animals were fasted over night and the test articles were administered as 1% gum acacia suspension by oral route. The dose used in this study is selected from one of the four levels 5 mg/kg, 50 mg/kg, 500 mg/kg and 2000 mg/kg *i.e.* the dose that produced evident toxicity but not mortality from sighting study. The animals were observed for signs and symptoms of toxicity apart from the cage side observations.

2.9. Adjuvant induced arthritis

Arthritis was induced on day 0 by a single injection of 0.1 ml of Freund's complete adjuvant with 10 mg/ml of mycobacterium tuberculosis H37Ra (Difco, USA) in to the sub plantar region of the right hind paw (Newbould, 1963). Test compounds and indomethacin were administered as gum acacia suspension by oral route at the dose of 100 and 2.5 mg/kg respectively on day of immunization with adjuvant (Prophylactic regimen). The drug treatments were continued daily on the same time after the challenge up to day 21. Rats were inspected daily for the onset of arthritis characterized by edema and erythema in the paws.

Disease severity was evaluated by arthritic scoring and measurement of hind paw volumes and body weights on day 0, 5, 12, 18 and 21 day. Arthritic scoring was done as per the method (Trentham et al., 1977) and lesions on both hind paws were graded from 0 to 4, based on the extent of erythema and edema on the paws. The maximum score was being 8 for each animal. The change in hind paw volume was measured using plethysmometer (Ugo, Basile, Italy) and expressed as mean paw volume of both hind paw of the rats. After the induction of arthritis (day 0), the increase in paw volume was calculated by subtracting the hind paw volume measured as the base line on day 0.

2.10. In vitro anti-inflammatory activity studies

The RAW 264.7 mouse macrophage cell line was procured from, National Center for Cellular Sciences, Pune, India. Cells were grown in Dulbecco's Modified Eagles Medium without phenol red supplemented with 10% heat inactivated serum, 100 μg/ml penicillin, 200 μg/ml streptomycin, 2 mmol L-glutamine, 1% antibiotic/Anti mycotic solution (GIBCO/SRL) under 5% Co_2 with 85% relative humidity at 37 °C. Cell count and viability was performed using a standard trypan blue dye exclusion method. The cell concentration was adjusted to 1×10^5 cells/ml in each well with the same media in the 12 well plates. After 24 h, the medium in each well was replaced with fresh medium containing (i) Normal control-comprising cell with medium only (ii) control comprising with 10 μg/ml of LPS only. (iii) Test compounds at a dose of 50, 25 and 12.5 μg/ml along with 10 μg/ml of LPS and (iv) Positive control comprising the dexamethasone along with 10 μg/ml of Lippolysaccharide. After stimulation and treatment, volume of 200 μl of media from each well was collected at 6 h for the estimation of TNF-α, after 24 h for IL-1 β and Nitric Oxide respectively (Singh et al., 2005). TNF-alpha and IL1 beta in the collected cell culture supernatants were measured by sandwich ELISA method (R&D Biosystems, USA). Nitric oxide levels were measured by using the Griess reagent (Dirsch et al., 1998). The adherent cells, which remained after removing the media, were subjected to MTT assay for determining the Cytotoxicity of the test compounds at selected concentrations.

2.11. Ulcerogenic assay

The compounds BN-4, BN-14 and BN-16 at dose of 200 mg/kg and indomethacin at dose of 30 mg/kg were administered per oral to the overnight fasted rats (n = 4) as gum acacia suspension. Six hours later, rats were sacrificed and the stomach was removed from each animal and opened along the greater curvature according to the method given (Cashin et al., 1997). The opened stomach was washed with normal saline and observed for ulceration. Lesions on the mucosal surface were scored (Bania et al., 2000) according to arbitrary scale: 0 = No lesion; 0.5 = hyperaemia; 1 = one or two lesions; 2 = severe lesions; 3 = very severe lesions; and 4 = Mucosal full of lesions.

2.12. Statistical analysis

Data are expressed as mean ± S.E.M. The results of all *in vitro* and *in vivo* experiments are expressed as a percentage of inhibition from control (pre-drug) values. Differences between vehicle control and treatment groups were tested using one-way ANOVA followed by Dunnett's multiple comparison as post hoc procedure. Significance was evaluated at P value of <0.05 (probability of 95%).

