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Exploring the anti-inflammatory activity of a novel 2-phenylquinazoline analog with protection against inflammatory injury

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ABSTRACT

Inflammation is a protective immune response against harmful stimuli whose long time continuation results in host disease. Quinazolinones are nitrogen containing heterocyclic compounds with wide spectrum of biological activities. The anticancer effect of a 3-(arylideneamino)-phenylquinazoline-4(3H)-one derivative was reported earlier. The anti-inflammatory effect of these quinazolinone derivatives has now been examined in endotoxin stimulated macrophages and in different in vivo models of inflammation by measuring the proinflammatory cytokines (TNF-α, IL-1β and IL-6), mediators NO and NF-κB (by ELISA and western blot), and translocation of the nuclear factor kB (by immunocytochemical analysis). To elucidate the in vivo effect, mice endotoxin model was and the various levels of edema, inflammatory pain and vascular permeability were studied. One of the quinazolinone derivatives showed significant anti-inflammatory activity in stimulated macrophage cells by inhibiting the expression of TNF-α, IL-1β, IL-6, iNOS, COX-2, p-IκB and NF-κBp65. Significant ($P<0.01$) improvement was observed in the mortality of endotoxemic mice. The carrageenan and formalin-induced paw edema thicknesses were found to be reduced significantly ($P<0.01$) along with the reduction of pain, vascular permeability and edema induced by complete Freund's adjuvant ($P<0.01$). These findings indicate that 3-(arylideneamino)-phenylquinazoline-4(3H)-one derivative as a potential anti-inflammatory agent.

Introduction

Inflammation is an adaptive response triggered by noxious stimuli and conditions such as infection and tissue injury (Ahmed, 2011; Medzhitov, 2008). In inflamed condition, various inflammatory mediators such as bioactive lipids, reactive oxygen and nitrogen species, cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6 are released (Fujiwara and Kobayashi, 2005). Overproduction of these inflammatory mediators has been implicated in the pathophysiology of many inflammatory diseases including rheumatoid arthritis, atherosclerosis, chronic hepatitis, pulmonary fibrosis and others (Mörmann et al., 2008; Szekanecz and Koch, 2007). The macrophages play an important role in releasing proinflammatory mediators, which are regulated by several transcription factors. One of the most ubiquitous transcription factors is nuclear factor κB (NF-κB) that regulates the expression of many genes involved in the immune and inflammatory responses (Kopp and Ghosh, 1995). It is a heterodimer of p50 and p65 of the Rel family, normally present in the cytosol as an inactive complex due to its binding with an inhibitory protein known as inhibitor κB or IκB (Baeuerle, 1998). Therefore, a NF-κB inhibitor may be useful in the development of therapeutic agents to control inflammation associated diseases.

It is well known that septic shock resulting from Gram-negative bacterial infections causes death of many patients (Parillo, 1993). The major reasons for these shocks are endotoxins or lipopolysaccharides (LPS) of the Gram-negative bacteria cell wall, which induce disturbance of immune and inflammatory responses resulting in tissue damage, septic shock or even death (Mayeux, 1997; Silva et al., 2004). LPS is recognized by toll-like receptor 4 (TLR4) on cell membrane of macrophages and regulates the acute and early release of proinflammatory mediators through TLR4-NF-κB signaling pathways (Miller et al., 2005). Quinazolinones are bicyclic compounds consisting of a pyrimidine system fused at 5,6 with a benzene ring and possess a broad spectrum of biological and pharmacological activities such as antibacterial and antifungal (Alagarsamy et al., 2006), antiviral and antihypertensive (Pandey et al., 2004), anticonvulsant (Kornet, 1992), anti-inflammatory (Farghaly et al., 1990), and CNS depressant (Jatav et al., 2008). Due to the biological significance of the quinazolinone nucleus, several groups of researchers are engaged in the design and synthesis of new quinazolinone derivatives and screening them for bioactivities. The antimicrobial activity

Abbreviations: CFA, complete Freund's adjuvant; ELISA, Enzyme-linked immunosorbent assay; I-κB, inhibitor of kappa B; IL, Interleukin; LPS, lipopolysaccharide; NF-κB, Nuclear factor-κB; NO, Nitric oxide; PBS, Phosphate buffer saline; PGE2, Prostaglandin-E2; RAW 264.7, murine macrophage cell line; TLR4, toll-like receptor 4; TNF, Tumor necrosis factor.

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of 2-phenylquinazoline derivatives has been reported (Nanda et al., 2007). As infection always leads to inflammation, it can be expected that these compounds may have anti-inflammatory or immunomodulating potency to sustain the defense mechanism. Recently we have reported that 2-phenylquinazoline analogs are potent anticancer agents (Das et al., 2012). We therefore decided to evaluate the anti-inflammatory efficacy of these 2-phenylquinazoline analogs, using RAW 264.7 murine macrophage cells and a murine endotoxic shock model in LPS-induced production of early inflammatory cytokine. These 2-phenylquinazoline analogs were also tested in the acute models of inflammation, i.e. carrageenan and formalin induced inflammation with inflammatory pain responses. The findings are detailed below.

Materials and methods

Chemicals. Anthranilic acid, acetic acid, formalin solution, *Escherichia coli* lipopolysaccharide (LPS, 0111:B4), carrageenan, complete Freund's adjuvant (CFA), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), Evans blue and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, Mo, USA). Pyridine, anisaldehyde, salicylaldehyde, and p-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) system were obtained from SRL, India. Benzoyl chloride, sodium bicarbonate, hydrazine hydrate, 4-nitrobenzaldehyde, and vanillin were from Merck, India. RAW 264.7 cell (murine macrophage) was obtained from the American type Culture Collection (ATCC). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin–neomycin (PSN), trypsin, and ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco BRL (Grand Island, NY, USA). Tissue culture plasticwares were purchased from NUNC (Roskilde, Denmark). Bradford protein assay reagent was purchased from Fermentus (European Union) and DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) from Invitrogen, CA. Rabbit anti-TNF-α, IL-1β, IL-6, NF-kB/p65, p-IkB, COX-2, and iNOS polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Nuclear extraction kit was purchased from Cayman (Michigan, USA) and assay kits for TNF-α, IL-1β and IL-6 were purchased from GE Healthcare Bio-Sciences (NJ, USA).

Preparation of 3-(arylideneamino)-2-phenylquinazolin-4(3H)-ones. 3-(Arylideneamino)-2-phenylquinazolin-4(3H)-one derivatives were synthesized as described earlier (Das et al., 2012). Briefly, anthranilic acid was treated with benzoyl chloride in the presence of pyridine to form 2-phenyl-4H-benzo[d][1,3]oxazin-4-one, which on condensation with hydrazine hydrate yielded 3-amino-2-phenylquinazolin-4(3H)-one. This was then treated with different substituted benzaldehydes like nitrobenzaldehyde, vanillin, anisaldehyde, and salicylaldehyde separately in ethanol to form the corresponding 3-(arylideneamino)-2-phenylquinazolin-4(3H)-ones, which were named as P(3a), P(3b), P(3c), and P(3d) respectively. The substituents present in the phenyl ring are shown in Table 1 and the synthetic route is summarized in Scheme 1.

Animals. All experiments were performed in accordance with the guidelines for the Care and Use of Laboratory animals. BALB/C adult mice, female, weighing approximately 18 to 22 g, were obtained from the animal house of Indian Institute of Chemical Biology. The mice were housed in micro isolator cages at laboratory temperature of 24 ± 1 °C with 40–80% relative humidity, and received food and water ad libitum. The animals were allowed to adapt to the experimental environment for 5 to 7 days before experimentation. Care and maintenance of animals were done in adherence to the guidelines of the Institutional Animal Care and Use Committee.

Cell culture. RAW 264.7 murine macrophage cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate at 37 °C under a humidified atmosphere of 5% CO_2.

