Anti-inflammatory and analgesic effect of plumbagin through inhibition of nuclear factor-kappa B activation

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Running title: Plumbagin suppresses inflammation via NF-κB

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No. of text pages: 32
No. of tables: 1
No. of figures: 6
No. of references: 40
No. of words in Abstract: 233
No. of words in Introduction: 591
No. of words in Discussion: 1473

Abbreviations: ANOVA, analysis of variance; AP-1, activator protein 1; COX, cyclooxygenase; ECL, enhanced chemiluminescence; IκBα, inhibitor of κB; IKK, IκBα kinase; iNOS, inducible nitric oxide synthase; IRAK, interleukin-1 receptor-associated kinase; LSD, least significant difference; NF-κB, nuclear transcription factor kappa-B; NO, nitric oxide; NSAIDs, nonsteroidal anti-inflammatory drugs; PGE2, prostaglandin E2; TBST, Tris-buffered saline plus Tween-20; TNF-α, tumor necrosis factor; IL, interleukin; TNFR, tumor necrosis factor receptor; PL, plumbagin

Recommended Section: Inflammation, Immunopharmacology, and Asthma
Abstract

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (PL) is a naturally occurring yellow pigment found in the plants of the Plumbaginaceae, Droseraceae, Ancistrocladaceae, and Dioncophyllaceae families. It has been reported that PL exhibits anti-carcinogenic, anti-inflammatory and analgesic activities. However, the mechanism underlying its’ anti-inflammatory action remains unknown. In the current study, we investigated and characterized the anti-inflammatory and analgesic effects of PL orally administrated in a range of dosages from 5 to 20 mg/kg; we also examined the role of NF-κB and pro-inflammatory cytokines and mediators in this effect. The results showed that PL significantly and dose-dependently suppressed the paw edema of rats induced by carrageenan and various pro-inflammatory mediators, including histamine, serotonin, bradykinin and prostaglandin E2. PL reduced the number of writhing episodes of mice induced by intraperitoneal injection of acetic acid. But it did not reduce the writhing episode numbers induced by MgSO4 in mice and it did not prolong the tail flick reaction time of rats to noxious thermal pain either. Mechanistic studies showed that PL effectively decreased the production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. It also inhibited the expression of pro-inflammatory mediators iNOS and COX-2 while did not inhibit the expression of COX-1. Further studies demonstrated that PL suppressed IκBα phosphorylation and degradation and thus inhibited the phosphorylation of p65 subunit of NF-κB. This study suggests that PL has a potential to be developed into an anti-inflammatory agent for treating inflammatory diseases.
Introduction

Inflammation has been demonstrated at the root of almost all chronic diseases, such as cancer, cardiovascular diseases, and autoimmune diseases, and huge efforts and resources are dedicated to the development of anti-inflammatory drugs. Nuclear transcription factor kappa-B (NF-κB) plays a critical role in the pathogenesis of inflammation and a variety of drugs designed to treat human inflammatory disease are focused on the inhibition of NF-κB activation (Tak and Firestein, 2001). Under normal conditions, NF-κB is present in the cytoplasm as an inactive heterotrimer consisting of three subunits: p50, p65, and IκBα (inhibitor of κB). Upon external stimuli, such as mitogens, inflammatory cytokines, ultraviolet irradiation, ionizing radiation, viral proteins, bacterial lipopolysaccharides, and reactive oxygen species, IκBα undergoes phosphorylation which is mediated through the activation of the IκBα kinase (IKK) complex (Ducut Sigala et al., 2004) and ubiquitination dependent degradation by the 26S proteasome, thus exposing nuclear localization signals on the p50-p65 heterodimer, leading to nuclear translocation and binding to DNA. The binding of NF-κB with DNA results in transcription of the NF-κB-regulated genes (Aggarwal, 2004) and induces the transcription of proinflammatory mediators, such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 (Baeuerle and Baltimore, 1996). These mediators play important roles in mediation, propagation, and extension of a local or systemic inflammatory process and can cause further activation of NF-κB and subsequently increase further production of these proinflammatory mediators via positive feedback mechanisms (Sonis, 2002). Inhibition of these mediators is beneficial for the treatment of inflammatory diseases and has become an important strategy to suppress inflammation as the case in non-steroidal anti-inflammatory drugs.
Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (PL) is a naturally occurring yellow pigment found in the plants of the Plumbaginaceae, Droseraceae, Ancistrocladaceae, and Dioncophyllaceae families. Available reports on PL mainly focused on its anticancer activity as well as the underlying mechanisms. Animal and cell studies demonstrated that PL has anticancer, antiproliferative, chemopreventive, radiosensitizing and antimetastatic activities (Singh and Udupa, 1997; Sugie et al., 1998; Devi et al., 1998; Prasad et al., 1996; Wang et al., 2008). Mechanistic studies revealed that these activities of PL are related to its ability to modulate nuclear transcription factor kappa-B (NF-κB) activation pathway which in turn induces S-G2/M cell cycle arrest through the induction of p21 (an inhibitor of cyclin-dependent kinase) (Jaiswal et al., 2002), changes redox status of cell (Srinivas et al., 2004), and inhibits the enzyme NADPH oxidase (Ding et al., 2005).