3. Results

3.1. Effect of thienopyridine derivatives on inflammation induced by carrageenan

All the test compounds starting from BN-1 to BN-19 were evaluated for their anti inflammatory in carrageenan induced rat paw edema model as a primary screening protocol.

As shown in Table 1 the compounds BN-2, BN-4, BN-13, BN-14, BN-15 and BN-16 in the series significantly ($P<0.05$) reduced the paw swelling compared to the control group. The percent inhibition of paw swelling by the compounds BN-4, BN-14 and BN-16 at a dose of 100 mg/kg was found to be comparable ($P>0.05$) with that of the standard drug indomethacin at dose of 10 mg/kg. Dose response study was conducted for active compounds. Results indicated that compounds BN-4, BN-14 and B-16 have shown anti-inflammatory potential in a dose dependent manner and the maximum effect was observed at the dose of 200 mg/kg. The percent inhibition of paw volume obtained for the compounds BN-4, BN-14 and B-16 at dose of 200 mg/kg was found to be 53.2%, 56.45% and 57.53% respectively as shown in Fig. 2.

3.2. Effect of thienopyridine derivatives on dextran induced paw edema model

The compounds BN-4, BN-14 and BN-16 have shown significant potent anti-inflammatory potential in a preliminary study (Table 1). These compounds were selected for dose response study at different doses starting from 50 mg/kg to 200 mg/kg. Oral administration of test compounds (BN-4, BN-14 and BN-16) at the doses of 100 and 200 mg/kg significantly ($P<0.01$) decreased the paw volume compared with that of control group. The test compounds BN-14 and BN-16 have shown significant ($P<0.05$) decrease in paw swelling compared to control even at the dose of 50 mg/kg. The percent inhibition of paw volume by the compounds BN-4, BN-14 and B-16 at dose of 200 mg/kg was found to be 32.71%, 38.54%, 43.75% respectively as shown in Fig. 3.

Table 1
Anti inflammatory effect of thienopyridine derivatives on acute inflammation by carragenan, dextran and arachidonic acid induced paw edema models.

Compound code	R1	R2	n	Inhibition mean ± S.E		
				Carrageenan model	Dextran model	Arachidonic acid model
BN-1	CH_3	CH_3	1	24.67 ± 5.67	4.21 ± 2.19	8.43 ± 4.23
BN-2	CH_3	CH_3	2	20.64 ± 2.05	4.91 ± 0.61	12.85 ± 1.75
BN-3	CH_3	CH_3	2(m-CH3)	23.17 ± 5.15	6.67 ± 0.93	14.86 ± 1.75
BN-4	CH_3	CH_3	3	35.68 ± 3.77$_a$	18.5 ± 2.14	25.64 ± 3.39$_a$
BN-5	$-(CH_2)_4-$		1	4.32 ± 1.19	8.07 ± 3.06	11.65 ± 3.14
BN-6	$-(CH_2)_4-$		2	3.14 ± 1.96	4.91 ± 2.74	9.24 ± 3.14
BN-7	$-(CH_2)_4-$		2(m-CH3)	0.70 ± 0.70	12.98 ± 2.30	5.22 ± 1.61
BN-8	$-(CH_2)_4-$		3	1.20 ± 0.87	11.23 ± 2.30	9.24 ± 2.01
BN-13	$-(CH_2)_3-$		1	12.30 ± 1.95	8.07 ± 1.86	8.03 ± 1.45
BN-14	$-(CH_2)_3-$		2	33.47 ± 6.70$_a$	24.83 ± 2.78	35.90 ± 4.85$_a$
BN-15	$-(CH_2)_3-$		2(m-CH3)	22.13 ± 9.28	9.12 ± 2.18	12.85 ± 3.43
BN-16	$-(CH_2)_3-$		3	44.90 ± 5.86$_a$	30.90 ± 4.32	37.18 ± 3.92$_a$
BN-17	$-(CH_2)_3-$		4	6.87 ± 1.25	6.10 ± 3.39	11.11 ± 1.71
BN-18	$-(CH_2)_5-$		1	17.47 ± 0.27	5.16 ± 1.69	7.26 ± 3.34
BN-19	$-(CH_2)_5-$		2	3.20 ± 0.27	3.16 ± 0.47	12.39 ± 0.43

Symbol a indicates Active molecules of the thienopyridine series.

