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Anti-inflammatory effects and gastrointestinal safety of NNU-hdpa, a novel dual COX/5-LOX inhibitor

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are associated with a risk of serious adverse events. Now, the development of dual inhibitors of cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) has become a hot area in searching for safer NSAIDs. NNU-hdpa, 2-(4-hydroxylphenyl)-3-(3,5-dihydroxylphenyl) propenoic acid, a newly synthesized compound, is expected to have COX/5-LOX dual inhibition with an improved gastrointestinal profile. In this study, NNU-hdpa was subjected to *in vitro* and *in vivo* experiment protocols. *In vitro* COX/5-LOX inhibition assays showed that NNU-hdpa exhibits a dual inhibitory activity against the COX and 5-LOX enzymes. Anti-inflammatory activity *in vivo* was evaluated using two animal edema model tests. Pretreatment with NNU-hdpa (p.o.) dose-dependently inhibited the xylene-induced ear edema in mice and carrageenan-induced paw edema in rats respectively. In gastric lesion test, NNU-hdpa was gastric-sparing in that it elicited markedly fewer stomach lesions as compared to the stomach lesions caused by aspirin in rats. In further studies, NNU-hdpa was found to significantly inhibit the productions of PGE_2 and LTB_4 in LPS-challenged RAW 264.7, which is parallel to its prevention of the nuclear translocation of the NF-κB p50 and p65 subunits. These data indicate that NNU-hdpa comprises a novel class of dual inhibitors of COX and 5-LOX having therapeutic potential with an enhanced gastric safety profile.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used medications in the world because of their demonstrated efficacy in reducing pain and inflammation (Laine, 2001). NSAIDs comprise both traditional nonselective NSAIDs that nonspecifically inhibit both COX-1 and COX-2, and selective COX-2 inhibitors.

Although effective at relieving pain and inflammation, both NSAIDs are associated with a significant risk of serious adverse events when used chronically. As for traditional NSAIDs, they are reported to be associated with an increased risk of gastrointestinal ulcers, including gastrointestinal hemorrhage, perforation and obstruction, due to COX-2 as well as COX-1 inhibition. Treatment of these complications has brought a heavy burden to social fortune. As for selective COX-2 inhibitors, they are reported to have an improved gastrointestinal tolerability profile compared with traditional NSAIDs; however, serious cardiovascular effects of some selective COX-2 inhibitors emerged from clinical studies and pharmacosurveillance in recent years, which force the drug companies to withdraw rofecoxib and valdecoxib from the market (Ong et al., 2007). Thus, global pharmaceutical industry and researchers have dedicated their efforts to the search for safer NSAIDs. Now, a renewed interest, still under evaluation, including dual COX and 5-lipoxygenase (5-LOX) inhibitors (Fiorucci et al., 2001), NO-donor NSAIDs (Wallace and Cirino, 1994; Wallace and Del Soldato, 2003; Del Soldato et al., 1999; Hoogstraate et al., 2003), H_2S-donating NSAIDs (Wallace et al., 2007) has appeared. Overall, these new strategies or ideas are all related to the modulation of pro-inflammatory factors.

The development of inflammatory diseases may be accompanied by increased production of leukotrienes and prostaglandins from arachidonic acid (Coruzzi et al., 2007). COX-1 and COX-2 are responsible for the production of prostaglandins and LOX for leukotrienes. Inhibition of cyclooxygenase by nonsteroidal anti-inflammatory drugs and selective COX-2 inhibitors reduces the levels of prostaglandins, resulting in a reduction in pain and inflammation. However, this inhibition can cause alternative processing of arachidonic acid via the 5-lipoxygenase (5-LOX) pathway, resulting in increased production of pro-inflammatory and gastrotoxic leukotrienes. The dual inhibitors of COX and 5-LOX decrease the production of both leukotrienes and prostaglandins, and as such, they should theoretically display enhanced anti-inflammatory effects and improved gastric tolerability (Wallace et al., 1990; Gyömber

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et al., 1996; Martel-Pelletier et al., 2003). Thus, the development of novel dual inhibitors of COX and 5-LOX has currently attracted most attention.

Currently, it has been widely shown that many plant-derived compounds present significant anti-inflammatory effects (Shankar et al., 2007). For this reason, they represent potential molecules for the development of new drugs, especially designed for the treatment and/or control of chronic inflammatory states such as rheumatism, asthma, inflammatory bowel diseases, atherosclerosis, etc. Among these compounds, resveratrol (3,5,4′-trihydroxystilbene) has been identified as a naturally-occurring substance with anti-inflammatory activity and low side-effects. Furthermore, the researchers have established that the inhibitory activity exerted by resveratrol lies in its dual inhibition of COX and 5-LOX (Martinez and Moreno, 2000; Yoshiyuki et al., 1995). But till now, the potential of natural products-derived dual inhibitors has not been well estimated.