Recently, the anti-inflammatory and analgesic activities of leaves of *Plumbago zeylanica* and plumbagin were reported by Sheeja (Sheeja et al., 2010) in bioassay-guided isolation of anti-inflammatory and antinociceptive compound from this plant. In Sheeja’s report, plumbagin inhibited carrageenan induced rat paw edema, prolonged hot plate reaction time in mice, and shortened duration of pain response in formalin induced nociception. However, the mechanism underlying the anti-inflammatory action of plumbagin remains unknown. Because NF-κB plays a pivotal role in inflammation and PL has the ability to modulate NF-κB in cancer cells, we therefore hypothesize that PL could suppress experimental inflammation through inhibition of NF-κB activation.

In this report, the anti-inflammatory activity of PL was examined in the rat paw edema models induced by commonly used carrageenan and other phlogistic agents.
and the role of NF-κB pathway and proinflammatory mediators COX, iNOS, TNF-α, IL-1β, and IL-6 were examined. In addition, the analgesic activity of PL was also investigated in inflammatory and non-inflammatory pain models. This research will provide a solid foundation for the use of PL as an anti-inflammatory agent for therapeutic purpose.
Methods

**Experimental animals.** Male ICR mice weighing 17-23g and male SD rats weighing 200-250g were purchased from the Laboratory Animal Services Center, the Chinese University of Hong Kong, Hong Kong. The animals were acclimated for $\geq 1$ week under 12 hours light and 12 hours dark cycle at room temperature of $22 \, ^\circ\text{C} \pm 1^\circ\text{C}$. Chow diet and water were provided *ad libitum*. Rats and mice were fasted 24 h before experiment. After completion of experimental testing, animals were injected with Dorminal which contains 20% pentobarbital and then sacrificed by cervical dislocation. Animal care and treatment procedures conformed to the Institutional Guidelines and Animal Ordinance (Department of Health, Hong Kong Special Administrative Region).

**Drugs and reagents.** Plumbagin (purity: 99%), indomethacin, aspirin, carrageenan, Tween ®80, histamine, serotonin, prostaglandin E$_2$ (PGE$_2$), bradykinin, acetic acid and magnesium sulfate (MgSO$_4$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rotundine, an analgesic drug derived from medical plant in China, was purchased from Guangzhou Shiqiao Pharmaceutical Co., Ltd., Guangzhou, China. Morphine hydrochloride injection was purchased from Northeast Pharmaceutical Group Co. Plumbagin, indomethacin and rotundine were dissolved in 100% ethanol and then resuspended with 0.5% carboxymethyl-cellulose for animal oral
administration (final concentration of ethanol: 10%). Vehicle was prepared in the same method without drugs added.

**Induction of acute inflammation in rat hind paws.** The acute inflammation in the hind paws of rats was induced by subcutaneous injection of phlogistic agents, including carrageenan, histamine, serotonin, bradykinin or PGE₂, into right hind paws of rats according to our previous methods (Zhou et al, 2006). In brief, two distinct schemes of treatment have been adopted, PL (5, 10, 20 mg/kg), indomethacin (reference drug, 10 mg/kg), or vehicle was orally administrated 1 h before inflammation induction for the prophylactic scheme. At induction, each rat was injected with freshly prepared solutions of carrageenan (0.1ml, 1% w/v), histamine (0.05ml, 1% w/v), serotonin (0.05ml, 1% w/v), bradykinin (0.05ml, 1% w/v), or PGE₂ (20 μg in 0.05ml) in physiological saline (0.9% w/v NaCl) into subplantar tissues of the right hind paw of rats. In the therapeutic scheme, the animals received PL (5, 10, 20 mg/kg), indomethacin (reference drug, 10 mg/kg), or vehicle, 60 min after the injection of carrageenan. The left hind paws without injection were used as controls. The volumes (ml) of both hind paws of each animal were measured using a plethysmometer (7150, UGO Basile, Italy) at 1 h before inflammation induction and at different time intervals designed from 0.5 to 6 h after injection of the phlogistic
agents. The percentage of increase in paw volume (paw edema) of the right hind paws of each rat at each time point were calculated by the following equation: percentage of increase (\%) = \frac{(A-B)}{B} \times 100, where A represents the paw volumes at different time points after injection, and B represents the paw volume before injection. At the end of experiment, the paws injected with carrageenan were collected to obtain paw exudates. Three to five samples of paw exudates were randomly selected for western blot analysis.