3.3. Effect of thienopyridine derivatives on arachidonic acid induced paw edema model

The compounds BN-4, BN-14 and BN-16 also showed significant inhibition of paw volume in the arachidonic acid induced Paw edema model. The percent inhibition of paw volume by test compounds at a dose of 100 mg/kg was comparable ($P > 0.05$) with that of the standard drug prednisolone at a dose of 5 mg/kg as shown in the (Table 1). These compounds were also tested for dose response study at different doses starting from 50 mg/kg to 200 mg/kg.

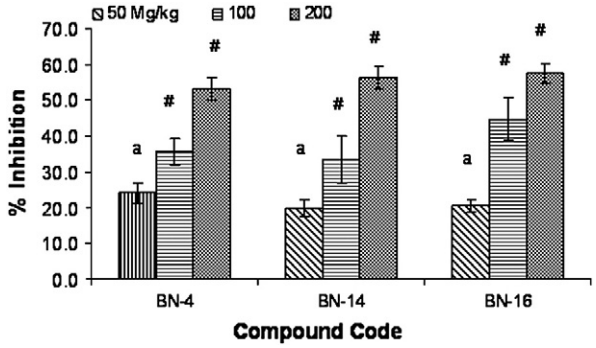

Fig. 2. Anti-inflammatory activity of thienopyridine derivatives by carrageenan induced edema model — dose response study. Values are expressed as Mean ± S.E.M, (n = 6). * represents the no statistical significance ($P > 0.05$) compared to indomethacin at the dose of 10 mg/kg (ANOVA followed by Dunnett's test).

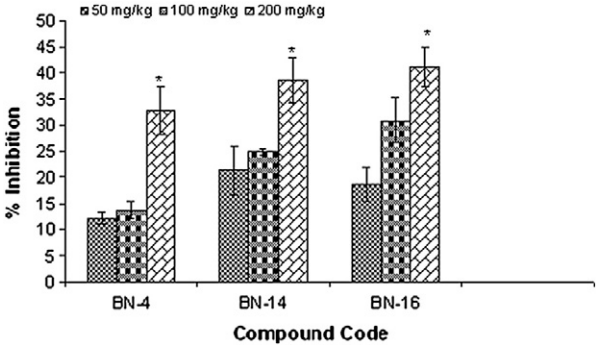

Fig. 3. Anti-inflammatory effect of thienopyridines by dextran induced edema — dose response study. Values are expressed as Mean ± S.E.M, (n = 6).* represents the no statistical significance difference ($P > 0.05$) compared to indomethacin group (ANOVA followed by Dunnett's test).

Results demonstrated that the compounds BN-4, BN-14 and BN-16 significantly ($P < 0.05$) reduced the percentage of swelling in dose dependent manner as shown in Fig. 4. The percentage inhibition of paw volume by the compounds BN-4, BN-14 and B-16 at dose of 200 mg/kg was found to be 44.92%, 41.37%, 45.63% respectively.

3.4. Effect of thienopyridine derivatives on cotton pellet granuloma in rats

The sub acute anti-inflammatory effect of the thienopyridine derivatives was studied employing cotton pellet-induced granuloma formation in rats. Results showed that the compounds BN-4, BN-14 and BN-16 significantly reduced the both wet and dry weights of granuloma formation as shown in Table 2. The percentage of decrease in granuloma tissue formation by the compounds BN-4, BN-14 and BN-16 at a dose of 100 mg/kg was comparable ($P > 0.05$) with that of standard drug indomethacin at dose of 5 mg/kg.

3.5. Acute oral toxicity

The compounds BN-4 and BN-16 have not shown any toxic symptoms and mortality at the doses 5, 50, 500, and 2000 mg/kg in sighting study. Based on sighting study, a dose 2000 mg/kg was selected for the main study in rats and mice for these compounds. They have not shown any toxic symptoms at the dose of 2000 mg/kg in main study. Therefore > 2000 mg/kg was determined as maximum tolerated dose. The compound BN-14 was found to be toxic at the doses 2000 and 1250 mg/kg in rats and 2000 mg/kg in mice. Based on this the

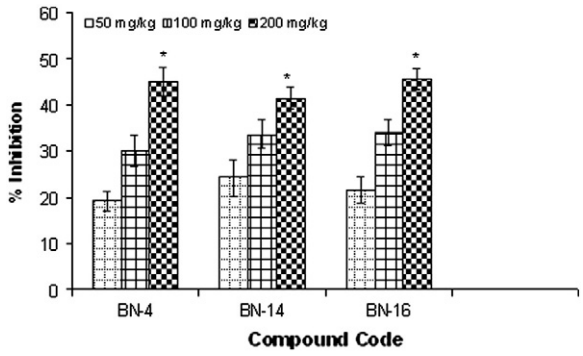

Fig. 4. Anti-inflammatory effect of thienopyridines by arachidonic acid induced edema — dose response study. Values are expressed as mean ± S.E.M, (n = 6). * represents $P > 0.05$ when compared to indomethacin (ANOVA followed by Dunnett's test).

