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Benzothiazolium compounds: novel classes of inhibitors that suppress the nitric oxide production in RAW264.7 cells stimulated by LPS/IFNγ

Huan-Yi Tseng,[a,†] Ssu-Hui Wu,[a,†] Wen-Hsin Huang,[b] Shao-Fang Wang,[a] Yung-Ning Yang,[a] Neeraj Mahindroo,[a] Tsu Hsu,[a] Weir-Torn Jiaang[a] and Shiow-Ju Lee[a,*]

[a]*Division of Biotechnology and Pharmaceutical Researches, National Health Research Institutes, 35, Keyan Road, Zhunan Town, Miaoli County, Taiwan, ROC*
[b]*School of Pharmacy, National Defense Medical Center, Taipei, Taiwan, ROC*

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Abstract—A series of benzothiazolium compounds were identified as novel classes of inhibitors of nitric oxide production in a cell culture system. They exhibited ~1600 folds potency with IC_{50} at ~50 nM to several μM as compared to IC_{50} 88.4 μM of L-NMMA, a known inhibitor of nitric oxide synthase. The mechanistic studies suggest that decreased iNOS protein synthesis and mRNA transcription, at least in part, were related to the inhibitory activity of effective benzothiazolium compounds. The correlation of in vivo and in vitro activities using mouse paw edema model was also demonstrated.

1. Introduction

Three distinct isoforms of nitric oxide synthase (NOS), neural (nNOS), endothelial (eNOS), and inducible (iNOS), utilize the L-arginine and oxygen as co-substrates and convert them into L-citrulline and nitric oxide (NO).[1–3] Among them, iNOS is induced to express and generate large quantities of nitric oxide upon the stimuli of endotoxins or cytokines involved in pathological responses at the quantities of nM in contrast to pM from nNOS and eNOS.[4] However, NO is a double-edged molecule. The improper overproduced NO by iNOS results in acute and chronic inflammation related diseases. Overproduction of NO by iNOS has been implicated in various pathological processes including septic shock, tissue damage following inflammation, and rheumatoid arthritis.[5–9] Selective inhibition of iNOS may be beneficial in various forms of shock and inflammation.

A lot of work has been put into design of selective NOS inhibitors since NG-methyl-L-arginine (L-NMMA) was identified as the first inhibitor of NOS.[10–13] Recently, much progress in terms of potency and selectivity has been obtained since most of the effort has been focused in finding selective non-amino-acid based analogue's NOS inhibitors which include L-arginine competitive inhibitors, analogues of tetrahydrobiopterine, and inhibitors that interfere with the access of the NOS active sites etc.[11,12] Most of these works determined the compound's potency using recombinant NOS enzymes with a few of them demonstrating in vivo activity.[10,12] Another approach using LPS-stimulated RAW264.7 macrophage cells has also been exploited to evaluate inhibitory activity of compounds in iNOS.[14–17]

2. Results and discussion

Herein, we have utilized the cell-based assay system of LPS/IFNγ stimulated RAW264.7 cells to identify a novel class of iNOS inhibitors.[18] This assay system has a broad spectrum for finding agents for suppressing NO production in acute or chronic inflammatory conditions. The high throughput screening of a library of compounds indicated that compounds with benzothiazolium

Keywords: Anti-inflammation; Benzothiazolium compound; Nitric oxide; IFNγ; Inducible nitric oxide synthase; iNOS; RAW264.7 cells; L-NMMA; LPS; Paw edema.
* Corresponding author. Tel.: +886 37 246 166x35715; fax: +886 37 586 456; e-mail: slee@nhri.org.tw
† Equal contribution authors.

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moiety inhibit nitric oxide (NO) production generated from RAW264.7 cells stimulated by LPS/IFNγ. Therefore, we collected about two hundreds of benzothiazolium compounds to further study this class of compounds. The inhibitors identified from herein are not limited to specifically inhibit iNOS enzyme per se. They may ultimately include those molecules directly or indirectly affect the transcription or translational events of iNOS expression or activity.

The compounds showed different degrees of potency in inhibiting nitric oxide (NO) production generated from RAW264.7 cells stimulated by LPS/IFNγ. In this assay system, L-NMMA, an archetypal competitive NOS inhibitor to which other inhibitors are often compared,[19–21] exhibited an IC_{50} of 88.4 +/− 15.4 μM. Based on the potency and structure, the representative benzothiazolium compounds shown herein were divided into five classes.

The A and B classes possess a 2-vinylbenzothiazolium and a 2-[(4-aminophenyl)vinyl]benzothiazolium core structure respectively. They exhibited weak activity with most potent compounds showing IC_{50} of 3.6 and 2.2 μM, respectively.