**Visceral nociceptive model induced by acetic acid and MgSO\textsubscript{4} in mice.** The abdominal writhing test induced by chemical stimulation of acetic acid was performed in mice as originally described by Siegmund (Siegmund et al., 1957). Briefly, PL (5, 10, 20 mg/kg), aspirin (200 mg/kg), or vehicle was orally administrated 2 h before acetic acid injection. After intraperitoneal injection of 0.2ml acetic acid (0.8% w/v) in physiological saline, the animals were isolated for observation. The numbers of abdominal writhing syndrome/events, which consisted of the contraction of abdominal area with extension of hind legs, were accurately recorded during a 15 min period in each animal.

The abdominal writhing test induced by MgSO\textsubscript{4} was performed in mice as originally described by Gyires (Gyires and Torma, 1984) with minor modifications. Briefly, 1h
before MgSO₄ intraperitoneal injection (120 mg/kg, 10ml/kg), PL (5, 10, 20 mg/kg), or vehicle was orally administrated and morphine (10 mg/kg) was administrated subcutaneously. After intraperitoneal injection of MgSO₄, the animals were isolated in transparent cage for observation. After the first writhing movement appeared the animals were kept under observation for 5 min and the number of writhing was counted during this period.

**Central Nociceptive Model Induced by Radiant Heat Stimulation in Rats**

The antinociceptive effects of PL and the reference drug, expressed as the time required for rat tail flick after exposure to a source of radiant heat, were evaluated according to the description of Zhou (Zhou et al, 2006) Briefly, animals were placed in a Plexiglas box that allowed their tails to be free, and then the box was placed on IITC model 336 tail flick analgesia meter (IITC Inc., U.S.A.) with the tail occluding a slit over a photocell for radiant heat stimulation generated by a power lamp mounted in a reflector. The tail-flick response was elicited by applying radiant heat to the point 1/3 of length away from the tip of the tail. The apparatus was arranged so that when the operator turned on the lamp a timer was activated. When the rat felt pain and flicked its tail, light fell on the photocell such that the timer was automatically stopped. The intensity of the heat stimulus in the tail-flick test was adjusted so that the animal
flicked its tail within 3 to 5 s. A 20-s cut-off time was set in order to prevent tail tissues from damage. Before the experiments, the heat stimulation latency of all animals was tested, those with response time to heat stimulation <2s or >6s were excluded. The tail-flick response was measured at 1, 2 and 3 h after oral administration of PL (5, 10, 20 mg/kg), or rotundine (100 mg/kg) as reference drug, or the vehicle.

Western blot analysis of IκBα, p-NF-κB p65, iNOS, COX-1, COX-2, TNF-α, IL-1β and IL-6 protein expressions. To obtain paw exudates, the rats were sacrificed by diethyl ether asphyxiation. Then, each hind paw injected with carrageenan was cut at the level of the calcaneus bone and several transversal cuts were made with a scalpel. Each paw was then centrifuged at 10000 g for 10 min at 4 °C to collect tissue exudates (edema fluid). For iNOS, COX-1, COX-2, TNF-α, IL-1β and IL-6 protein analysis, the edema fluid was vortexed in RIPA buffer (cat# CS9806, Cell Signaling, Danvers, MA, USA). For IκBα and p-NF-κB p65 protein analysis, the edema fluid was vortexed in ice-cold lysis buffer (sucrose 250mM, Tris-HCl 50mM, sodium EDTA 2mM, beta-mercaptoethanol 2mM, sodium fluoride 5 mM, sodium orthovanadate 1 mM, aprotinin 10 μg/ml, leupeptin 10 μg/ml, pH7.2) for 1 min. The RIPA or lysis buffer suspensions were immediately centrifuged at 14,000 g for 20 min at 4 °C, and the supernatant was gently collected. The contents of total protein in the supernatants were determined by using a protein kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts (100 μg) of protein were boiled in sample loading buffer for 5 min before loading on 10% sodium dodecyl sulfate-polyacrylamide gel for
electrophoresis and transferred onto Immobilon-P membrane for blotting (Pore size: 0.45μm, Millipore, USA). The nonspecific binding sites on the membrane were blocked with 5% non-fat milk in Tris-buffered saline plus 0.1% Tween-20 (TBST) at 4 °C overnight and then the membranes were incubated with specific primary antibodies, including IκB-α (Cat #SC-371, Santa Cruz, CA, USA), p-NF-κB p65 (Cat #SC-166748, Santa Cruz, CA, USA), iNOS (Cat #610329, BD Biosciences, New Jersey, USA), COX-1 (Cat #160110 Cayman, Ann Arbor, MI, USA), COX-2 (Cat#160106, Cayman, Ann Arbor, MI, USA), TNF-α (Cat #CS37071, Cell Signaling, Danvers, MA, USA), IL-1β (Cat #SC-1252, Santa Cruz, CA, USA), IL-6 (Cat #ab6672, Abcam, Cambridge, USA) and β-actin (Cat #SC-1615, Santa Cruz, CA, USA). Membranes were subsequently incubated with peroxidase-conjugated secondary antibodies in 5% non-fat milk in TBST for 1h at room temperature. The membranes were washed six times and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) method using hyperfilm and ECL reagent (Amersham, USA) according to the manufacturer’s instructions. Band intensities were quantified using a densitometer analysis system and expressed as an arbitrary unit (Quantity One software, Bio-Red).