Previous studies have found that some derivatives of trans-phenylpropenoic acids have significant anti-inflammatory effects possessing simultaneous blockade of COX and 5-LOX (Kneen et al., 1986). In light of the above discoveries and under the illumination of combination principles, a new type of lead compound, α-substituted-3,5-dihydroxylphenyl propenoic acids, has been developed and expected to exhibit characters of dual inhibitors of COX and 5-LOX. Further research into the development of synthetic analogues has resulted in the discovery of several active molecules. The present study describes the preliminary pharmacological activity of NNU-hdpa (2-(4-hydroxylphenyl)-3-(3,5-dihydroxylphenyl) propenoic acid, Fig. 1) and investigates its modulation of pro-inflammatory mediators as well as associated intracellular signaling pathways. We conclude that NNU-hdpa exerts an obvious anti-inflammatory activity without significant gastric ulcerogenic liability, probably via dual inhibition of COX and 5-LOX and inactivation of NF-κB.

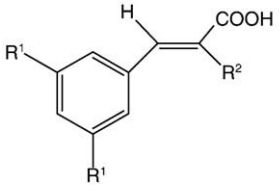
2. Materials and methods

2.1. Animals

Male Kunming mice weighing 25 ± 2 g and Sprague–Dawley rats weighing 150 ± 10 g were used. The animals were housed in a 12 h light/dark cycle in a temperature-controlled room (21–24 °C). Food and water were available *ad libitum*. Before treatment, animals were fasted overnight, with free access to water. The experimental protocol was approved by the institutional committee for animal care of Nanjing Normal University and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Cell line and cell culture

RAW 264.7 murine macrophages were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in 75- or 150-cm^2 flasks with Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated in a 5% CO_2 incubator at 37 °C.



R¹ =hydroxyl, R²=4-hydroxylphenyl

Fig. 1. Chemical structure of NNU-hdpa.

2.3. In vitro cyclooxygenase inhibition assays

The ability of NNU-hdpa and resveratrol (0.01, 0.1, 1, 10, 30, and 100 μM) to inhibit ovine COX-1 and COX-2 (IC_{50} value, μM) was determined using an enzyme immuno assay (EIA) kit according to previously reported method (Uddin et al., 2004). The solution of the test compound was prepared immediately before starting the assay.

2.4. In vitro lipoxygenase inhibition assays

The ability of NNU-hdpa and resveratrol (0.01, 0.1, 1, 10, 30, and 100 μM) to inhibit potato 5-LOX (IC_{50} values, μM) was determined using an enzyme immunoassay (EIA) kit according to previously reported method (Rao et al., 2006). The solution of the test compound was prepared immediately before starting the assay.

2.5. Xylene-induced ear edema test

In order to test whether NNU-hdpa possesses anti-inflammatory property, the xylene-induced ear edema test was performed as previously described with minor modifications (Olajide et al., 2000). NNU-hdpa was administered orally, as finely homogenized suspension in 0.5% carboxymethylcellulose (2 ml per 100 g body weight), at doses of 10, 20, and 40 mg/kg 1 h before application of xylene. A total of 20 μl of xylene was applied to the inner surface of the right ear of each mouse. The left ear remained untreated. Control animals received the vehicle (0.5% carboxymethylcellulose). Resveratrol (200 mg/kg, homogenized in 0.5% carboxymethylcellulose) was used as reference drug. The animals were sacrificed by cervical dislocation 1 h later, and two ear plugs (7 mm in diameter) were removed from both the treated ear and the untreated ear. Weights of treated and untreated ear plugs were measured with an electronic balance. The difference in weight of the two ear plugs was taken as a measure of edematous response.

2.6. Carrageenan-induced edema in rats

Groups of nine rats each were used. Paw swelling was induced by sub-plantar injection of 0.05 ml 1% sterile lambda carrageenan in saline into the right hind paw (Winter et al., 1962). NNU-hdpa at doses of 5, 10, 20 mg/kg were administered p.o. 1 h before carrageenan injection. Resveratrol (100 mg/kg) was used as described above. Control group received the vehicle only (5 ml/kg p.o). The inflammation was quantified by measuring the volume displaced by the paw, using a plethysmometer (YLS-7A, Zhenghua Biotechnology Co. Ltd., China) at time 1, 2, 3, 4, 5 and 6 h after carrageenan injection. The difference between the left and the right paw volumes (indicating the degree of inflammation) was determined and the percent inhibition of edema was calculated in comparison to the control rats.

2.7. PGE_2 and LTB_4 levels in inflamed paws

After determining the extent of the paw edema (0 h and 6 h), the animals were sacrificed by cervical dislocation. The right hind paws were removed below the ankle and degloved to remove the bone. The tissues were then homogenized in 5 ml of ice-cold saline, and sonicated on an ice bath for 12 s. The tissue homogenates were centrifuged at 2000 *g* at 4 °C for 5 min and aliquots of the supernatant were used to determine the PGE_2 and LTB_4 levels by ELISA with a microplate reader (Bio-rad, CA, USA) according to the manufacturer's instruction.