Table 2
Effects of thienopyridine derivatives on sub-acute inflammation by cotton pellet granuloma in rats.

Compound code	Dose (mg/kg)	Wet weight of granuloma (mg)	% inhibition	Dry weight of granuloma (mg)	% inhibition
Control	–	463.12 ± 12.52	–	116.42 ± 2.93	–
BN-4	100	366.08 ± 3.50[a]	22.23 ± 1.20	83.33 ± 3.59[a]	28.42 ± 3.09
BN-14	100	374.42 ± 4.69[a]	20.21 ± 0.58	86.75 ± 2.20[a]	25.48 ± 1.89
BN-16	100	365.08 ± 6.39[a]	23.09 ± 1.59	84.16 ± 2.11[a]	27.70 ± 1.82
Indomethacin	5	338.01 ± 10.38[a]	26.93 ± 2.24	78.25 ± 2.19[a]	32.78 ± 1.88

Values are expressed as mean ± SEM, (n = 6). Symbol a indicates statistical significance (P<0.05) compared to control group.

maximum tolerated doses of BN-14 were found to be 1000 mg/kg and 1250 mg/kg in rats and mice respectively.

3.6. Effect of thienopyridine derivatives on Freund's complete adjuvant induced arthritis

After Freund's complete adjuvant injection on the rat hind paw, there was significant (P<0.05) increase in paw swelling in the control group compared to vehicle alone treated group as shown in Fig. 6.

Test compounds BN-4, BN-14 and BN-16 at a dose of 100 mg/kg, displayed significant inhibition of paw volume on 12th, 18th and 21st (P<0.05) day and activity was comparable (P>0.05) with that of standard drug (indomethacin) treated group at different time intervals.

Arthritic scoring was also inhibited significantly (P<0.05) in test groups compared to arthritic control as shown in Fig. 5. As the incidence and severity of arthritis increased, the changes in the body weight of arthritic rats significantly reduced (7.42%) during the course of the experimental period compared to normal control. Normal group rats gained significant percent increase in body weight (38.32%). All test groups of rats (28.86%, 27.91% and 31.29%) gained significant percent increase (P<0.01) in body weight compared to arthritic control and the increase was comparable with normal control group.

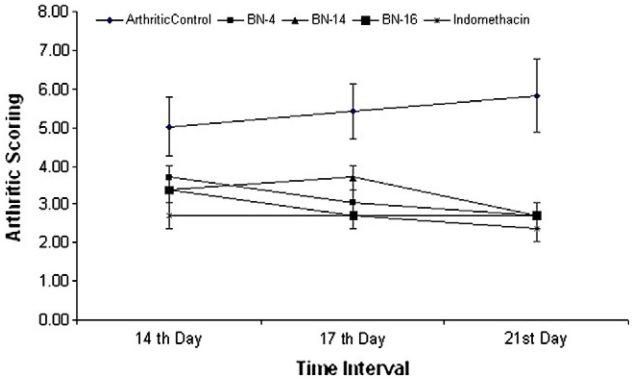

Fig. 5. Effect of thienopyridine derivatives on paw volume in Freund's complete adjuvant induced arthritis model in rats. Graph shows the arthritic scoring at different time intervals of test compounds.

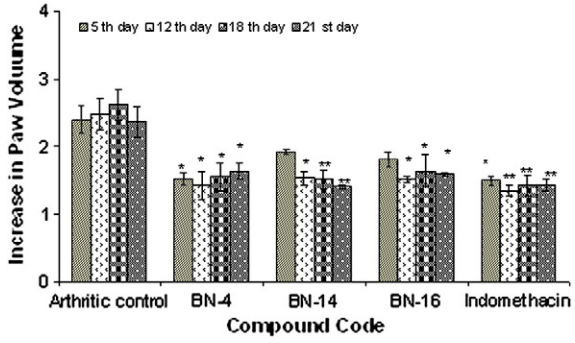

Fig. 6. Effect of thienopyridine derivatives on paw volume in Freund's complete adjuvant induced arthritis model in rats. Symbol * represents P<0.05 compared to arthritic control. ** represents P<0.01 compared to arthritic control. Values are expressed as Mean ± S.E.M, (n = 6).