**Statistical analysis.** All values are expressed as means ± S.E.M. Statistical significance of the difference was assessed by repeated measures ANOVA (analysis of variance) test, followed by post hoc test with LSD (least significant difference) method for acute inflammation and tail flick test. Statistical significance of the difference was assessed by one way ANOVA followed by post hoc test with LSD (least significant difference) method for other tests. p values lower than 0.05 were considered statistically significant.
Results

Plumbagin inhibited paw edema induced by different phlogistic agents in rats

Fig. 1A shows the prophylactic effect of PL on inhibition of the acute paw edema in rats evoked by carrageenan injection into the subplantar tissues of right hind paws. The maximum phlogistic response of carrageenan was observed at 4-6 h after the injection in the control animals. The paw volumes from PL-treated animals with dosages of 10 and 20 mg/kg at 2-6 h after induction of paw edema showed marked decrease in comparison with the data of non-treated animals at the same time points. Reference drug, indomethacin, also significantly suppressed the paw edema. Rats treated with 5 mg/kg PL did not differ from the control group. These results indicate that the anti-acute inflammatory effect of PL in rats was dose dependent. Fig. 1B shows the therapeutic effect of PL on inhibition of the acute paw edema in rats evoked by carrageenan. The paw volumes from PL-treated animals with dosages of 10 and 20 mg/kg showed marked decrease in comparison with the data of non-treated animals. Indomethacin also demonstrated significant suppression to the paw edema. For normal group, very slight increase in paw edema was found at 4 hour after 0.1 ml saline injection (2.67%±2.53%, n=3).

In the case of histamine, serotonin, bradykinin and PGE₂ induced rat paw edema, all measurements were conducted at time intervals of 0.5, 1, 2, 3 and 4 h after injection of the above phlogistic agents. Fig. 2 shows that PL can dose-dependently inhibit the acute inflammatory responses evoked by histamine, serotonin, PGE₂, or bradykinin. However, the figures also show that the anti-inflammatory effect of PL, while dose-dependent, also varies according to inflammatory agents. In rat paw edema induced by histamine, bradykinin and PGE₂, the paw volumes from PL-treated animals with dosages of 10 and 20 mg/kg showed marked decrease throughout the
experiments (Fig. 2A, 2C, and 2D); while in the paw edema induced by serotonin, only higher dose (20 mg/kg) significantly reduced edema (Fig. 2B). Indomethacin (10mg/kg) also showed anti-inflammatory effects in all animals (Fig. 2A-D).

**Plumbagin alleviated pain induced by acetic acid but not MgSO₄ and radiant heat stimulation**

The writhing assay induced by peritoneal injection of acetic acid in mice was employed in the study. Fig. 3A shows the numbers of the abdominal writhing episodes evoked by intraperitoneal injection of acetic acid in mice as well as the anti-nociceptive effect of PL. It can be seen that treatment with PL could dose-dependently reduce the number of writhing episodes of mice in comparison with that of vehicle-treated animals; while the reference drug aspirin had stronger effect than PL did.

Fig. 3B shows the numbers of the abdominal writhing episodes evoked by intraperitoneal injection of MgSO₄ in mice. It can be seen that treatment with PL did not reduce the number of writhing episodes of mice in comparison with that of vehicle-treated animals; while the reference drug, morphine, had significant effect in reducing MgSO₄ induced writhing episodes.

Table 1 shows that the tail flick reaction time of the control animals was around 5 s at 1, 2 and 3 h after orally taking the vehicle. Rats treated with PL did not differ from the control group while rotundine, a positive analgesic agent (Zhou et al., 2006), prolonged the reaction time of the animals and demonstrated significant anti-nociceptive action.

**Plumbagin suppressed elevation of iNOS and COX-2 expression induced by carrageenan in paw edema fluid**
It can be seen in Fig. 4 that PL dose-dependently attenuated the protein expression of iNOS and COX-2 in carrageenan-injected paw tissues. Around 45% and 50% reduction in iNOS protein expression was achieved by treatment with PL at dosages of 10 and 20 mg/kg, respectively, while no difference was found at the dosage of 5 mg/kg. Around 60-70% reduction in COX-2 protein expression was achieved by treatment with PL at all three dosages. Examination of COX-1 protein expression showed that the level of expression was not suppressed by administration of PL at any dosage. These results suggest that PL may have a selective inhibitory effect on COX-2 and iNOS protein expression. However, indomethacin at a dosage of 10 mg/kg demonstrated significant inhibition on iNOS, COX-1 and COX-2 protein expressions. No significant increase in iNOS and COX-2 protein expressions was found in normal animals in which no carrageenan was injected into the paw.