2.8. Gastric lesion assessment

Drug preparation was performed as already described in Section 2.5. Male rats were fasted overnight and then subjected to a daily oral

Table 1
In vitro COX-1/COX-2 and 5-LOX enzyme inhibition assay data for NNU-hdpa and resveratrol.

	IC_{50} (μM)		
	COX-1	COX-2	5-LOX
Resveratrol	10	3.3	10
NNU-hdpa	3.1	0.36	0.5
Aspirin	1.2	2.9	–

Values are means of two determinations acquired using an ovine COX-1/COX-2 and potato 5-LOX assay kits and the deviation from the mean is <10% of the mean value.

dose of NNU-hdpa, resveratrol and aspirin for seven successive days. The control rats were administered an equivalent volume of the vehicle. 24 h after the last dose the rats were sacrificed so that the stomach could be removed, opened along the greater curvature and cleaned gently by dipping in saline. The mucosal damage was examined by light microscopy. Gastric lesion score is calculated by summing the length of all lesions in a given stomach and expressed in mm of lesion (Kitagawa et al., 1990; Tam et al., 2000). NNU-hdpa was tested at a oral dose of up to 100 mg/kg, p.o., which is comparable to aspirin and resveratrol.

2.9. LTB_4 and PGE_2 measurement in LPS-stimulated RAW 264.7 cells

To further investigate the anti-inflammatory mechanism of NNU-hdpa, LTB_4 and PGE_2 productions in LPS-stimulated RAW 264.7 cells were examined. For LTB_4 and PGE_2 determination, RAW 264.7 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated for 18 h. Cells were pretreated with 500 μM of aspirin for 3 h to inactivate endogenous cyclooxygenase-1 (COX-1). Then, cells were washed twice with phosphate buffered saline (PBS) and pretreated with various concentrations of NNU-hdpa (0.1, 1, and 10 μM) and resveratrol (10 μM) for 2 h before further incubated for 16 h in fresh DMEM with or without 1 μg/ml of LPS. After incubation, supernatants were collected to measure LTB_4 and PGE_2 concentration by ELISA with a microplate reader (Bio-rad, CA, USA) as specified by the manufacturer.

2.10. Preparation of nuclear/cytosolic fractions and evaluation of NF-κB activity

Nuclear and cytosolic fractions from RAW 264.7 murine macrophages (about 5×10^6 cells) were performed by using a Nuclear Extract Kit (Active Motif Europe, Belgium), according to the manufacturer's instructions. The supernatant was aliquoted and stored at −80 °C until use for p50/p65 assays. Protein concentration was determined by using a protein assay.

The effect of NNU-hdpa (0.1, 1, and 10 μM), resveratrol (10 μM) on the activation of NF-κB was evaluated by commercially available ELISA kits for p50 and p65 subunits. Nuclear and cytosolic extracts were prepared as described above and evaluated for the presence of p50 and p65/RelA subunits using Trans AM™ NF-κB p50 Chemi and NF-κBp65 Chemi Transcription Factor Assay kits, according to the manufacturer's instructions. An equal amount (1 μg) of lysate was used for each sample. These assay kits specifically detected bound NF-κB p65 or p50 subunits in human extracts; activities of p50 and p65 were measured by a microplate reader and expressed as RLU (Relative Luminescence Unit). The amount of translocated p50 and p65 subunits is evaluated as the nuclear/cytoplasm (N/C) ratio (Bardelli et al., 2005).

2.11. Materials

Lipopolysaccharide (*Escherichia coli* serotype O_{127}:B_8, LPS), (Sigma Chemical Co., St. Louis, MO, USA); ovine COX-1/COX-2, potato 5-LOX, PGE_2/LTB_4 enzyme inmmunoassay kits (Cayman Chemical, Ann Arbor, MI, USA); Dulbecco's modified eagle medium (DMEM) (Gibco BRL, USA); fetal bovine serum (FBS), aspirin, xylene, carrageenan from seaweed (a mixture of lambda and kappa-carrageenans) (Sigma Chemical Co., St. Louis, MO, USA); Trans AM ™ NF-κB p50 Chemi and NF-κB p65 Chemi Transcription Factor Assay kits (Active Motif Europe, Belgium); resveratrol (Chemram Chemical, Nanjing, China), penicillin, streptomycin, carboxymethylcellulose (Sunshine biotechnology, Nanjing, China); NNU-hdpa, 2-(4-hydroxylphenyl)-3-(3,5-dihydroxylphenyl)propenoic acid, was supplied by the Department of Medicinal Chemistry, College of Pharmacy, Soochow University, China.

2.12. Statistical analysis

All results were expressed as mean ± S.D. of at least three independent experiments or with the number of observations indicated in the text. Statistical analysis was either by oneway ANOVA followed by *post hoc* Tukey test or, where appropriate, by Student's test. $P<0.05$ indicated significant difference.