3.7. Effect of thienopyridine derivatives on release of proinflammatory cytokines from macrophage cells

The test compounds BN-4, BN-14, and BN-16 significantly (P<0.05 and P<0.01) inhibited the release of pro-inflammatory mediators such as TNF-α, IL-1 β and Nitric Oxide release in macrophage cell line when treated with different concentrations (12.5 μg/ml, 25 μg/ml and 50 μg/ml) compared to LPS control (Figs. 7–9).

3.8. Effects of thienopyridine derivatives on gastric irritation in rats

Oral administration of test compounds at the dose of 200 mg/kg did not produce ulceration in all the rats as shown in Table 3. Animals in the standard (Indomethacin) showed significant ulceration in the stomach. Ulcer scoring was significantly (P<0.05) less in test groups (BN-4, BN14 and BN-16) of rats compared to those in indomethacin group.

4. Discussion

Present study was aimed at evaluation of thienopyridines as potential general antiinflammatory agents by following standard *in vitro* and *in vivo* protocols related to inflammation. Edema induced by carrageenan is mainly due to the activation of kinins and complement cascades which results in the release of histamines 5-hydroxytryptamine and prostaglandins. The release of these chemical substances results in increase in vascular permeability, accumulation of neutrophils and macrophages. Carrageenan induced inflammation is believed to be biphasic: the first phase involves the release of serotonin and histamine and the second phase is mediated mainly by prostaglandins and cyclooxygenase products (Vinegar et

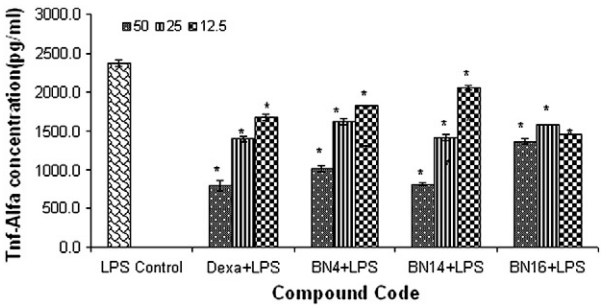

Fig. 7. Effect of thienopyridines on the production of Tnf-α from murine macrophage cell line. Values are expressed as mean ± S.E.M. * represents significant statistical difference (P<0.01) compared to LPS control (ANOVA followed by Dunnett's test).

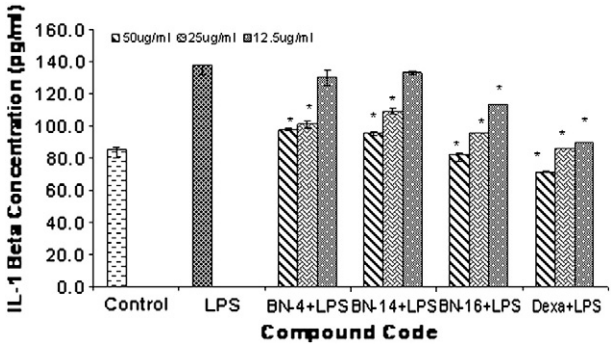

Fig. 8. Effect of thienopyridines on production of IL-1β from murine macrophage cell line. Values are expressed as mean ± S.E.M.* represents significant statistical difference ($P<0.01$) compared to LPS control.