**Plumbagin reversed change of IκBα and p-NF-κB p65 expression induced by carrageenan in paw edema fluid**

The appearance of IκBα in cell lysate was investigated by immunoblot analysis. A basal level of IκBα was detectable in the normal animals in which no carrageenan was injected into the paw. At 4 h after carrageenan administration, IκBα level was substantially reduced (Fig. 5) in the control group. Pre-treatment with PL prevented carrageenan-mediated IκBα degradation. In fact, the IκBα band intensity of PL in the pre-treatment group remained around 50% compared with the normal animals at 4 h after carrageenan administration (Fig. 5). In carrageenan-treated animals, the level of p65 NF-κB subunit was increased as compared with the normal animals (Fig. 5). However, administration of PL (20 mg/kg) significantly reduced p65 band intensity. Reference drug indomethacin at 10 mg/kg also reversed the change of IκBα and p65
NF-κB subunit induced by carrageenan in the paw edema fluid of rats.

Plumbagin inhibited elevation of TNF-α, IL-1β and IL-6 expression induced by carrageenan in paw edema fluid

The pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in the carrageenan-injected paw edema fluid were examined by immunoblot analysis (Fig. 6). A basal level of TNF-α and IL-6 was detectable in the normal group; while IL-1β wasn’t detected in normal rat paw fluid. Four hours after injection, carrageenan induced an obvious increase in the expression of TNF-α and IL-1β and a mild increase of IL-6 of paw edema fluid. Both PL and indomethacin attenuated the increased expression of TNF-α, IL-1β and IL-6.
Discussion

Although PL has been reported to have several biological functions, mostly related to anti-carcinogenic activity (Sugie et al., 1998; Wang et al., 2008), the anti-inflammatory and analgesic effect and the underlying mechanism of PL have not yet been investigated. In the present study, we provided the first evidence showing the anti-inflammatory and analgesic effects of PL in vivo through inhibition of NF-κB activation.

The pharmacological results of our current studies revealed that PL elicited significant anti-inflammatory activities in the carrageenan model in both prophylactic and therapeutic schemes. PL administrated before or after the carrageenan injection can still inhibit the paw edema (Figure 1A & 1B). In fact, carrageenan induced paw edema is one of the most commonly used models for inflammation investigation. This model has been widely accepted as a useful phlogistic tool for new anti-inflammatory agents screening. Development of paw edema of rats induced by carrageenan is highly correlated with the early exudative stage of inflammation (Ozaki, 1990). After carrageenan injection, a sudden elevation of paw volume can be observed which is correlated with vascular permeability induced by the action of histamine and serotonin (Vinger et al., 1987). Inflammation begins to be severe at approximately 1 h after induction and paw edema gradually elevates to a peak during 4-6 h after induction, which is the second phase due to the liberation and over-production of bradykinin, prostaglandins and kinins in paw tissue accompanied by leukocyte migration (Vinger et al., 1987). The inflammatory pattern in our present study is in close accordance with previous reports while the dose-dependent inhibition of inflammation by PL from 1-6 h after the induction of inflammation suggests that PL may act in both the earlier and later phases of inflammation.
Since PL can inhibit both first and second phase in carrageenan-induced edema, these results suggest that the anti-inflammatory activity of PL could be related to the impairment of pro-inflammatory mediators in the cyclooxygenase pathway, because most of the NSAIDs inhibit the production of pro-inflammatory mediators including eicosanoids. Thus, different inflammatory mediator, *i.e.* histamine, serotonin, prostaglandin E\(_2\) and bradykinin, were used for paw edema study, so as to further elucidate the anti-inflammatory effect of PL. The results here show that PL had marked dose-dependent inhibitory effect with different pharmacological intensities on various inflammatory models induced by histamine, serotonin, prostaglandin E\(_2\) or bradykinin. The results suggest that the underlying anti-inflammatory mechanisms of PL are possibly associated with the inhibition of either the synthesis, or the release, or the actions of those pro-inflammatory mediators.

Analgesic effect usually accompanies by anti-inflammation. We therefore examine the analgesic effect of PL with three nociceptive animal models: the tail flick test of rats evoked by radiant heat stimulation (noxious thermal pain), acetic acid-induced and MgSO\(_4\)-induced abdominal writhing assay in mice. The tail flick test is more sensitive in centrally acting analgesics whereas the acetic acid-induced abdominal writhing assay is commonly used for detecting both central and peripheral analgesia (Dewey et al., 1970; Fukawa et al., 1980; Schmidt et al., 2009, Won et al., 2006). Acetic acid was injected into the peritoneal cavity of mice to cause nociception in abdomen due to the release of various substances that excite pain nerve endings (Raj, 1996). And the MgSO\(_4\)-induced abdominal writhing assay is used as a model of non-inflammatory, prostaglandin-independent pain reaction. With the tail flick test model, it was found that PL did not have a significant ability of prolonging the response latencies to the treatment of noxious thermal pain (Table 1). In the writhing
response model, PL showed an ability of diminishing the numbers of the writhing episodes in a dose-dependent manner, indicating significant inhibition of the acetic acid-induced visceral nociception, while it did not inhibit MgSO₄-induced pain. These results suggest that the analgesic effect of PL is prostaglandin-dependent and PL might not be effective for the treatment of noxious thermal pain. It can be speculated that PL could inhibit cyclooxygenase pathway as shown in Figure 4, thus, further interfering with the mechanism of transduction in primary afferent nociceptors in a prostaglandin-dependent manner (Fields, 1987).