3. Results

3.1. Effect of NNU-hdpa on COX and 5-LOX activities

NNU-hdpa inhibited the activities of COX-1, COX-2 and 5-LOX *in vitro* in the different extents as shown in Table 1. Resveratrol used as reference drug gave the IC_{50} values of 10 μM on COX-1, 3.3 μM on COX-2 and 10 μM on 5-LOX, respectively. The NNU-hdpa showed an inhibition on COX-1, COX-2 and 5-LOX with the IC_{50} values of 3.1, 0.36 and 0.5 μM, respectively. NNU-hdpa exhibited a higher selective inhibitory effects on COX-2 compared to resveratrol. In addition, NNU-hdpa has a greater potency with respect to 5-LOX inhibition. These findings indicated that both NNU-hdpa and resveratrol have dual COX/5-LOX inhibition although there are differences in the extent of inhibition. Further, NNU-hdpa appears to have a better well-balanced inhibition of the COX-2 and 5-LOX than resveratrol.

3.2. Effect of NNU-hdpa on xylene-induced ear edema in mice

Anti-inflammatory activity of NNU-hdpa was evaluated as the inhibition of the xylene-induced ear edema in mice. Topical application of xylene induced cutaneous inflammation at the ears of mice, which caused a significant increase in ear plug weight of the right ear when compared to the vehicle-treated left ear. As a reference drug, resveratrol (200 mg/kg) inhibited the change in ear plug weight. Treating the mice with resveratrol resulted in a percent inhibition of 43.3% in ear plug weight (Table 2). When NNU-hdpa was orally administered at 10, 20 and 40 mg/kg, it produced an inhibitory effect in xylene-induced ear edema formation in a dose-dependent fashion (Table 2) and was more potent than resveratrol at the highest dose. These data indicate that NNU-hdpa contains reasonable anti-inflammatory activity.

Table 2
Anti-inflammatory effect of NNU-hdpa on xylene-induced ear edema in mice.

	Dosage (mg/kg)	Edema weight (mg)	Inhibition (%)
Control	–	9.0 ± 1.9	–
Resveratrol	200	5.2 ± 1.7[a]	43.3
NNU-hdpa	10	4.2 ± 1.8[a]	53.3
	20	3.5 ± 1.2[a]	61.1
	40	3.0 ± 1.1[a,b]	66.7
Aspirin	200	3.2 ± 1.0[a,b]	64.4

Values represent mean ± S.D. for 10 mice. Inhibition percentages indicate relative degree of inhibition with respect to the control treated with the vehicle and xylene.
[a] $P<0.05$ compared with control mice.
[b] $P<0.05$ compared with resveratrol treated mice.

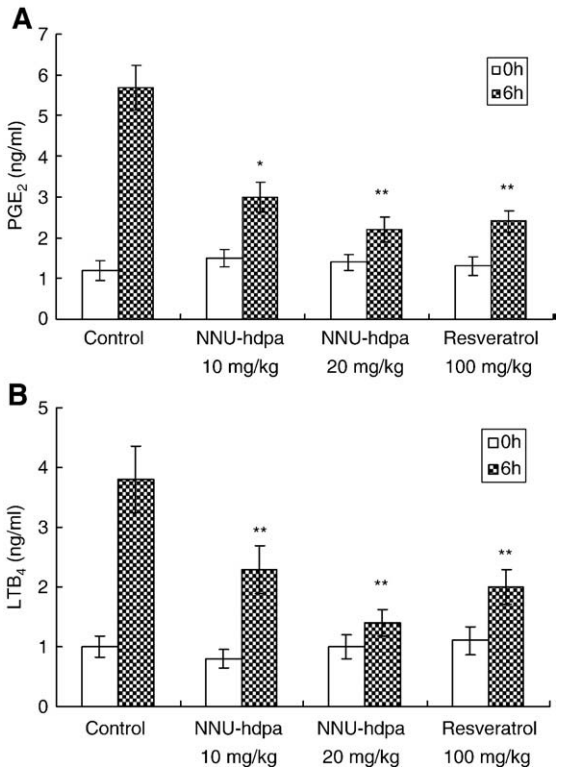


Fig. 2. Effects of NNU-hdpa on PGE_2 and leukotrienes B_4 levels in paw tissues at 6 h after injection of carrageenan (or saline) in the right hind paw. The animals were pretreated with NNU-hdpa (5 mg/kg was not shown) or resveratrol 1 h before the carrageenan injection. The animals were sacrificed 6 h later and the contents of PGE_2 (A) and LTB_4 (B) were determined in the supernatant prepared from the paws collected at time 0 and 6 h after carrageenan administration. The values are expressed as the means ± S.D. ($n=6$). *$P<0.05$ and **$P<0.01$ compared to the control group at the same time point (receive vehicle and saline injection).