al., 1969). In fact, paw edema was significantly inhibited by the compounds BN4, BN-14 and BN16 following 3 h of carrageenan administration. Edema developed at 3 h of carrageenan administration is mainly due to prostaglandins, indicating that the compounds BN-4, BN-14 and BN-16 might be inhibiting the prostaglandins and cyclooxygenase enzymes which are the important mediators for inflammation. We have also studied the effect of thienopyridine compounds on arachidonic acid and dextran induced paw edema models at a dose of 100 mg/kg. The paw edema induced by arachidonic acid is a widely used to distinguish between 5-lipooxygenase and cyclooxygenase inhibitors. The paw edema induced by dextran is mainly due to the release of histamines and serotonins from the mast cells (Chawla et al., 1987). Of all the compounds (BN-1 to BN-19) tested BN-4, BN-14 and BN-16 have shown significant inhibition of paw edema in the three acute antiinflammatory models of inflammation. Compounds BN-4, BN-14 and BN-16 have shown higher inhibition of paw edema in carrageenan and arachidonic acid induced paw edema models compared to dextran induced paw edema model. These results indicate that BN-4, BN-14 and BN-16 might be preferentially inhibiting of prostaglandin synthesis (Carrageenan induced paw edema) and 5-Lipoxygenase enzymes (Arachidonic acid induced paw edema) and eliciting the anti-inflammatory response. In the second part of our study, the effect of thienopyridine derivatives on sub acute phase of inflammation was investigated by cotton pellet granuloma test. Cotton pellet granuloma method has been widely used to assess the transudative, exudative and proliferative component of chronic inflammation. Inflammation produced in this method is due to the proliferation of macrophages, neutrophils, fibroblasts and synthesis of collagen and mucopolysaccharides which leads to the formation of granuloma tissue (Ionac et al., 1996). In our study, the decrease in the weight of granuloma tissue formation indicated

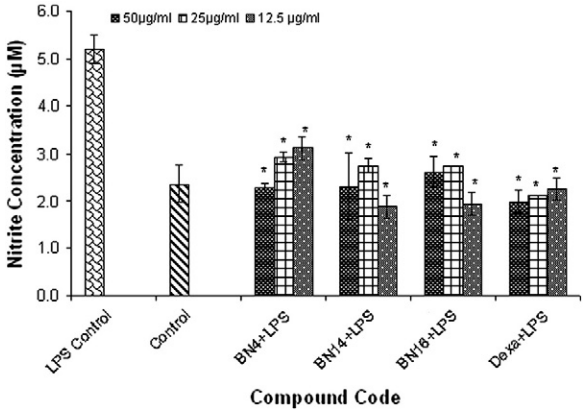

Fig. 9. Effect of Thienopyridine derivatives on the release of Nitric Oxide from macrophage cell line (Raw 264.7). Values are expressed as Mean ± S.E.M. * represents statistically significant difference ($P<0.01$) compared to LPS control (ANOVA followed by Dunnett's test).

Table 3
Effect of thienopyridine compounds on ulcerogenic activity.

Compound code	Dose (mg/kg)	Ulcer incidence	Ulcer score
Control	–	0/4	0.00 ± 0.00
BN-4	200	1/4	0.25 ± 0.12
BN-14	200	1/4	0.37 ± 0.10
BN −16	200	0/4	0.25 ± 0.12
Indomethacin	30	4/4	2.25 ± 0.41

Lesions on the mucosal surface were scored according to an arbitrary scale: 0 = no lesions; 0.5 = hyperemia; 1 = one or two lesions; 2 = severe lesions; 3 = very severe lesions; 4 = mucosa full of lesions.

that the proliferative phase was effectively suppressed by the compounds BN-4, BN-14 and BN-16. The test compounds were evaluated for toxicity evaluation by employing OECD-420 guideline which revealed that BN-4 and BN-16 were found to be safe even at the dose of 2000 mg/kg in rats and mice. The maximum tolerated doses of BN-14 were found to be 1000 mg/kg and 1250 mg/kg in rats and mice respectively.

Freund's complete adjuvant induced arthritis has been used as chronic model for inflammation. One of the reasons for the wide utilization this model is due to strong correlation between efficacy of therapeutic agents in this model and rheumatoid arthritis in human. Our results revealed pronounced and reproducible anti-arthritic activity of BN-4, BN-14 and BN-16 at the dose of 100 mg/kg. Among these compounds, BN-16 at the dose of 100 mg/kg significantly ($P<0.05$) inhibited the development phase of chronic joint swelling induced by Freund's complete adjuvant on both the paws. To evaluate the effect of compounds on the development of arthritis, body weight was considered as an indirect index of health status and recovery from the disease. In our study, there was a significant reduction in body weight of rats in arthritic control group compared to vehicle treated group. Significant restoration and gain in body weight were observed in the rats treated with BN-4, BN-14, BN-16 and indomethacin.