The introduction of a quinoline, giving C class with a 2-[(quinoline)vinyl]benzothiazolium core structure, resulted in ~10–20 folds increase in activity as compared to the most potent compounds of classes A and B. Compounds **C1**–**4**, with alkyl groups at R1 and R2, exhibited IC_{50}s ranging from 200 to 300 nM, irrespective of the length of the alkyl substitution. However, when both R1 and R2 were replaced with $(CH_2)_3SO_3H$ (**C5**), the activity decreased by 225–300 folds (IC_{50} of 64.6 μM).

Further, replacement of the quinoline in C class with a benzothiazolium moiety gave the D class with a 2-[(benzothiazol)vinyl]benzothiazolium core structure. Compound **D2** with ethyl groups at R1 and R2 was ~12-folds more potent than **D1** which had methyl substitutions instead. In addition when R2 ethyl group of **D2** was replaced with $(CH_2)_2OH$ or $(CH_2)_2COOH$, the activity decreased significantly by 26–28 folds (**D3** and **D4**).

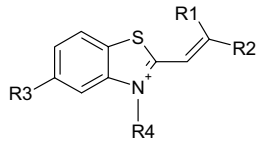
The E class having a 1-benzothiazol-3-benzothiazoliumpropenyl core structure exerted potent activity with IC_{50} ranging from 55 to 847 nM. Compounds with either methyl or ethyl substitution at R1, R2, and R3 (**E1**–**E3**) were more potent than the compounds with R2 as cyclobutane, methoxyl, thiolmethyl or amine (**E4**–**7**) by factors ranging from 4 to 15. Furthermore, the replacement at both R1 and R3 with $(CH_2)_3SO_3H$, also significantly decreased the compound's potency with a factor of ~60 (**E7** to **E10**) in a manner similar to the above analysis for **B6**, **C5**, **C18**, **D3**, and **D4**. Similar results were also obtained with **E11** and **E12**, as compared to **E2** and **E3** respectively.

In summary, classes C, D, and E are more potent than classes A and B in suppressing NO production. It appeared that the alkyl substitutions at the iminium or enamine positions of these classes of benzothiazolium compounds favor high potency.

In consideration of the effects in growth inhibition or cytotoxicity of these compounds would mislead the interpretation of their potency, measurement for relative viable cell numbers using MTS assay was performed. Most of these compounds at the concentration of 10 μM did not show significant growth inhibition or cytotoxicity while some of them showed slight to moderate effects with **C4**, **C6**, **C10**, **E1**, **E3**, **E4**, **E8**, and **E9** exhibiting GI_{50}s ranging from 5.5 to 9.3 μM, respectively (Tables 1–5).

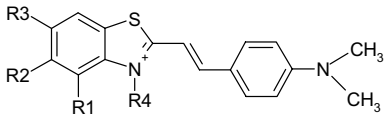
To elucidate the possible mechanisms of active benzothiazolium compounds for inhibition of the NO production. Representative active compounds **A7**, **B4**, **C1**, **C7**, **E3**, and **E7** were examined in relation to iNOS protein expression along with L-NMMA in the LPS/IFNγ stimulated RAW264.7 cells.[22] The iNOS protein expression was enormously induced upon stimulation of LPS/IFNγ in RAW264.7 cells (Fig. 1, lane 2 compared to lane 1). The concurrent treatment of effective benzothiazolium compounds with LPS/IFNγ inhibited the iNOS protein expression in correlation with their potency. For example, **C1** and **E3** (IC_{50}s 216 and 62 nM, respectively) almost completely inhibited the iNOS protein expression while **B4** (IC_{50} 2.3 μM) exhibited about

Table 1. Structure and activity of the class A compounds with a 2-vinyl-benzothiazolium core structure



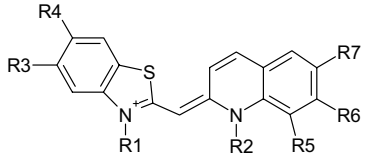
Compd	R1	R2	R3	R4	NO suppression IC_{50} (nM)	MTS GI_{50} (μM)
A1	CH_2CH_3	SCH_3	OCH_3	CH_2CH_3	3993 ± 1624	>10
A2	SCH_3	CH_2CH_3	OCH_3	CH_2CH_3	3636 ± 867	>10
A3	SCH_3	CH_3	OCH_3	CH_2CH_3	18,449 ± 2360	>10
A4	SCH_3	CH_3	H	CH_2CH_3	24,235 ± 793	>10
A5	CH_2CH_3	SCH_3	OCH_3	$(CH_2)_3SO_3$	66,113 ± 9436	>10
A6	CH_3	SCH_3	OCH_3	$(CH_2)_3SO_3$	69,500 ± 2345	>10
A7	CH_3	$NHCH_2C_6H_5$	H	CH_2CH_3	7611 ± 995	>10
A8	CH_3	NHC_6H_5	H	CH_2CH_3	44,762 ± 4987	>10

Table 2. Structure and activity of the class B compounds with a 2-[(4-amino-phenyl)-vinyl]-benzothiazolium core structure