3.3. Effect of NNU-hdpa on carrageenan-induced paw edema in rats

In the carrageenan-induced paw edema, there was a gradual increase in the edema paw volume in the control group during the whole experiment (6 h). As shown in Fig. 5, maximal edema formation was observed 6 h after 1% carrageenan injection, and treatment with NNU-hdpa dose-dependently inhibited carrageenan-induced paw swelling. In particular, treatment with NNU-hdpa at 20 mg/kg (p.o.) significantly suppressed edema formation 2–6 h after edema induction, the maximal inhibitory percent being 50.0%. As a reference drug, resveratrol (100 mg/kg, p.o.) produced a smaller inhibition (24.0%–37.1%) of edema development than NNU-hdpa 3–6 h after the carrageenan injection (Fig. 5).

3.4. Effect of NNU-hdpa on PGE_2 and LTB_4 levels in paw tissues during carageenan-induced edema

In addition to inhibition of hind paw swelling, the anti-inflammatory effect of both NNU-hdpa and resveratrol was also assessed using a range of biochemical assays. Thus, carrageenan-induced hind paw swelling was associated with a pronounced increase in PGE_2 and LTB_4 levels in the homogenate of an inflamed paw. A 3.8-fold increase of PGE_2 over the measurements at time 0 was observed 6 h after the carrageenan injection (Fig. 2A). The changes for LTB_4 were observed with a less increase than PGE_2 at the same time point (Fig. 2B). Both NNU-hdpa and resveratrol pretreatment reduced the rise in hind paw PGE_2 and leukotriene B_4 level observed in carrageenan-injected hind paws. NNU-hdpa (at high dose) was more potent than resveratrol as an inhibitor of leukotriene B_4 (Fig. 2A) and PGE_2 formation (Fig. 2B).

Table 3
Gastric lesion studies in rats.

	Dosage (mg/kg)	Gastric lesion score (mm)
Control	–	0
Aspirin	100	42.1 ± 5.2[a]
Resveratrol	100	0.9 ± 0.2[b]
NNU-hdpa	25	0.8 ± 0.4[b]
	50	1.1 ± 0.7[b]
	100	1.2 ± 0.5[b]

Values represent mean ± S.D. for nine rats. Gastric lesion score is calculated by summing the length of all lesions in a given stomach and expressed in mm of lesion. NNU-hdpa was tested at the doses of 25, 50 and 100 mg/kg p.o.
[a] $P<0.01$ compared with the control group (receive the vehicle).
[b] $P<0.05$ compared with the aspirin group.

3.5. Gastric lesion test

To characterize the gastrointestinal safety profiles of NNU-hdpa, experiments were performed to measure the extent of NNU-hdpa-induced stomach lesions. Aspirin at an oral dose (100 mg/kg) caused significant stomach lesions with a lesion score of >40 mm, while resveratrol and NNU-hdpa (at a high oral dose) caused negligible lesions (Table 3). Examination of stomach specimens under light microscopy revealed that in rats treated with this compound there is no injury observed in stomach mucosa. The stomach of aspirin-treated rats is characterized by appreciable damage of the protective mucosal layer.

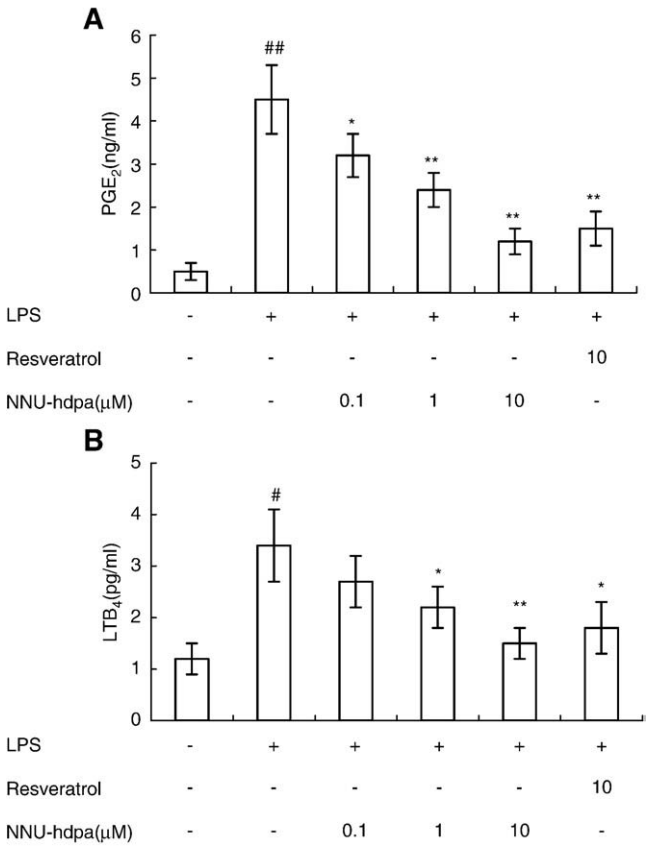


Fig. 3. Effect of NNU-hdpa on PGE_2 (A) and LTB_4 (B) production in LPS-challenged RAW 264.7 cells. Cells were treated with 1 μg/ml LPS, 10 μM resveratrol, and the indicated concentrations of NNU-hdpa. The amounts of PGE_2 and LTB_4 in the culture medium were analyzed by enzyme immunoassay. The data represent means ± S.D. of three independent experiments performed in triplicate. #$P<0.05$, ##$P<0.01$ compared to the control group, while *$P<0.05$, **$P<0.01$ compared to the LPS-challenged groups.