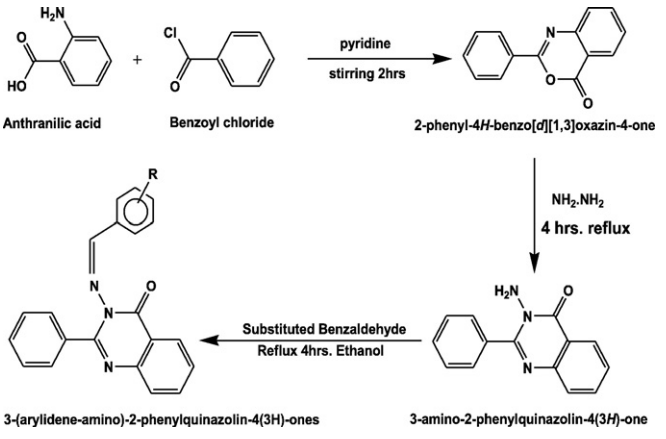
Table 1
Chemical nature of 3-(arylideneamino)-2-phenylquinazoline-4(3H)-one derivatives.

Compound(s)	R group	Molecular formula
3-{[(3-Nitrophenyl)methylene]imino}-2-phenylquinazolin-4(3H)-one P(3a)	3″-NO_2	$C_{21}H_{14}N_4O_3$
3-{[(3-Hydroxyphenyl)methylene]imino}-2-phenylquinazolin-4(3H)-one P(3b)	2″-OH	$C_{21}H_{15}N_3O_2$
3-{[(4-Methoxyphenyl)methylene]imino}-2-phenylquinazolin-4(3H)-one P(3c)	4″-OCH_3	$C_{22}H_{17}N_3O_2$
3-{[(4-Hydroxy-3-methoxyphenyl)methylene]imino}-2-phenylquinazolin-4(3H)-one P(3d)	3″-OCH_3 4″-OH	$C_{22}H_{17}N_3O_3$

Determination of PGE2 levels. To investigate the effect of 2-phenylquinazoline analogs on PGE2 levels in LPS stimulated cells, RAW 264.7 cells (1×10^6 cells/well) seeded on 24-well plates. Derivatives were dissolved in 0.05% of DMSO. Cells were treated in the presence or absence of 10, 20, 40 and 80 μM of P(3a), P(3b), P(3c) and P(3d) for 1 h, and then stimulated with LPS (1 μg/mL) for 24 h at 37 °C in a 5% CO_2 incubator. The concentrations of PGE2 in the supernatants of RAW 264.7 cell cultures were determined using an EIA kit (R&D Systems, Minneapolis, MN, USA). Concentration values were determined for two wells of each sample and the experiments were performed in triplicate.

Measurement of cell viability. Cell viability was evaluated using the MTT assay. RAW 264.7 cells (4×10^5 cells/well) were seeded in each well of 96-well plates for 4 h. After 24 h incubation with various concentrations (0 to 80 μM) of the test compounds in the presence or absence of LPS (1 μg/mL), the MTT solution (5 mg/mL in PBS) was added at the rate of 10 μL/well. The plate was incubated for additional 4 h at 37 °C and the formazan crystals produced were dissolved in 100 μL of DMSO. Absorption was measured by an ELISA reader at 595 nm (Mosmann, 1983).

NO assay. RAW 264.7 cells (1×10^6 cells/well) were plated onto 24-well plates, pretreated with the indicated concentrations of P(3a) for 1 h, and subjected to stimulation with of LPS (1 μg/mL) for 4, 12 and 24 h. The sample supernatants were mixed with equal volumes of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) and then incubated at room temperature for 10 min. The absorbance was measured at 540 nm on a microplate reader. Nitrite concentration was determined using a dilution of sodium nitrite as a standard. Concentration values were determined for two wells of each sample and the experiment was performed in triplicate.



Scheme 1. Synthetic route of quinazolinone derivative.

Cytokine assays in vitro. To determine the effect of P(3a) on the production of cytokines from LPS-stimulated cells, RAW 264.7 cells were plated onto 24-well plates (1×10^6 cells/well), pretreated in the presence or absence of 10, 20 and 40 μM of P(3a) for 1 h, and then stimulated with LPS (1 μg/mL) for 4, 12 and 24 h at 37 °C in a 5% CO_2 incubator. At each time point, cell-free supernatants were collected and the concentrations of cytokines TNF-α, IL-1ß and IL-6 were measured by sandwich ELISA, using commercially available assay kit from GE Healthcare Bio-Sciences (NJ, USA) according to the manufacturer's instructions.

Extraction of nuclear proteins and assay of NF-kB p65. RAW 264.7 cells were plated onto 24-well plates (1×10^6 cells/well), pretreated in the presence or absence of 10, 20 and 40 μM of P(3a) for 1 h, and stimulated with LPS (1 μg/mL) for 12 h. After centrifugation, it was resuspended in 400 μL of ice cold hypotonic buffer for 10 min, vortexed, and centrifuged at 15,000 *g* at 4 °C to get the supernatant containing nuclear protein (Majumdar et al., 2008). Aliquots of this were added to incubation wells precoated with the NF-kB p65 DNA-binding consensus sequence, and the translocated p65 subunits present in the nuclear lysate were assayed according to the recommendations of the manufacturer of the NF-kB assay kit (Cayman, Michigan, USA).

Western blot analysis. The cell lysates were separated by 10–15% SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA) using standard electroblotting procedures. Blots were then blocked and immunolabeled with primary antibodies overnight at 4 °C. NBT-BCIP and alkaline phosphatase conjugated secondary antibodies were used as chromogenic substrates (Towbin et al., 1979).

Immunofluorescence microscopy. The effect of P(3a) on LPS induced nuclear translocation of NF-κB was measured by immunocytochemical analysis. Raw 264.7 cells cultured on chambered plastic slides were fixed with ethanol for 30 min at 4 °C and the detergent was extracted with 3% Triton X-100 for 10 min at room temperature. After washing with PBS and blocking with 3% bovine serum albumin (BSA) for 30 min, samples were incubated overnight with a primary antibody at 4 °C. Excess primary antibody was removed by washing with PBS and samples were incubated with FITC-conjugated secondary antibody for 2 h at room temperature. After washing with PBS, slides were mounted using DAPI to visualize the nuclei. Specimens were covered with coverslips and evaluated under a confocal laser scanning microscope (Nikon A1R).

Acute toxicity study. Acute toxicity studies were carried out in female BALB/c mice (n = 20). P(3a) was solubilized in 0.2% Tween 80, suspended in phosphate buffer saline, and administered intraperitoneally (ip) in 1–200 mg/kg doses. The animals were observed for signs and symptoms of toxicity and the number of mortality was recorded from 24 h to 15 days following treatment. The LD_{50} was determined according to the method of Litchfield and Wilcoxon (Irwin, 1962; Litchfield and Wilcoxon, 1949).

Survival study. BALB/c mice were divided into six groups (n = 10). The first group (control group) received vehicle only, the second group received only P(3a), the third group received LPS at the lethal dose of 32 mg/kg ip, and the remaining three groups received P(3a) at doses of 5, 10 and 15 mg/kg ip 2 h prior to LPS administration. Mice were monitored for survival twice in a day throughout 7 days.

Analysis of serum cytokines from P(3a) treated BALB/c mice. For the analysis of serum cytokines, mice were randomly assigned into six groups (n = 10). Mice in the control group received the vehicle, the second group received only P(3a), the third group received LPS at 32 mg/kg ip, and the animals of the remaining three groups received P(3a) at doses of 5, 10 and 15 mg/kg ip 2 h prior to LPS administration. The serums were collected from the animals at 1st, 4th and 6th hour, and cytokines TNF-α, IL-1β and IL-6 were measured by sandwich ELISA using commercially available reagents according to the manufacturer's instructions.

Liver histopathology. Six hours after LPS injection, livers were isolated, pre-treated without or with P(3a) at the dose of 10 mg/kg ip, and fixed in 10% formalin for 24 h. Formalin-fixed livers were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). To estimate the extent of damage, the specimens were observed under a light microscope. This histological alteration was quantitatively analyzed as an index of the severity of tissue injury. The index was determined by counting the numbers of polymorphonuclear neutrophils (PMN) in 10 randomly selected high-power fields and by noting the histological changes.

Liver function analysis. After 6 h of treatment with LPS and P(3a) at dose 10 mg/kg ip, blood was collected by retrorbital puncture and the serum sample was analyzed for glutamate-pyruvate transaminase (GPT, a specific marker for hepatic parenchymal injury) and glutamateoxalacetate transaminase (GOT, a nonspecific marker for hepatic parenchymal injury) using the kits of Biovision (California) as per the manufacturers' protocol.