In the past two decades, a number of anti-inflammatory botanical-derived medicines have been developed but only a few of them have been studied with the goal of elucidating the molecular mechanisms of their actions (Surh et al., 2001). To address this issue, we firstly evaluated the anti-inflammatory and analgesic properties of PL, and then determined the molecular mechanisms relevant to these actions, focusing on several key molecular targets, including IκBα, NF-κB, COX-2, iNOS, and proinflammatory cytokines TNF-α, IL-1β, and IL-6.

It has been widely accepted that the formation of proinflammatory cytokines (e.g. TNF-α, IL-1β, and IL-6) and the overproduction of vasoactive mediators (e.g. nitric oxide (NO) by iNOS or eicosanoids via COX-2) play important roles in the pathophysiology of inflammation. The expression of inducible genes leading to the formation of these proteins relies on transcription factors, which are either controlled by (other) inducible genes and, hence, require de novo protein synthesis or alternatively, by so-called ‘primary transcription factors’. Among the latter, NF-κB has received a considerable amount of attention because of its unique mechanism of activation, its active role in cytoplasmic/nuclear signaling, and its rapid response to pathogenic stimulation. Activation of NF-κB is centrally involved in the local or
systemic inflammation (Ruetten and Thiemermann, 1997). Binding of NF-κB to the respective binding sequence on genomic DNA encoding for iNOS, COX-2, TNFα, IL-1β and IL-6 results in a rapid and effective transcription of these genes (Collart et al., 1990; Xie et al., 1994). The anti-carcinogenic, apoptotic and radiosensitizing effects previously described suggest that PL mediates its effects by suppressing NF-κB activation which is due to the interaction of PL with the cysteine residue of both IKK and p65 directly (Sandur et al., 2006). In the current study, we found that PL indeed suppressed IκBα phosphorylation and degradation, and as a result, inhibited phosphorylation of p65 subunit of NF-κB in carrageenan induced paw edema in vivo. This resulted in suppression of NF-κB regulated reporter gene transcription and gene products involved in inflammation, i.e. TNF-α, IL-1β, IL-6, COX-2, and iNOS in this study.

As far as the COXs are concerned, COX-2 is the predominant cyclooxygenase isoform in all stages of inflammation, including facilitation of the production of proinflammatory prostanoids and of inflammatory prostaglandins (Vane and Botting, 1998). Inflammation is induced or potentiated by the over-production of prostaglandins (Harris et al., 2002), while selective COX-2 inhibitors can suppress inflammatory conditions through inhibition of the inflammatory prostaglandins (Dannenberg et al., 2001). NO is crucially involved in the regulation of COX pathway and can modulate eicosanoid production by acting at several levels (Mollace et al, 2005). Thus, in inflammatory conditions where both the iNOS and COX-2 systems are induced, there is a NO-mediated induction of COX-2 leading to increased formation of proinflammatory prostaglandins. This results in an exacerbated inflammatory response (Mollace et al, 2005). Thus, inhibition of NO production by the suppression of the enzyme activity of iNOS is one of the major pathways for
anti-inflammatory effect. Moreover, Salvemini et al reported that peripheral or central administration of iNOS inhibitors could effectively inhibit carrageenan-induced hyperalgesia in rats which means that NO produced by iNOS is involved in maintenance of the carrageenan-evoked inflammatory response (Salvemini et al., 1996). In the present studies, we demonstrated that PL can significantly suppress the de novo expressions of inducible NOS and COX-2 enzymes, but it cannot inhibit COX-1 enzyme, this is believed to be one of the mechanisms by which PL reduces carrageenan-induced paw edema of rats.

Other than being regulated by NF-κB, proinflammatory cytokines TNF-α, IL-1β and IL-6 help to propagate the extension of a local or systemic inflammatory process by activating NF-κB, forming a positive feedback mechanism to exaggerate the inflammatory process (Sonis, 2002). IL-1β is a potent pro-inflammatory cytokine that exerts its effects by binding to its receptor (IL1-R1) on the plasma membrane. This binding induces phosphorylation of the IKK complex, a crucial step in NF-κB activation based on the recruitment of the interleukin-1 receptor-associated kinase (IRAK) (Wang et al., 2001). Like IL-1β, TNF-α is a potent pro-inflammatory cytokine that plays a crucial role in inflammation (Tracey and Cerami, 1993). It binds to its cellular receptor TNFR1, which triggers signalling cascades that activate NF-κB and AP-1 (activator protein 1) transcription factors. It has also been demonstrated that IL-6 can induce activation of NF-κB in the intestinal epithelia (Wang et al., 2003). Therefore, the inhibition of the production of TNF-α, IL-1β and IL-6 by PL described in the present study could also likely attribute to the inhibitory effect of PL on the activation of NF-κB.