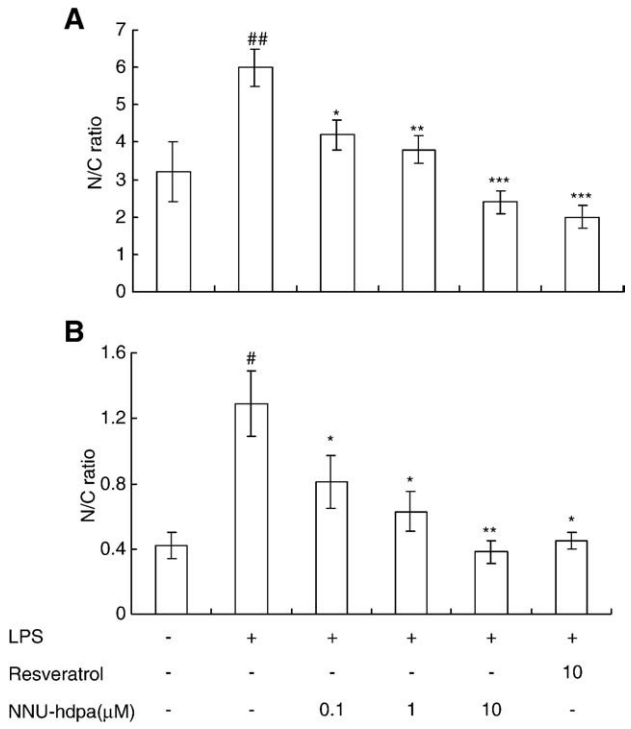


Fig. 4. NNU-hdpa inhibits NF-κB translocation in murine macrophage RAW 264.7 cells. NNU-hdpa inhibits, in a concentration-dependent manner, the nuclear translocation of activated p50 subunit (A) and p65 subunit (B) in cells challenged by LPS 1 μg/ml. The effects of resveratrol (10 μM) are demonstrated for comparison. Results are expressed as nuclear/cytoplasmic (N/C) ratio. Data are the mean ± S.D.; $n = 5$. $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared to control cells; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to LPS-challenged cells.

3.6. Effect of NNU-hdpa on PGE_2 and LTB_4 levels in LPS-challenged RAW 264.7 cells

The levels of LTB_4 and PGE_2 were significantly upregulated in LPS-challenged RAW 264.7 cells (Fig. 3A and B). After induction of LPS, the accumulation of PGE_2 and LTB_4 in RAW 264.7 cells increased from 0.5 to 4.5 ng/ml and 1.2 to 3.4 pg/ml respectively. However, NNU-hdpa inhibited the LPS-induced accumulation of PGE_2 and LTB_4 in a concentration-dependent manner. At a concentration of 10 μM, NNU-hdpa significantly decreased almost 73% and 56% of the PGE_2 and LTB_4 production in LPS-challenged RAW 264.7 cells, while resveratrol exhibited 67% and 47% inhibition of prostaglandin PGE_2 and LTB_4 production respectively. Our findings suggested that NNU-hdpa inhibited both leukotriene and prostaglandin production stimulated by LPS and had a slight higher inhibitory effects than those of resveratrol.

3.7. NNU-hdpa inhibits NF-κB activation

To ensure a quantitative evaluation, we assessed the translocation of p65 and p50 subunits in murine macrophage RAW 264.7 cells, by using a commercially available ELISA kit (Fig. 4). In un-stimulated macrophages, a low basal activation of NF-κB is detected; conversely, LPS at 1 μg/ml potently stimulates p50 (Fig. 4A) and p65 nuclear translocation (Fig. 4B). NNU-hdpa inhibits, in a concentration-dependent manner (0.1 to 10 μM), the nuclear translocation of the NF-κB p50 subunit: at the highest 10 μM concentration, LPS-induced p50 translocation is inhibited by about 63% in RAW 264.7 cells (Fig. 4A). NNU-hdpa is about as effective as resveratrol, which has been used as a reference drug (Fig. 4A). As depicted in Fig. 4B, NNU-hdpa does not significantly affect p65 translocation in un-stimulated cells, but it dose-dependently inhibits the LPS-induced one. At the maximum 10 μM concentration, NNU-hdpa is even more effective than resveratrol. (Fig. 4B).