Carrageenan-induced paw edema. Animals were divided into four groups (n = 10). In all groups, inflammation was induced by single sub-plantar injection of 0.02 mL of freshly prepared 1% carrageenan in normal saline (Chattopadhyay et al., 2002). The group treated with carrageenan alone served as control. Three groups received P(3a) at doses of 5, 10 and 15 mg/kg ip, 30 min before the carrageenan injection. The paw thickness was measured using vernier calipers and the difference between paw volumes was calculated every hour after carrageenan injection.

Formalin-induced paw edema. The experiment was the same as described before for carrageenan induced paw edema except that a single dose of 0.02 mL of formalin (2%) was used as the inflammation inducer (Ajith and Janardhanan, 2001).

Induction of arthritis. Complete Freund's adjuvant (CFA) was introduced into the subplantar tissue of the right posterior paw of mice for the induction of arthritis (Newbold, 1963). In the positive control, subplantar injection of CFA produced a definite edema within a few hours with progressive arthritis up to 14th day of inoculation. Treatments with P(3a) at the doses of 5, 10 and 15 mg/kg ip were initiated from the 2nd day and continued till the 14th day at 3 day intervals. The percent inhibition was calculated as follows:

$$\%\text{Inhibition of paw edema} = [(\text{Control}-\text{Test})/\text{Control}] \times 100.$$

Acetic acid-induced inflammatory response in mice. Acetic acid (0.6%) induced abdominal constriction model was used in five groups of mice (n = 10). The control animals received vehicle, one group received only P(3a), and the rest were treated with P(3a) at 5, 10 and 15 mg/kg ip 15 min before the injection of acetic acid. Pairs of mice were then placed in separate cages, and the numbers of abdominal constrictions were cumulatively counted (Koster et al., 1959).

Vascular permeability in mice. Mice were pretreated with P(3a), 5, 10 or 15 mg/kg ip. After 15 min, 100 μL of a 10% Evans blue solution in saline was intravenously injected along with 100 μL of acetic acid (0.7%) ip. The peritoneal fluid was collected 30 min after acetic acid injection in a sterile PBS (1 mL) solution, and the total collection was evaluated by optical density assay at 620 nm (Aurella et al., 1967).

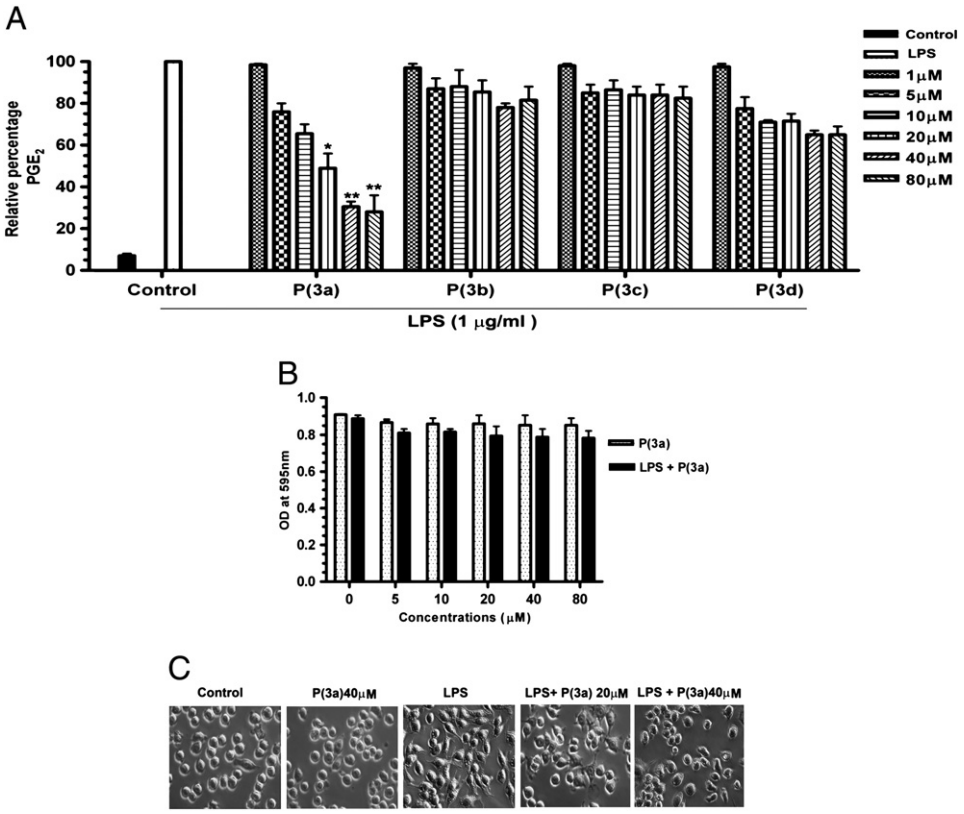


Fig. 1. Effect of quinazolinone derivatives on PGE2 production. A) The cells were incubated with P(3a), P(3b), P(3c), or P(3d) in the presence or absence of LPS for 24 h and the optical density was determined by ELISA method. The data are reported as the mean ± SEM of triplicate experiments. B) The effect of P(3a) on cell viability measured by MTT assay. Cells were incubated with or without LPS (1 μg/mL) for 24 h and OD measured at 595 nm. The data are reported as the mean ± SEM of triplicate experiments. (C) Morphological changes in RAW 264.7 cell visualized by optical microscopy (200×) in the presence and absence of LPS with 20 and 40 μM of P(3a). Mean ± SEM (*P<0.05, **P<0.01).

Statistical analysis. Results were expressed as mean ± SEM. Statistical analyses were performed with one-way analysis of variance (ANOVA) followed by Dunnett's test. P values less than 0.05 (P<0.05) were considered as indicative of significance.

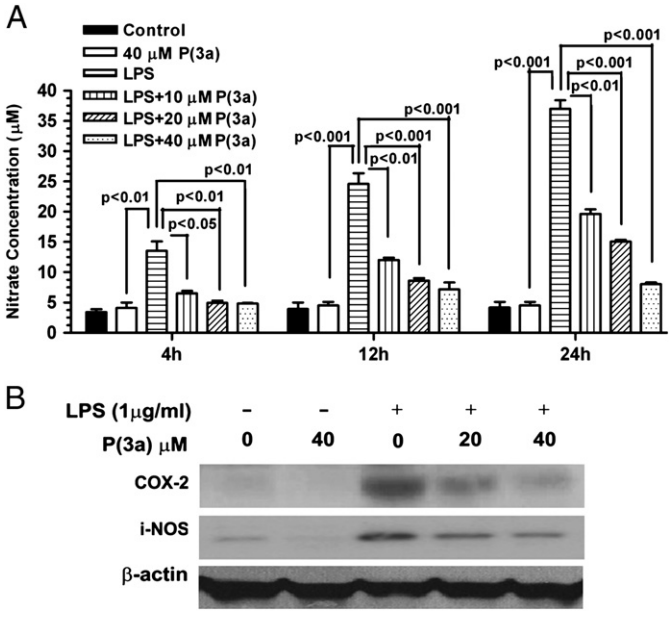


Fig. 2. The effect of P(3a) on LPS induced NO production (A) and iNOS and COX-2 expression (B) in RAW 264.7 cells. Cells were pre-treated with different concentrations (10, 20 and 40 μM) of P(3a) for 1 h, then incubated with LPS (1 μg/mL) for 4, 12 and 24 h for NO production and for 12 h for iNOS and COX-2 expression. The data are reported as mean ± SEM of triplicate experiments.

Results

Effects of P(3a) on LPS-induced PGE2 production, viability, and morphology of RAW 264.7 cells

P(3a) inhibited LPS-induced PGE2 production more significantly than the other derivatives, and in a concentration-dependent manner (Fig. 1A). Treatment with P(3a) at 20 and 40 μM reduced LPS-induced PGE2 production by 55 and 78%, respectively. So, the compound was selected for further study using the concentration range of 0–40 μM. The cytotoxic effects evaluated in RAW 264.7 cells using MTT assay revealed that P(3a) did not affect cell viabilities at the concentrations used (1 to 80 μM) to inhibit PGE2 production (Fig. 1A). Fig. 1C shows the change in the morphology of macrophage cells observed under optical microscope (200×) after 12 h of P(3a) treatment in the presence or absence of LPS (1 μg/mL). The control cells were rounded whereas LPS stimulated RAW 264.7 cells had changed to an irregular form with accelerated spreading and pseudopodia formation. Co-treatment of LPS with P(3a) at 40 μM concentration reduced the level of cell spreading and pseudopodia formation by suppressing cell differentiation.