TNF-α and Interleukin-1 β are important primary mediators, which plays a critical role in both acute and chronic inflammation (Holtmann et al., 2002). The formation of a number of small molecular mediators of inflammation is linked with TNF-α and contributes to the inflammation. TNF-α facilitates inflammatory cell infiltration by promoting the adhesion of neutrophils and lymphocytes to endothelial cells (Dore and Sirois, 1996). Nitric oxide is a highly soluble free radical, having numerous promiscuous roles. Nitric oxide synthesis is greatly amplified during inflammation. Several studies have demonstrated that inflammation correlates with the level of Nitric oxide (Miller and Grisham, 1995). To determine the possible mechanism of action, the test compounds were screened for *in vitro* antiinflammatory activity by LPS challenged macrophage cell culture assay. After treatment with test compound along with LPS, biochemical parameters such as TNF-α and IL-1 β and Nitric Oxide levels were measured at specific time intervals. The test compounds significantly (BN-4, BN-14 and BN-16) inhibited the release of primary inflammatory mediators TNF-α and IL-1 β and secondary mediator (NO) from macrophage cell line. These *in vitro* study results revealed the anti-inflammatory activity of thienopyridine analogs.

Since the test compounds were found to be potential anti-inflammatory agents, there is a possibility of side effects such as gastric ulceration, toxic manifestations typical of nonsteroidal anti-inflammatory drugs. Hence ulcerogenic potential of BN-4, BN-14 and BN-16 was evaluated in rats. Results indicated that the oral administration of these compounds did not produce ulcers in stomach compared to indomethacin group. The low toxic effect by the compounds BN-4, BN-14 and BN-16 might be due to preferential inhibition of 5-Lipoxygenase in arachidonic acid induced inflammation in *in vivo* studies. Structural relationship studies revealed that compound BN-16 with cyclopentane ring attached to thiophene and cycloheptane ring

attached to pyridine ring was found to be highly potent among all the molecules in the series. The cyclopentane-fused thiophenes on the whole, were found to be more active as anti-inflammatory agents. The order of potency of these derivatives was increasing with the increase in the size of the ring attached to pyridine up to cycloheptane. Cyclohexanone fused thiophene derivatives were found to be less potent as anti-inflammatory agents. Thus thienopyridine derivatives were found to possess potent anti-inflammatory activity which may find use for the development of new anti-inflammatory agents.

5. Conclusion

In conclusion, the results of this study showed that new thienopyridine analogs BN-4, BN-14 and BN-16 exhibited potent anti-inflammatory activity against acute and chronic phases of inflammation. Potent anti-inflammatory activity and less gastric toxicity may provide advantage for these analogs for further future development. Further studies in detail are needed to verify the mechanism of anti-inflammatory activity of these derivatives.

Acknowledgment

Authors wish to thank Dr J.S. Yadav, Director IICT for providing necessary facilities and encouragement. We also thank the Principal, Kakatiya University, Warangal for providing test compounds.

References

Bakhite, E.A., Abdel-Rahman, A.E., Mohamed, O.S., Thabet, E.A., 2002. Synthesis and reactions of New thienopyridines, pyridothienopyrimidines and pyridothienotriazines. Bull. Korean Chem. Soc. 23, 1709–1714.

Bania, S., Kaul, A.K., Jaggia, B.S., Surib, K.A., Surib, O.P., 2000. Anti-inflammatory activity of hydro soluble fraction of Euphorbia royleaana latex. Fitoterapia 71, 655–662.

Boneu, B., Destelle, G., 1996. Platelet anti-aggregating activity and tolerance of clopidogrel in atherosclerotic patients. Thromb. Haemost. 76, 939–943.

Cashin, C.H., Dawson, W., Kitchen, E.A., 1997. The pharmacology of benoxaprofen (2-[4- chlorophenyl]-alpha-methyl-5-benzoxazole acetic acid), LRCL 3794, a new compound with antiinflammatory activity apparently unrelated to inhibition of prostaglandin synthesis. J. Pharm. Pharmacol. 1977, 330–336.

Chawla, A.S., Singh, M., Murthy, M.S., Gupta, M.P., Singh, H., 1987. Anti-inflammatory action of Ferulic acid and its esters in carrageean induced paw oedema model. Indian J. Exp. Biol. 25, 187–189.

DiMatrino, M.J., Campbel Jr., G.K., Wolff, C.E., Hanna, N., 1987. The pharmacology of arachidonic acid-induced rat paw edema. Agents Actions 2, 303–305.