4. Discussion

NNU-hdpa was designed and expected to have dual COX/5-LOX inhibition based on the idea of combination principle. In order to test whether the synthesized compound has achieved our goals, it was first subjected to *in vitro* COX/5-LOX inhibition assays by an enzyme immuno assay (EIA) kit. Inhibitions of COX-1,COX-2 and 5-LOX by NNU-hdpa were evidenced by their IC_{50} of 3.1, 0.36, and 0.5 μM respectively. In addition, NNU-hdpa is more potent than resveratrol in COX-1,COX-2 and 5-LOX suppression and has a better balanced profile in COX-2 and 5-LOX inhibition. Although these findings indicated that NNU-hdpa is a promising COX/5-LOX dual inhibitor, its real value still needs to be proved by *in vivo* experimental data. Then we use two well known animal models to evaluate its anti-inflammatory activity.

In xylene-induced ear edema test, NNU-hdpa at 10, 20 and 40 mg/kg, significantly suppressed xylene-induced ear edema formation in a dose-dependent fashion (Table 2). In another inflammatory model, the paw edema induced by carrageenan (in which peak edema is characterized by the presence of prostaglandins (Yang et al., 1996)), NNU-hdpa had potent inhibitory effects on paw swelling and the production of PGE_2 and LTB_4 in a homogenate of the inflamed paw. In Fig. 5, NNU-hdpa displayed its effect a little earlier than the reference drug resveratrol. Furthermore, we found that NNU-hdpa appeared to be more effective than resveratrol, the maximal inhibitory rate being 66.7% and 50.0% (43.3% and 37.1% for resveratrol) respectively (Fig. 5 and Table 2).

However, Jang et al. (1997) reported that resveratrol is a selective COX-1 inhibitor. We think that this result is questionable. It is now known to us that major side-effects of NSAIDs on the gastrointestinal system are due to inhibition of COX-1. If resveratrol is a selective COX-1 inhibitor, serious gastric injury should be observed or identified after its chronic administration. But histopathologic examination of the organs did not reveal any alterations (Juan et al., 2002). This paper also showed that aspirin inhibited the cyclooxygenase activity of COX-1 with median effective dose ED_{50} of 880 μM. A large amount of evidence has confirmed that aspirin is a typical nonselective NSAIDs, which significantly inhibit both COX-1 and COX-2. The ED_{50} of 880 μM demonstrates that aspirin has almost negligible COX inhibition because 880 μM is difficult to be achieved in tissue or plasma. Thirdly, this paper showed that resveratrol significantly reduced pedal edema in the carrageenan-induced inflammation in rats. We know that the

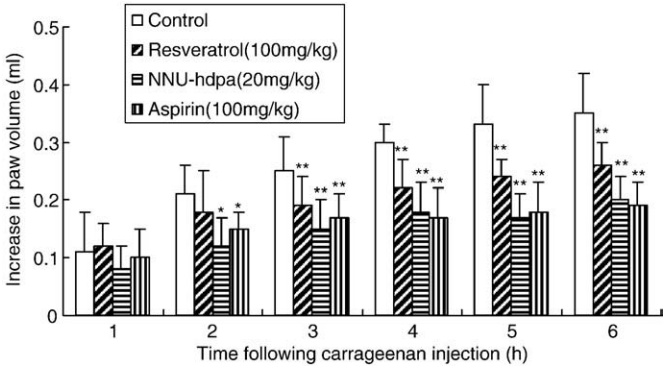


Fig. 5. Effect of NNU-hdpa on carrageenan-induced paw edema in rats. NNU-hdpa (10 mg/kg and 20 mg/kg, p.o., 5 mg/kg was not shown) was administered 1 h min before carrageenan injection. Reference control animals were treated with resveratrol (100 mg/kg, p.o.). Paw edema was induced 1 h later by sub-plantar injection of 1% carrageenan, 0.05 ml per rat. The volume of the paw was measured at intervals of 1, 2, 3, 4 5 and 6 h post-injection. The values are expressed as the means ± S.D. ($n = 9$). $^{*}P<0.05$ and $^{**}P<0.01$ indicate significant differences from the vehicle control group.

paw edema induced by carrageenan is mainly associated with COX-2 induction at the site of carrageenan injection. If resveratrol is not able to inhibit COX-2, how can the similar edema-suppressing activity to indomethacin be identified? From these points, we find the results in our paper are in the vicinity of facts and are more reasonable. These observations are also consistent with other reports (Das and Das, 2007; Kim et al., 2006; Martín et al., 2006).

One thing to be worthy of being mentioned was that in gastric lesion test NNU-hdpa did not cause significant gastric ulceration at the highest dose of 100 mg/kg for successive 7 days. While in aspirin group, the stomach of rats was characterized by complete damage of protective mucosal layer. These findings indicate that NNU-hdpa contains remarkable anti-inflammatory activity and a more favorable toxicity profile compared with aspirin treatment.