Effects of P(3a) on LPS induced NO production as well as iNOS and COX-2 expression

P(3a) has been found to inhibit NO production by reducing iNOS protein expression. As shown in Fig. 2A, pretreatment with 10, 20 and 40 μM concentrations of P(3a) markedly inhibited LPS induced NO production after 4, 12 and 24 h incubation. To determine whether the inhibitory effect of P(3a) on proinflammatory mediators (NO and PGE2) was related to the modulation of iNOS and COX-2 expression, western blot analysis was performed. LPS remarkably upregulated iNOS and

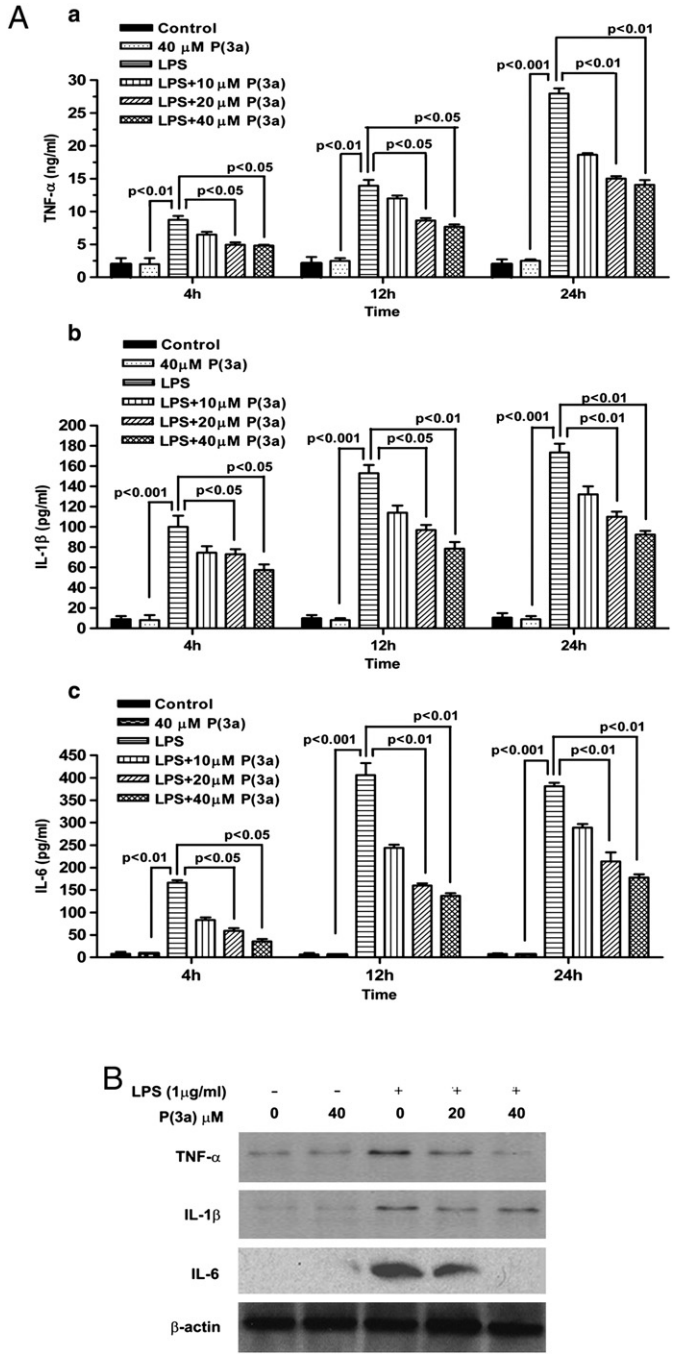


Fig. 3. (A) Effect of different concentrations of P(3a) on production of proinflammatory cytokines TNF-α (a), IL-1β (b), and IL-6 (c) in LPS-induced RAW 264.7 cells. The cells were treated with 1 μg/mL LPS alone or with different concentrations (10, 20 and 40 μM) of P(3a) for 4, 12 and 24 h. The values are means ± SEM of three independent experiments. (B) The proinflammatory cytokine expression level was determined after treatment with P(3a) at concentrations 20 and 40 μM following 12 h incubation in the presence of LPS.

COX-2 protein levels, and pre-treatment with P(3a) inhibited these upregulations (Fig. 2B). These results indicate that the inhibitory effect of P(3a) on iNOS and COX-2 protein expression is one of the mechanisms responsible for the anti-inflammatory action.

Effects of P(3a) on LPS induced TNF-α, IL-1β and IL-6 production in Raw 264.7 cells

LPS stimulated RAW 264.7 cells were treated with 10, 20 and 40 μM of P(3a) and the concentrations of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were measured by sandwich ELISA (Fig. 3A). The cells receiving only LPS showed significant increase in cytokine accumulation ($P<0.01$), whereas the concentrations of proinflammatory cytokines significantly decreased in the cell supernatant co-treated with LPS and P(3a) after incubation for 4, 12 and 24 h ($P<0.05$ or 0.01).

LPS-induced inflammatory stress is known to cause activation of the transcriptional factor NF-κB and of the subsequent proinflammatory mediators including TNF-α, IL-1β, and IL-6. Activation of NF-κB requires the phosphorylation of IκB, which results in targeted degradation of IκB. Therefore we first evaluated the effect of P(3a) on the expression of TNF-α, IL-1β, and IL-6 protein levels by western blot analysis after 12 h incubation with P(3a) and LPS. The expressions of NF-κB fractions including both the subunits p65 (nuclear) and p65 (cytosol) and of phosphorylated Iκβ in total cell protein extracts were then determined. As shown in Fig. 4, exposure to LPS for 1 h led to increased expression of NF-κB p65 subunit and simultaneous elevated expression of proinflammatory cytokines TNF-α, IL-1β, and IL-6, while the expressions of NF-κB p65 subunit and phosphorylated IκB were increased in LPS induced cells. Conversely, P(3a) at 20 and 40 μM concentrations significantly inhibited the expressions of the cytokines and the NF-κB p65 subunit, while phosphorylation and degradation of IκB were suppressed (Fig. 4B). P(3a) inhibited the levels of translocation of NF-κB p65 in a concentration and time dependent manner, as shown in Fig. 4A. Immunocytochemistry studies also provided evidence that the translocation of p65 subunit of NF-κB is inhibited in LPS stimulated with P(3a) (Fig. 4C). In accordance with our findings in Figs. 4A and C, nuclear content of NF-κB p65 was observed to remain low up to 4 h of experiment when cells were treated with P(3a) before LPS treatment in comparison to only LPS treated cells (Supplementary Fig. 1). Besides these, to validate our finding on Raw 264.7 cells, we have also demonstrated the effect of P(3a) on LPS stimulated primary macrophage and it was found to suppress the proinflammatory cytokines including TNF-α, IL-1β, and IL-6 (Supplementary Fig. 2).

Acute toxicity study

Administered intraperitoneally (ip), compound P(3a) was non toxic till 100 mg/kg mice. The experimental mice were observed for the first 24 h and monitored for the next 15 days. However, no toxic symptom or abnormal behavior was observed. Thus, one-tenth of this dose, i.e. 10 mg/kg ip was taken as the experimental dose; the two other selected doses were 5 and 15 mg/kg ip.

Improvement in survival rate of BALB/c mice challenged with lethal dose of LPS and effect of P(3a) on cytokine production

The effect of P(3a) in LPS induced death was assessed by measuring the survival rate of BALB/c mice, as shown in Figs. 5A and B. The induction of endotoxemia with 32 mg/kg ip of LPS resulted in 100% mortality. In contrast, when mice received P(3a) at the doses of 5, 10 and 15 mg/kg ip prior to LPS administration, the survival rates were 15.2, 71.6 and 56.7% respectively. The survival rate significantly improved in mice treated with P(3a) at 10 mg/kg dose compared to those receiving only LPS ($P<0.01$).