In conclusion, PL inhibits NF-κB, resulting in decrease of proinflammatory cytokines TNF-α, IL-1β and IL-6, COX-2 and iNOS expression, and thus gives its
anti-inflammatory effect in animal models. This result suggests that PL has a potential
to be developed into an anti-inflammatory agent for treating inflammatory diseases.
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JPET #170852


Salvemini D, Wang ZQ, Wyatt PS, Bourdon DM, Marino MH, Manning PT and


cells. *Mol Carcinog* 40:201-211.


Footnotes

† P.L. and Y.F.W contribute equally to this research.

Acknowledgement: This research was supported by the Central Allocation Grant of Research Grant Committee of Hong Kong [HKBU2/07C]
Legends for Figures

Fig. 1. Effect of plumbagin (PL) on carrageenan-induced paw edema of rats in prophylactic (A) and therapeutic (B) schemes. The chemical structure of PL (5-hydroxy-2-methyl-1,4-naphthoquinone) is shown on the left upper corner. PL at dosages of 5 (◆), 10 (▼) and 20 (▲) mg/kg, the reference drug indomethacin at dosage of 10 mg/kg (△), and the vehicle (○) were orally administrated to rats at 1 h before carrageenan injection in prophylactic scheme (A) or at 1 hr after carrageenan injection in therapeutic scheme (B). The percentage of increase in paw volume (paw edema) of the right hind paws of each rat at each time point were calculated by the following equation: the percentage of increase (%) = (A-B)/B × 100, where A represents the paw volumes at different time points after injection, and B represents the paw volume before injection. Each point represents the mean ± S.E.M. (n=9). p value, compared with the vehicle control animals at the corresponding time point. For the prophylactic scheme, the basal paw volume at 0 h for vehicle, indomethacin, 5, 10, 20 mg/ml is 1.53 ± 0.04 ml, 1.47 ± 0.04 ml, 1.50 ± 0.02 ml, 1.56 ± 0.02 ml, and 1.54 ± 0.03 ml respectively. For the therapeutic scheme, the basal paw volume at 0 h for vehicle, indomethacin, 5, 10, 20 mg/ml is 1.34 ± 0.04 ml, 1.38 ± 0.01 ml, 1.40 ± 0.03 ml, 1.38 ± 0.02 ml, and 1.38 ± 0.03 ml respectively. Each data represents the mean ± S.E.M.

Fig. 2. Effect of plumbagin (PL) on histamine (A), serotonin (B), bradykinin (C), and prostaglandin E2 (PGE2) (D) induced paw edema of rats. PL at dosages of 5 (◆), 10 (▼) and 20 (▲) mg/kg, the reference drug indomethacin at dosage of 10 mg/kg (△), and the vehicle (○) were orally administrated to rats at 1 h before the injection of histamine,
serotonin, bradykinin, or PGE₂. Each point represents the mean ± S.E.M. (n=8-9). p
group, compared with the vehicle control animals at the corresponding time point. For
histamine induced paw edema, the basal paw volume at 0 h for vehicle, indomethacin,
5, 10, 20 mg/ml is 1.47 ± 0.03 ml, 1.54 ± 0.03 ml, 1.47 ± 0.03 ml, 1.57 ± 0.02 ml, and
1.51 ± 0.03 ml respectively. For serotonin induced paw edema, the basal paw volume
at 0 h for vehicle, indomethacin, 5, 10, 20 mg/ml is 1.35 ± 0.02 ml, 1.34 ± 0.02 ml,
1.31 ± 0.02 ml, 1.32 ± 0.02 ml, and 1.36 ± 0.04 ml respectively. For bradykinin
induced paw edema, the basal paw volume at 0 h for vehicle, indomethacin, 5, 10, 20
mg/ml is 1.38 ± 0.02 ml, 1.40 ± 0.03 ml, 1.39 ± 0.03 ml, 1.37 ± 0.01 ml, and 1.44 ±
0.03 ml respectively. For PGE₂ induced paw edema, the basal paw volume at 0 h for
vehicle, indomethacin, 5, 10, 20 mg/ml is 1.45 ± 0.03 ml, 1.49 ± 0.02 ml, 1.46 ± 0.02
ml, 1.51 ± 0.02 ml, and 1.39 ± 0.03 ml respectively. Each data represents the mean ±
S.E.M.