To explore the mechanism underlying potentially beneficial effects of NNU-hdpa, its effects on macrophage functions related to inflammation were investigated. Here, we found that NNU-hdpa dose-dependently inhibited LPS-induced pro-inflammatory molecules including PGE_2 and LTB_4. These inhibitions of the releases of PGE_2 and LTB_4 may be explained by the inhibitions of COX and 5-LOX activity as shown in *in vitro* COX/5-LOX inhibition assays.

NF-κB is a redox-sensitive transcription factor that comprises RelA (p65), NF-κB1 (p50 and p105), NF-κB2 (p52 and p100), c-Rel and RelB. Although different homo- and heterodimeric forms of this factor have been described, NF-κB is usually composed of the p50/p65 heterodimer (De Winther et al., 2005; Li and Verma, 2002). Growing evidence have demonstrated that NF-κB is known to play a critical role in the regulation of genes involved in cell survival, and in the coordination of the expressions of pro-inflammatory enzymes including LOX and COX-2 (Surh et al., 2001; Lappas et al., 2002). Therefore, we examined the p50 and p65 (Fig. 4) nuclear translocation of NF-κB to confirm that whether the releasing inhibitions of PGE_2 and LTB_4 were influenced by the NF-κB signaling pathway. The results obtained indicate that the p65 and p50 NF-κB subunits to the nucleus is simultaneously inhibited by NNU-hdpa in a concentration-dependent manner, and that this inhibition corresponds with the inhibitions of PGE_2, and LTB_4 production.

Leukotriene and prostaglandins are produced by the activity of three enzymes, namely 5-lipoxygenase (5-LOX), cyclooxygenase (COX)-1 and COX-2, as part of the arachidonic acid (AA) pathway (Martel-Pelletier et al., 2003; Parente, 2001; Bertolini et al., 2002). COX-1 converts AA to, among other molecules, thromboxanes, such as thromboxane A_2, and prostaglandins, such as prostaglandin D_2, E_2, F_2 and prostacyclin. The activity of COX-2 leads to production of a narrower spectrum of prostaglandins, specifically PGE_2 and prostacyclin. The prostaglandins, play a major role in many aspects of human physiology, including vascular homeostasis, gastroprotection, and pathophysiological processes, including pain and inflammation. 5-LOX (together with other enzymes) converts AA to the leukotrienes B_4, C_4, D_4 and E_4. Leukotrienes are extremely potent vasoactive and leucotactic compounds, that are in some respects more inflammogenic than prostaglandins. LTB_4, in particular, induces recruitment of leukocytes to inflamed sites, lysosomal release in neutrophils, adhesion molecule expression and subsequent plasma leakage.

It has been proposed that the inhibition of one or both COX enzymes, while reducing the levels of gastroprotective prostaglandins, may result in alternative processing of AA via the 5-LOX pathway. This increases the production of cysteinyl leukotrienes and leukotriene B_4, which contribute to gastrointestinal toxicity by promoting the migration of leucocytes, breaking down the mucosal barrier and stimulating gastric acid secretion (Brune, 2004).

For this reason, developing compounds that will inhibit COX and 5-LOX at the same time could lead to an enhanced anti-inflammatory effect and reduce undesirable side-effects. Moreover, it can also be expected that dual COX and 5-LOX inhibition may originate an improved gastrointestinal safety profile, due to a number of adverse effects of leukotrienes in the gastrointestinal mucosa, which impair mucosal integrity and exacerbate the damaging effect of noxious stimuli.

In this study, NNU-hdpa was shown to be gastric-sparing relative to aspirin with potent anti-inflammatory activity and this activity may be associated with, at least in part, dual inhibition of COX and 5-LOX. Martinez and Moreno (2000) showed that resveratrol was able to inhibit arachidonic acid release induced by LPS. This may tell us that resveratrol can regulate the upstream of the AA metabolism. Together with the results of dual inhibition of COX and 5-LOX in this study, we may conclude that resveratrol is able to exert its anti-inflammatory effect through multiple ways.

The above results also suggest that NNU-hdpa prodrugs may present a potential promising safer chemical alternative for traditional NSAIDs and selective COX-2 inhibitors for the treatment of inflammatory diseases and pain. However, although the dual inhibition concept appears a rather logical approach and NNU-hdpa has an improved profile in gastrointestinal mucosa, the lesson from COX-2 inhibitors (withdrawal from market) warns us to be cautious before drawing definite conclusions about the pharmacological profile of a new class of drugs. Large clinical trials will establish in the future whether theoretical expectations on safety and efficacy of these drugs are achieved. Any potential advantage of dual COX/5-LOX inhibitors over standard NSAIDs or COX-2 inhibitors would need to be demonstrated in the clinic. This may tell us that the dual inhibitors of COX and 5-LOX still have a long way to go before they are available in market.

Conflict of interest

The authors state no conflict of interest.

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