The early responses generated by cytokines like TNF-α, IL-1β, and IL-6 play a critical role in moderating the physiological responses in the progression of inflammation. To evaluate the effect of P(3a) on the production of cytokines in mice linked with a fatal outcome, we determined the serum concentration of proinflammatory cytokines at different time intervals. After challenging with LPS, serum concentrations of cytokines in P(3a) treated mice were measured by ELISA method at 0, 1, 4 and 6 h (Figs. 5C, D and E). Higher levels of cytokines were produced in mice serums that are co-treated with LPS and 10 mg/kg of P(3a). Significant difference was observed at 1 h for TNF-α ($P<0.05$) (Fig. 5C), 4 and 6 h for IL-1β ($P<0.05$ or <0.01) (Fig. 5D), and 3 and 6 h for IL-6 ($P<0.05$ or <0.01) (Fig. 5E) in LPS

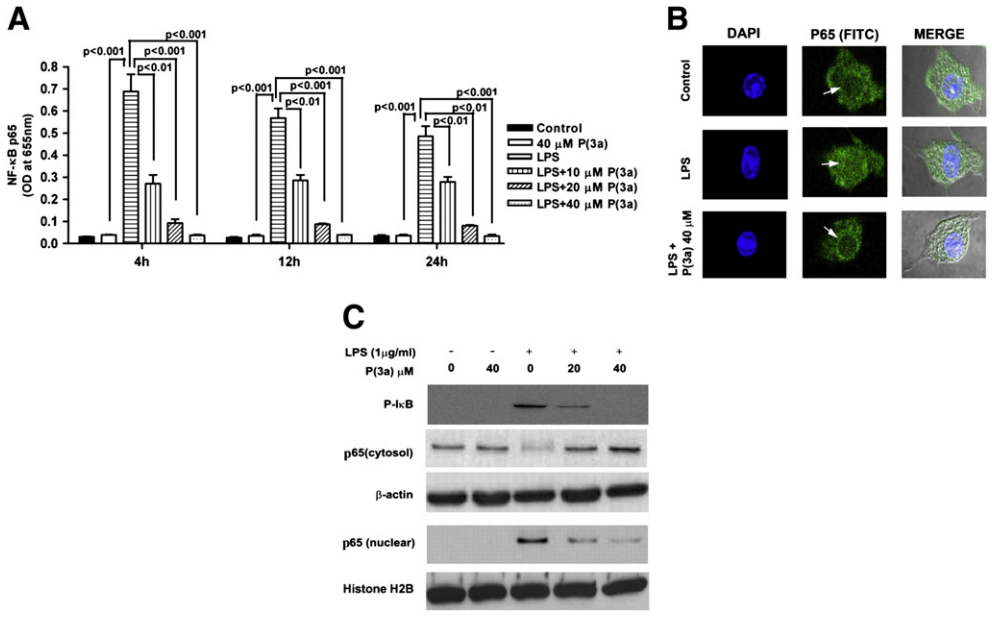


Fig. 4. (A) Effect of P(3a) on levels of translocated NF-kB p65 in RAW 264.7 cells after 4, 12 and 24 h incubation at concentrations 10, 20 and 40 μM in the presence and absence of LPS. (B) Nuclear and cytosolic NF-kB p65 level with p-IkB protein expression level was determined after treatment with P(3a) at concentrations of 20 and 40 μM after 12 h incubation in the presence of LPS. The data are reported as the mean ± SEM of triplicate experiments. (C) Effect of P(3a) for 4 h on LPS-induced NF-κB translocation. Immunofluorescence level measured by confocal laser scanning microscopy (magnification 600×); nucleus is identified with arrows.

treated mice. Further, P(3a) significantly reduced the production TNF-α at 1 h, but those of IL-1β and IL-6 only at 4 and 6 h, respectively. In addition to the concentration of TNF-α, those of IL-1β and IL-6 in serum of P(3a) group are comparable with those of the control.

Liver function (GOT, GPT, ALT and AST)

Baseline values of GOT, GPT, ALT and AST were not significantly different among the different groups (Fig. 5G). LPS caused a significant increase in the plasma levels of GOT, GPT, ALT and AST at the late stage (6 h). The rise plasma level of GOT, GPT, ALT and AST caused by LPS was reduced by pre-treatment of mice with P(3a) at the dose of 10 mg/kg.

Anti-inflammatory and anti-arthritic activity of P(3a) in BALB/c mice

To investigate the beneficial effects of P(3a) on inflammation models, mice were initially treated with P(3a) at the doses of 5, 10 and 15 mg/kg ip and the different experimental groups were subjected to carrageenan and formalin treatment for 5 h. The results showed that P(3a) at 10 mg/kg significantly inhibited paw edema (Figs. 6A and B). The carrageenan and formalin-induced paw thicknesses were reduced compared to control by 56 and 40% ($P<0.01$) respectively (48 and 34%, $P<0.05$, in the 15 mg/kg treated group).

Compared with the control, swellings of inflamed hind limb joints of CFA sensitized mice were reduced in the group treated with P(3a) at the dose of 10 mg/kg at day 14 (Fig. 6D). The administration of CFA caused swelling of hind paw from the 2nd day and this persisted up to the end of 14th day, but treatment with P(3a) at the doses of 10 ($P<0.01$) and 15 mg/kg produced significant reduction in paw volume.

The inflammatory pain and writhing responses were inhibited in mice pretreated with P(3a) at the doses of 5, 10 and 15 mg/kg ip, administered 15 min before chemically-induced inflammatory constrictions with acetic acid (0.6%, 200 μL, ip). The inhibitory effect was significantly higher, at 87.4% ($P<0.01$) with 10 mg/kg and 62.7% ($P<0.01$) with 15 mg/kg, as compared with the control group (Fig. 6E).

Acetic acid-induced pain and carrageenan-induced paw edema are usually related to increased vascular permeability due to the release of cytokines from blood vessels to the injured tissues. To demonstrate if P(3a) can reduce permeability changes during inflammation, we performed vascular permeability assay in BALB/c mice using Evans blue and acetic acid. As shown in Fig. 6F, acetic acid increased the permeation of Evans blue from the vasculature to abdominal cavity, while pretreatment with P(3a) significantly reduced the OD value compared to the acetic acid-treated group (10 mg/kg, 79.3%, $P<0.01$;15 mg/kg, 71.5%, $P<0.01$), suggesting that P(3a) is able to inhibit vascular permeability and reduce inflammatory effusion.

Discussion

Macrophages are primary innate immune cells that play important roles in the host's immune defense during infection, recognize pathogen-associated molecules such as LPS in Gram-negative bacterial infections, and trigger innate immune response through TLR signaling (Morgensen, 2009). In response to the binding of LPS to TLR4 receptor, up-regulation of an intracellular tyrosine kinase system leads to activation of the transcriptional factor NF-κB, which eventually induces the various inflammatory mediators including TNF-α, IL-1β, and IL-6 (Cohen, 2002). Excessive production of proinflammatory mediators may lead to tissue damage and multiple organ failure (MOF), which are considered to be important initiators of inflammatory responses causing various inflammatory diseases (Cannon et al., 1990; Glauser, 1996; Mannel and Echtenacher, 2000; Marks et al., 1990). In the present study using LPS stimulated mouse macrophage (RAW 264.7) cells and mouse model of acute and chronic inflammation, we have demonstrated that among four 3-(arylideneamino)-2-phenylquinazolin-4(3H)-one derivatives P(3a), P(3b), P(3c) and P(3d) the nitro group containing P(3a) exerted anti-inflammatory activities in vitro as well as in vivo. Out of these, the nitro group containing compound P(3a) was found to have lower toxicity than the rest in RAW 264.7 cells.