Fig. 3. Effect of plumbagin (PL) on acetic acid-induced (A) and MgSO₄-induced (B)
writhing response of mice. PL, the reference drug aspirin, and the vehicle were orally
administered to mice at 2 h (A) or 1 h (B) before the peritoneal injection of acetic acid
(A) or Mg SO₄(B). The number of writhing episodes of each mouse in 15 min after
acetic acid injection or were recorded (A), or the number of writhing episodes of each
mouse was recorded for 5 min after the first writhing movement appeared (B). Each
bar represents the mean ± S.E.M. (n=8-11). * P<0.05, ** P<0.01, *** P<0.001
compared with the vehicle control animals.

Fig. 4. Effects of plumbagin (PL) on iNOS, COX-2 and COX-1 protein expressions in
carrageen-induced paw edema fluid of rats. PL at the dosages of 5, 10 and 20 mg/kg,
the reference drug indomethacin (indo) at dosage of 10 mg/kg, and the vehicle were orally administrated at 1 h before the carrageenan injection. Animal without carrageenan inject served as normal control. At 4 h after the injection, paws were removed and paw edema fluid was collected by centrifugation. Protein expressions of iNOS, COX-2 and COX-1 in the paw edema fluid were detected by Western blot analysis using β-actin as the loading control. Each bar represents the mean ± S.E.M. (n=3-5). *P<0.05, ***P<0.001 compared with the vehicle control animals. #P<0.05 compared with the indomethacin treated animals.

Fig. 5. Effects of plumbagin (PL) on IκBα, p-NF-κB p65 protein expressions in carrageenan-injected paw edema fluid of rats. The experiment was performed as described in Fig. 4. Protein expressions of IκBα, p-NF-κB p65 in the paw edema fluid were detected by Western blot analysis using β-actin as the loading control. Indo refers to indomethacin. Each bar represents the mean ± S.E.M. (n=3-5). * P<0.05, *** P<0.001 compared with the vehicle control animals.

Fig. 6. Effects of plumbagin (PL) on TNF-α, IL-6 and IL-1β in carrageenan-injected paw edema fluid of rats. The experiment was performed as described in Fig. 4. Protein expressions of TNF-α, IL-6 and IL-1β in the paw edema fluid were detected by Western blot analysis using β-actin as the loading control. Indo refers to indomethacin.
Table 1. Effect of plumbagin (PL) on radiant heat stimulation-induced tail flick reaction of rat.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage</th>
<th>Tail flick reaction time</th>
<th>p value</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
<td>2 h</td>
</tr>
<tr>
<td>A. Vehicle</td>
<td>5.8±0.34</td>
<td>5.8±0.57</td>
<td>4.8±0.25</td>
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<tr>
<td>B. Rotundine</td>
<td>100 mg/kg</td>
<td>15.0±1.76</td>
<td>14.6±1.32</td>
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<tr>
<td>C. PL</td>
<td>20 mg/kg</td>
<td>6.1±0.19</td>
<td>6.2±0.46</td>
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<tr>
<td>D. PL</td>
<td>10 mg/kg</td>
<td>6.2±0.44</td>
<td>6.2±0.36</td>
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<tr>
<td>E. PL</td>
<td>5 mg/kg</td>
<td>5.4±0.27</td>
<td>5.3±0.35</td>
</tr>
</tbody>
</table>

The tail-flick response was measured at 1, 2 and 3 h after oral administration of PL (5, 10, 20 mg/kg), or rotundine (100 mg/kg) as reference drug, or vehicle. Each data represents the mean ± SD (n=8). p value, compared with the vehicle control animals.
Fig. 3

A

B

Number of writhing episodes

Vehicle  PL  PL  PL  Aspirin  PL  PL  PL  Morphine

20 mg/kg  10 mg/kg  5 mg/kg  200 mg/kg

**  *  ***

Number of writhing episodes

Vehicle  PL  PL  PL  PL  Morphine

20 mg/kg  10 mg/kg  5 mg/kg  5 mg/kg

***
**Fig. 5**

A.  

Western blot analysis of IκBα and β-actin levels in different treatment groups: Vehicle, Indo, 10 mg/kg, 20 mg/kg, 10 mg/kg, 5 mg/kg, and Normal. The y-axis represents arbitrary units. The bars show a significant decrease in IκBα levels with increasing Plumbagin doses compared to Vehicle and Indo.

B.  

Western blot analysis of p-p65 and β-actin levels in different treatment groups: Vehicle, Indo, 10 mg/kg, 20 mg/kg, 10 mg/kg, 5 mg/kg, and Normal. The y-axis represents arbitrary units. The bars show a significant increase in p-p65 levels with increasing Plumbagin doses compared to Vehicle and Indo.

**Statistical Significance:**  
- *******: Significantly different from Vehicle and Indo.
- **** : Significantly different from Vehicle, Indo, and 10 mg/kg.

**Note:** The graph shows a clear trend of increasing p-p65 and decreasing IκBα levels with Plumbagin treatment.
Fig. 6

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Carrageenan</td>
<td></td>
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<tr>
<td>Indo (10mg/kg)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PL (20mg/kg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</table>

**Western Blots**

- **TNF-α**
- **IL-1β**
- **IL-6**
- **β-actin**