Dirsch, V.M., Stuppner, H., Vollmarl, A.M., 1998. The griess assay: suitable for a bio-guided fractionation of anti-inflammatory plant extracts? Planta Med. 64, 423–426.

Dore, M., Sirois, J., 1996. Regulation of P-selectin expression by inflammatory mediators in canine jugular endothelial cells. Vet. Pathol. 33, 662–671.

Harris, B.D., Nan, Z., Carolina, B.S.A., Haris, D., Biqi, W., 2004. Thieno [2, 3-*b*] pyridine-6-carbonitriles and thieno [2, 3-b] pyridine-5-carbonitriles as protein kinase inhibitors. PCT 2004/ 048386 A2.

Haung, W., Li, J., Tang, J., Liu, H., Shen, Jiang, H., 2005. Microwave-assisted synthesis of 2-amino-thiophene-3-carboxilic derivatives under solvent-free conditions. Synth. Commun. 35, 1351–1357.

Holtmann, M.H., Schuchmann, M., Zeller, G., Galle, P.R., Neurath, M.E., 2002. Arch. Immunol. Ther. Exp. (Warsz.) 50, 279–288.

Ikuo, K., Kijino, A., Imai, A., Yasumura, M., (Hodogaya Chemical Co., Ltd., Japan), JKXXAFJP 04134083 (1992). (As shown in the Japanese reference style).

Ionac, M., Parnham, M.J., Plauchithiu, M., Brune, K., 1996. Oxaceprol, a typical inhibitor of inflammation and joint damage. Pharmacol. Res 33, 367–373.

Miller, M.J.S., Grisham, M.B., 1995. Nitric Oxide as a mediator of inflammation?— You had better believe it. Mediators Inflamm. 4 (6), 387–396.

Murkherjee, P.K., Saha, K., Das, J., Pal, M., Saha, B.P., 1997. Studies on the anti-inflammatory activity of rhizomes of Nelumbo nucifera. Planta Med. 63, 367–369.

Newbould, B.B., 1963. Chemotherapy of arthritis induced in rats by mycobacterial adjuvant. Br. J. Pharmacol. Chemother. 21, 127–136.

Seguraa, L., Villa, R., Guptab, M.P., Esposito- Aveellac, M., Adzeta, T., Caniguerala, S., 1998. Antiinflammatory activity of Anthurium cerrocampanense Croat in rats and mice. J. Ethanopharmacol. 61, 243–248.

Shireesha, B., Raghuram Rao, A., Narsaiah, B., Ramakrishna, S., Madhusudhana, Yakaiah, T., 2010. Thienopyridines as Biologically Active Molecules. PCT/IN2010/000005.

Singh, U., Tabibian, J., Venugopal, S.K., Devaraj, S., Jialal, I., 2005. Development of an in vitro screening assay to test the anti-inflammatory properties of dietary supplements and pharmacologic agents. Clin. Chem. 51, 2252–2256.

Swingle, K.F., Shideman, F.E., 1972. Phases of the inflammatory response to subcutaneous implantation of cotton pellet and their modification by certain anti-inflammatory agents. J. Pharmacol. Exp. Ther. 183, 226–234.

Trentham, D.E., Townes, A.S., Kang, A.H., 1977. Autoimmunity to type II collagen an experimental model of arthritis. J. Exp. Med. 146, 857–868.

Vane, J.R., Bolting, R.M., 1995. New insights into the mode of action of anti- inflammatory drugs. Inflamm. Res. 11, 1–10.

Vasuma, T., Baba, A., Makino, H., Aoki, I., Nagata, T., 2010. Thienopyridine Derivatives and Their use as Anti-Inflammatory Agents. 2001; WO2001/064685.

Vinegar, R., Schreiber, W., Hugo, R., 1969. Biphasic development of carrageenin edema in rats. J. Pharmacol. Exp. Ther. 166, 96–103.

Winter, E.A., Risley, E.A., Nuss, G.W., 1963. Carrageenan induced paw edema in hind paw as an assay for anti-inflammatory drugs. J. Pharmacol. Exp. Ther. 141, 369–373.

Yamaguchi, M., Maruyama, N., Koga, T., Kamei, K., Akima, M., Kuroki, T., Hamana, M., Ohi, N., 1995. Novel antiasthmatic agents with dual activities of thromboxane A_2 synthetase inhibition and bronchodilation. Chem. Pharm. Bull.(Tokyo) 43, 236–240.