Several compounds containing nitro group(s) are reported to possess antibacterial, insecticidal, and anthelmentic activities (Dahiya et

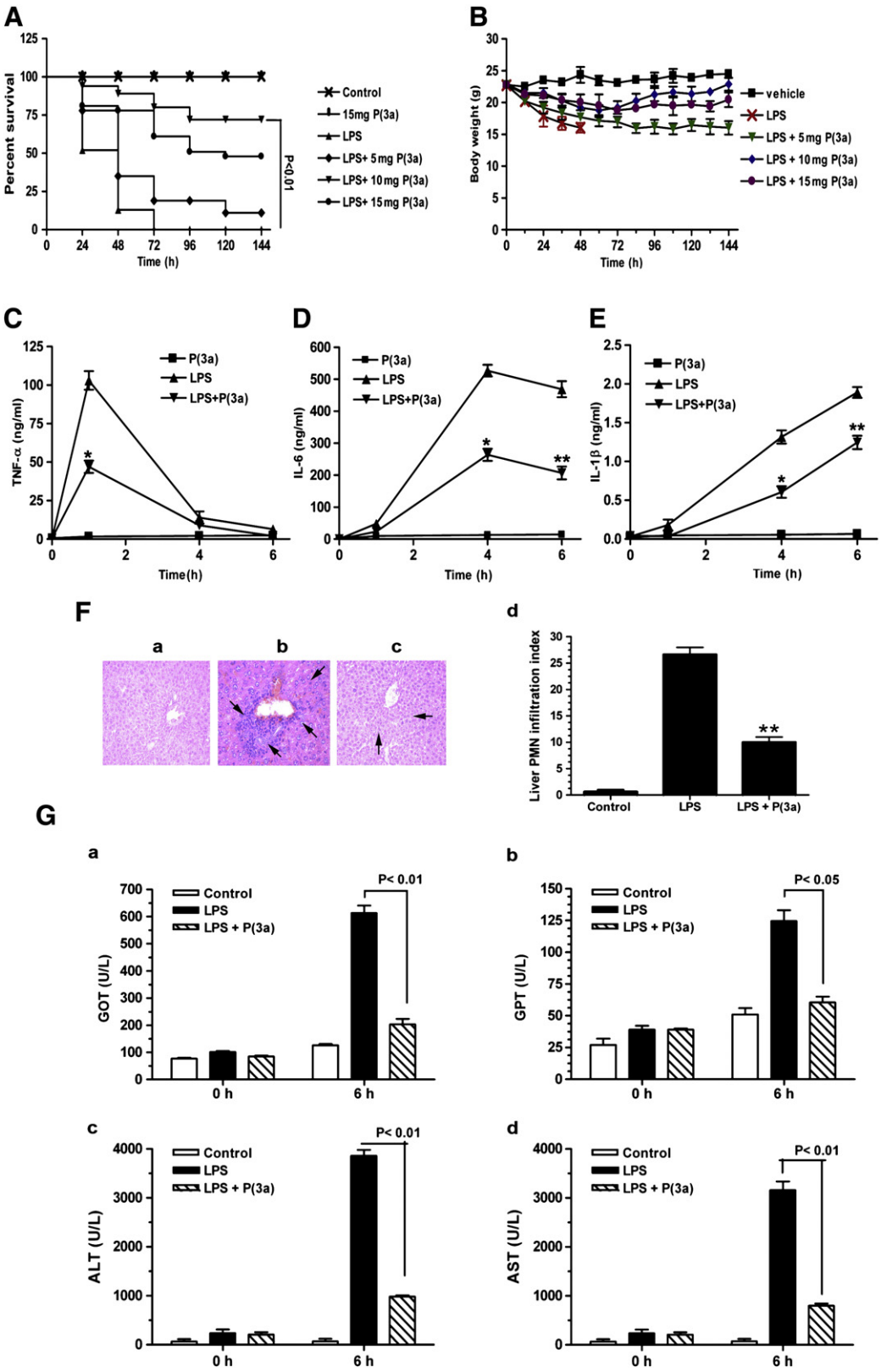


Fig. 5. Effect of P(3a) on (A) survival and (B) body weight of mice (n = 10) treated with LPS. Level of cytokines TNF-α (C), IL-6 (D), and IL-1β (E) was measured by ELISA at 1, 4 and 6 h upon treatment with P(3a) at dose 10 mg/kg, after challenging with LPS. Histopathological studies by light microscope morphologically showing liver tissues (F) from mice in the control group (a), infiltration of PMN (arrows indicated) in liver of LPS induced group (b), and minimal infiltration by PMN (arrows indicated) in liver of LPS induced mice treated with P(3a) at dose 10 mg/kg (c), and statistical analysis of the PMN index in liver (d). Tissue sections were stained with hematoxylin and eosin and viewed by light microscope (200×). Pre-treatment effects of P(3a) at the dose of 10 mg/kg on plasma level (G) of GOT (a),GPT (b) ALT (c) and AST (d) in mice treated with LPS. The data are reported as the mean ± SEM (*P<0.05, **P<0.01) of triplicate experiments.

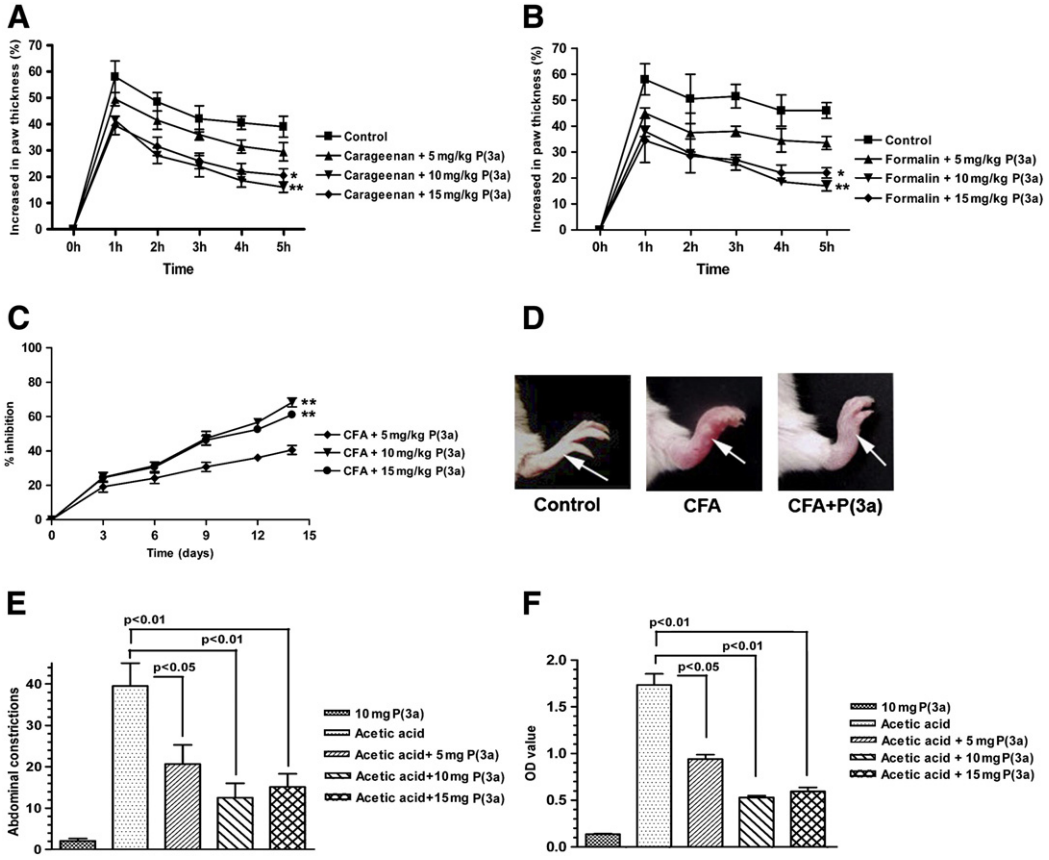


Fig. 6. Effect of P(3a) on carrageenan (A) and formalin (B) induced paw edema, and CFA (C) induced arthritis in mice, and the maximum inhibition shown on day 14 by P(3a) at the doses of 5, 10 and 15 mg/kg. D) Representative photographs showing joint inflammation in the hind limb on day 14 after CFA challenge in mice. Redness and swelling of toes are evident with respect to control in the 10 mg/kg P(3a) treated group. Acetic acid induced abdominal constriction (E) and vascular permeability (F) in mice after treatment with P(3a) at the doses of 5, 10 and 15 mg/kg, the data are reported as the mean ± SEM (*P<0.05, **P<0.01) of triplicate experiments.

al., 2008). Furthermore, compounds having nitro group attached to the aryl moiety of pyrazoles showed analgesic and anti inflammatory potential along with antimicrobial activity (Sahu et al., 2008). It was reported that isoxazole analogs of curcumin exhibit significant anti-inflammatory activity, while those with nitro group at 4-position of the isoxazole ring showed higher antioxidant activity (Madhavi et al., 2010). In contrast, derivatives carrying nitro group in oxadiazole ring have effective antiinflammatory activity (Bharathi et al., 2010). The earlier studies with non-steroidal antiinflammatory drug paracetamol showed it to have strong analgesic activity with less anti-inflammatory potency, but introduction of a nitro group in the parent structure (e.g., in nitro-paracetamol) imparted greater antinociceptive with anti inflammatory activity (Swayeh et al., 2000). Thus, the higher activity of P(3a) over the other analogs possibily owes it to the presence of nitro group.

Proinflammatory cytokines including TNF-α, IL-1β and IL-6 are known to induce innate immunological responses that mediate the development of various inflammatory diseases (Glauser, 1996; Mannel and Echtenacher, 2000). To decipher the molecular mechanism by which P(3a) inhibits inflammatory responses on Gram negative bacterial sepsis, we have evaluated the survival rate of mice in endotoxic induced murine shock model. Reports suggest that during endotoxemia there is loss of body weight (Voisin et al., 1996) and our results showed that P(3a) significantly improved the survival of mice challenged with the lethal dose of LPS and enhanced the body weight (Fig. 5A). Moreover, inhibition of the production of serums TNF-α, IL-1β, and IL-6 by P(3a) was consistent with our in vitro results (Fig. 3). Thus P(3a) mediated reduction of serum cytokines may be partly responsible for the improved mice survival rate.

LPS injection causes systemic inflammatory response such as multiple organ failure (MOF) and hepatic injury (O'Mahony et al., 2006), which is considered to be a major cause of mortality in LPS induced sepsis. Histological studies demonstrated that P(3a) suppressed LPS induced liver damage as shown in Fig. 5F. Thus, the alteration of cytokine profile by P(3a) is in agreement with its beneficial effect in mice endotoxemic model. It is known that bacterial endotoxins form a complex with TLR4-CD14 on the cell surface, which then activates the transcriptional factor NF-κB normally sequestered in the cytoplasm and translocates it to the nucleus to activate the transcriptional target genes including cytokines and other associator molecules (Hiransai et al., 2010; Jeon et al., 2010). The transcription factor NF-κB is maintained inactive in the cytoplasm by the inhibitory protein IκB; its activation requires phosphorylation of IκB on serine subunits. The dissociation of IκB causes the translocation of NF-κB p65 sub-unit from cytoplasm to the nucleus and triggers the inflammatory gene expression (Li and Verma, 2002).

During inflammation, vasodilatation leads to transient increases in capillary permeability producing extravasation of plasma proteins, and thereby causes tissue edema. Generally, these reactions are linked to painful perception or hyperalgesic sensitization (Correa and Calixto, 1993; Paulino et al., 2009). We found that P(3a) reduced both the inflammatory pain in the acetic acid model and the paw edema volume induced by carrageenan and formalin. Thus, these results indicated the efficacy of P(3a) to inhibit plasma extravasation from the blood vessel. These inflammatory models are known to cause local production of bradykinin (Lee et al., 2008) and of prostaglandins such as PGE2, leukotrienes as also derivatives of arachidonic acid. These prostanoids then bind the prostaglandin sub-type receptors, triggering the inflammatory and

hyperalgesic pathway in affected tissues. Bradykinin and related kinins represent a group of potent inflammatory mediators responsible for pain and inflammation. On the other hand, bradykinin receptors promote pain stimuli and inflammation by the NF-κB pathway (Moreau et al., 2007). Thus, the inhibition of NF-κB activation (Fig. 4) and inflammatory enzyme expression may contribute to these beneficial effects of P(3a). The acute peripheral antiinflammatory models are biphasic, in which the early phase is mediated by histamine, serotonin and increased synthesis of prostaglandin E_2. Recent studies have shown that carrageenan also induces peripheral release of nitric oxide (NO), sustained by TNF-α, IFN-γ and IL-1β. The late phase, on the other hand, is sustained by the release of prostaglandin and NO (Antônio and Souza Brito., 1998. Thus, the observed anti-inflammatory effect of P(3a) in the early phases could be related to the suppression of the pro-inflammatory cytokines IL-6 and TNF-α (Matsumori et al., 1997) and to the blockade of the TNF-α/NF-κB signaling pathway (Yang et al., 2005). Further, the reduction in paw volume and suppression of swelling caused by the assessed derivative P(3a) reveals that it may be effective in RA, as it interferes with the production of potent pro-inflammatory agents such as TNF-α, IL-1β, and IL-6. In addition to the above mechanism, P(3a) might suppress the activation of NF-κB also.

In conclusion, our study demonstrates for the first time that P(3a) markedly suppresses cytokine production in vitro and in vivo by inhibiting the NF-κB pathway, and consequently improves the survival of endotoxemic animals. The compound also significantly reduced paw edema (induced by carrageenan and formalin) and CFA mediated arthritic paw swelling with inflammatory pain responses. Thus it can be developed as a valuable protective agent against inflammatory diseases.

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Competing interests

The author(s) declare that they have no competing interests.

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References

Ahmed, A.U., 2011. An overview of inflammation: mechanism and consequences. Front. Biol. 6, 274–281.
Ajith, T.A., Janardhanan, K.K., 2001. Antioxidant and anti-inflammatory activities of methanol extract of *Phellinus rimosus*. Indian J. Exp. Biol. 39, 1166–1169.
Alagarsamy, V., Giridhar, R., Yadav, H.R., Revathi, R., Rukmani, K., De Clercq, E., 2006. Anti HIV, antibacterial and antifungal activities of some novel 1,4-disubstituted-1,2,4-triazolo[4,3-a] quinazolin-5(4H)-ones. Indian J. Pharm. Sci. 68, 532–535.
Antônio, M.A., Souza Brito., A.R., 1998. Oral anti-inflammatory and anti-ulcerogenic activities of a hydroalcoholic extract and partitioned fractions of *Turnera ulmifolia* (Turneraceae). J. Ethnopharmacol. 61, 215–228.
Aurella, J-Gábor A., Szoclsányi, J., Jancsó, N., 1967. A simple method for measuring the amount of Evans blue exuded into the skin in response to an inflammatory stimulus. J. Pharm. Pharmacol. 19, 486–487.
Baeuerle, P.A., 1998. IκB-NF-κB structures. Cell 95, 729–731.
Bharathi, D., Hemalatha, S., Devadass, G., Kumar, P.R., Shanmugasundaram, P., Aanandhi, M.V., 2010. Synthesis, characterisation and in vitro anti inflammatory and anthelmintic activities of 1,3,4-oxadiazole derivatives. IJCRGG 2, 1867–1870.
Cannon, J.G., Tompkins, P.G., Gelfand, J.A., Michie, H.R., Stanford, G.G., van der Meer, J.W., Endres, S., Lonnemann, G., Corsetti, J., Chernow, B., 1990. Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever. J. Infect. Dis. 161, 79–84.
Chattopadhyay, D., Arunachalam, G., Mandal, A.B., Sur, T.K., Mandal, S.C., Bhattacharya, S.K., 2002. Antimicrobial and anti-inflammatory activity of folklore: *Mallotus peltatus* leaf extract. J. Ethnopharmacol. 82, 229–237.
Cohen, J., 2002. The immunopathogenesis of sepsis. Nature 420, 885–891.
Correa, C.R., Calixto, J.B., 1993. Evidence for participation of B1 and B2 kinin receptors in formalin-induced nociceptive response in the mouse. Br. J. Pharmacol. 110, 193–198.
Dahiya, R., Kumar, A., Yadav, R., 2008. Synthesis and biological activity of peptide derivatives of iodoquinazolinones/nitroimidazoles. Molecules 13, 958–976.
Das, S., Chatterjee, N., Bose, D., Dey, S.K., Munda, R.N., Nandy, A., Bera, S., Biswas, S.K., Saha, K.D., 2012. Anticancer potential of 3-(arylideneamino)-2-phenylquinazoline-4(3H)-one derivatives. Cell. Physiol. Biochem. 29, 251–260.
Farghaly, A.M., Chaaban, I., Khalil, M.A., Bekhit, A.A., 1990. Nonsteroidal antiinflammatory agents. Synthesis of novel 2-pyrazolyl-4(3H)-quinazolinones. Arch. Pharmacol. 32, 3833–3836.
Fujiwara, N., Kobayashi, K., 2005. Macrophages in inflammation. Curr. Drug Targets Inflamm. Allergy 4, 281–286.
Glauser, M.P., 1996. The inflammatory cytokines. New developments in the pathophysiology and treatment of septic shock. Drugs 52, 9–17.
Hiransai, P., Ratanachaiyavong, S., Itharat, A., Graidist, P., Ruengrairatanaroj, P., Purintrapiban, J., 2010. Dioscorealide B suppresses LPS-induced nitric oxide production and inflammatory cytokine expression in RAW 264.7 macrophages: the inhibition of NF-kappaB and ERK1/2 activation. J. Cell. Biochem. 109, 1057–1063.
Irwin, S., 1962. Drug screening and evaluation procedure. Science 136, 123–132.
Jatav, V., Mishra, P., Kaswa, S., Stabes, J.P., 2008. Synthesis and CNS depressant activity of some novel 3-[5-substituted-1,3,4-thiadiazole-2-yl]-2-styryl quinazoline-4(3H)-ones. Eur. J. Med. Chem. 43, 135–141.
Jeon, K.I., Xu, X., Aizawa, T., Lim, J.H., Jono, H., Kwon, D.S., Abe, J., Berk, B.C., Li, J.D., Yan, C., 2010. Vinpocetine inhibits NF-kappaB-dependent inflammation via an IKK-dependent but PDE-independent mechanism. Proc. Natl. Acad. Sci. U. S. A. 107, 9795–9800.
Kopp, E.B., Ghosh, S., 1995. NF-kappa B and related proteins in innate immunity. Adv. Immunol. 58, 1–27.
Kornet, M.J., 1992. Synthesis and anticonvulsant activity of 3-alkyl-3,4-dihydro-2(1H)-quinazolinones. J. Heterocycl. Chem. 29, 103–105.
Koster, R., Anderson, M., De Beer, E.J., 1959. Acetic acid for analgesic screening. Fed. Proc. 18, 412–415.
Lee, C.H., Shieh, D.C., Tzeng, C.Y., Chen, C.P., Wang, S.P., Chiu, Y.C., Huang, C.Y., Hsu, C.J., Fong, Y.C., Tang, C.H., 2008. Bradykinin-induced IL-6 expression through bradykinin B2 receptor, phospholipase C, protein kinase Cdelta and NF-kappaB pathway in human synovial fibroblasts. Mol. Immunol. 45, 3693–3702.
Li, Q., Verma, I.M., 2002. NF-kappaB regulation in the immune system. Nat. Rev. Immunol. 2, 725–734.
Litchfield, J.T., Wilcoxon, F., 1949. A simplified method of evaluating dose effect experiments. J. Pharmacol. Exp. Ther. 96, 99–113.
Madhavi, K., Bharathi, K., Prasad, K.V.S.R.G., 2010. Synthesis and evaluation of 3-methyl-4-nitro-5-(substituted styryl) isoxazoles for antioxidant and anti-inflammatory activities. RJPBCS 1, 1073–1082.
Majumdar, K.N., Banerjee, A., Ratha, J., Mandal, M., Sarkar, R.N., Saha, K.D., 2008. Leishmanial lipid suppresses tumor necrosis factor alpha, interleukin-1beta, and nitric oxide production by adherent synovial fluid mononuclear cells in rheumatoid arthritis patients and induces apoptosis through the mitochondrial-mediated pathway. Arthritis Rheum. 58, 696–706.
Mannel, D.N., Echtenacher, B., 2000. TNF in the inflammatory response. Chem. Immunol. 74, 141–161.
Marks, J.D., Marks, C.B., Luce, J.M., Montgomery, A.B., Turner, J., Metz, C.A., Murray, J.F., 1990. Plasma tumor necrosis factor in patients with septic shock. Mortality rate, incidence of adult respiratory distress syndrome, and effects of methyl prednisolone administration. Ann. Rev. Respir. Dis. 141, 94–97.
Matsumori, A., Ono, K., Nishio, R., Igata, H., Shioi, T., Matsui, S., Furukawa, Y., Iwasaki, A., Nose, Y., Sasayama, S., 1997. Modulation of cytokine production and protection against lethal endotoxemia by the cardiac glycoside ouabain. Circulation 96, 1501–1506.
Mayeux, P.R., 1997. Pathobiology of lipopolysaccharide. J. Toxicol. Environ. Health 51, 415–435.
Medzhitov, R., 2008. Origin and physiological roles of inflammation. Nature 454, 428–435.
Miller, S.I., Ernst, R.K., Bader, M.W., 2005. LPS, TLR4 and infectious disease diversity. Nat. Rev. Microbiol. 3, 36–46.
Moreau, M.E., Bawolak, M.T., Morissette, G., Adam, A., Marceau, F., 2007. Role of nuclear factor-kappaB and protein kinase C signaling in the expression of the kinin B1 receptor in human vascular smooth muscle cells. Mol. Pharmacol. 71, 949–956.
Morgensen, T.H., 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin. Microbiol. Rev. 22, 240–273.
Mörmann, M., Thederan, M., Nackchbandi, I., 2008. Lipopolysaccharides (LPS) induce the differentiation of human monocytes to osteoclasts in a tumour necrosis factor (TNF) alpha-dependent manner: a link between infection and pathological bone resorption. Mol. Immunol. 45, 3330–3337.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Nanda, A.K., Ganguli, S., Chakraborty, R., 2007. Antibacterial activity of some 3-(arylideneamino)-2-phenyl-quinazoline-4(3H)-ones: synthesis and preliminary QSAR studies. Molecules 12, 2413–2426.
Newbold, B.B., 1963. Chemotherapy of arthritis induced in rats by mycobacterial adjuvants. Br. J. Pharmacol. Chemother. 21, 127–136.
O'Mahony, D.S., Liles, W.C., Altemeier, W.A., Dhanireddy, S., Frevert, C.W., Liggitt, D., Martin, T.R., Matute-Bello, G., 2006. Mechanical ventilation interacts with endotoxemia to induce extrapulmonary organ dysfunction. Crit. Care 10, R136.
Pandey, V.K., Tusi, S., Tusi, Z., Raghubir, R., Dixit, M., Joshi, M.N., 2004. Heterocyclic compounds thiazolyl quinazolones as potential antiviral and antihypertensive agents. Indian J. Chem. 43, 180–184.
Parillo, J.E., 1993. Pathogenetic mechanisms of septic shock. N. Engl. J. Med. 328, 1471–1477.
Paulino, N., Rodrigues, N.C., Pardi, P.C., Suarez, J.A., Santos, R.P., Scremin, A., Vogel, C., Feist, H., Michalik, D., 2009. Evaluation of anti-inflammatory effect of synthetic 1,5-bis(4-acetoxy-3-methoxyphenyl)-1,4-pentadien-3-one, HB2. Bioorg. Med. Chem. 17, 4290–4295.
Sahu, S.K., Banerjee, M., Samantray, A., Behera, C., Azam, M.A., 2008. Synthesis, analgesic, anti-inflammatory and antimicrobial activities of some novel pyrazoline derivatives. Trop. J. Pharm. Res. 7, 961–968.
Silva, E., Pedro, M.A., Sogayar, A.C., Mohovic, T., Silva, C.L., Janiszewski, M., 2004. Brazilian sepsis epidemiological study. Crit. Care 8, 251–260.
Swayeh, O.A., Futter, L.E., Clifford, R.H., Moore, P.K., 2000. Nitroparacetamol exhibits anti-inflammatory and anti-nociceptive activity. Br. J. Pharmacol. 130, 1453–1456.
Szekancecz, Z., Koch, E., 2007. Macrophages and their products in rheumatoid arthritis. Curr. Opin. Rheumatol. 19, 289–295.
Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354.
Voisin, L., Breuille, D., Combaret, L., Pouyet, C., Taillandier, D., Aurousseau, E., Obled, C., Attaix, D., 1996. Muscle wasting in a rat model of long-lasting sepsis results from the activation of lysosomal, Ca^{2+}-activated, and ubiquitin–proteasome proteolytic pathways. J. Clin. Investig. 97, 1610–1617.
Yang, Q., Huang, W., Jozwik, C., Lin, Y., Glasman, M., Caohuy, H., Srivastava, M., Esposito, D., Gillette, W., Hartley, J., Pollard, H.B., 2005. Cardiac glycosides inhibit TNF-alpha/NF-kappaB signaling by blocking recruitment of TNF receptor-associated death domain to the TNF receptor. Proc. Natl. Acad. Sci. U. S. A. 102, 9631–9636.