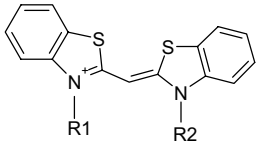
Compd	R1	R2	R3	R4	NO suppression IC_{50} (nM)	MTS GI_{50} (μM)
B1	H	H	H	CH_3	4802 ± 1786	>10
B2	H	H	H	CH_2CH_3	4937 ± 1813	>10
B3	H	H	CH_3	CH_2CH_3	2268 ± 953	>10
B4	H	CH_3	H	CH_2CH_3	2331 ± 75	>10
B5	R1R2 fused benzene		H	CH_3	2158 ± 819	>10
B6	H	$COOCH_3$	H	CH_2CH_3	10,832 ± 306	>10
B7	H	H	$NHCOCH_3$	CH_2CH_3	3307 ± 597	>10

Table 3. Structure and activity of the class C compounds with a 2-[(quinoline)-vinyl]-benzothiazolium core structure



Compd	R1	R2	R3	R4	R5	R6	R7	NO suppression MTS IC_{50} (nM)	GI_{50} (μM)
C1	CH_3	CH_2CH_3	H	H	H	H	H	216 ± 90	>10
C2	CH_2CH_3	CH_2CH_3	H	H	H	H	H	244 ± 143	>10
C3	CH_2CH_3	$(CH_2)_2CH_3$	H	H	H	H	H	288 ± 74	>10
C4	$(CH_2)_3CH_3$	CH_2CH_3	H	H	H	H	H	215 ± 51	5.5 ± 2.2
C5	$(CH_2)_3SO_3H$	$(CH_2)_3SO_3H$	H	H	H	H	H	64,557 ± 4133	>10
C6	CH_2CH_3	CH_2CH_3	CH_3	CH_3	H	H	H	258 ± 28	5.8 ± 0.8
C7	CH_2CH_3	CH_2CH_3	CH_3	H	H	H	H	295 ± 48	>10
C8	CH_2CH_3	CH_2CH_3	Br	H	H	H	H	241 ± 19	>10
C9	CH_2CH_3	CH_2CH_3	I	H	H	H	H	259 ± 50	>10
C10	CH_2CH_3	CH_2CH_3	OCH_3	H	H	H	H	1065 ± 418	9.3 ± 1.2
C11	CH_2CH_3	CH_2CH_3	OCH_3	OCH_3	H	H	H	684 ± 14	>10
C12	CH_2CH_3	CH_2CH_3	H	H	H	CH_3	H	183 ± 25	>10
C13	CH_2CH_3	CH_2CH_3	H	H	H	H	$NHCOCH_3$	13,345 ± 3139	>10
C14	CH_2CH_3	CH_3	H	H	H	CH_3	H	253 ± 35	>10
C15	CH_2CH_3	CH_3	H	H	H	H	CH_3	178 ± 29	>10
C16	CH_3	CH_2CH_3	H	H	CH_3	H	H	318 ± 42	>10
C17	CH_3	CH_2CH_3	H	H	H	CH_3	H	444 ± 86	>10
C18	CH_2CH_3	$(CH_2)_3SO_2OH$	H	H	H	CH_3	H	27,177 ± 3163	>10

Table 4. Structure and activity of the class D compounds with a 2-[(benzothiazol)-vinyl]-benzothiazolium core structure

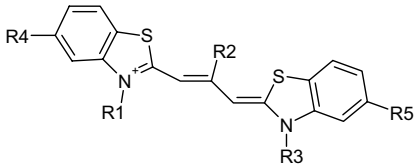


Compd	R1	R2	NO suppression IC_{50} (nM)	MTS GI_{50} (μM)
D1	CH_3	CH_3	4690 ± 1833	>10
D2	CH_2CH_3	CH_2CH_3	403 ± 232	>10
D3	CH_2CH_3	$(CH_2)_2OH$	10,706 ± 401	>10
D4	CH_2CH_3	$(CH_2)_2COOH$	11,453 ± 1286	>10

20% inhibition. In addition, L-NMMA at the concentration of 10 μM did not inhibit iNOS protein expression as expected (Fig. 1, lane 3).

In addition, the mRNA expression affected by the treatment of these compounds was examined by RT-PCR[23] (Fig. 2). The results also showed great correlation to their potency in terms of NO suppression though not completely corresponding to respective counter protein expression levels as detected by Western analysis. Taking together results from Western and RT-PCR analyses, the inhibitory effects of active benzothiazolium

Table 5. Structure and activity of the class E compounds with a benzothiazol-propenyl-benzothiazolium core structure



Compd	R1	R2	R3	R4	R5	NO suppression IC_{50} (nM)	MTS GI_{50} (μM)
E1	CH_3	CH_2CH_3	CH_3	H	H	55 ± 22	5.5 ± 3.9
E2	CH_2CH_3	CH_3	CH_2CH_3	H	H	198 ± 98	>10
E3	CH_2CH_3	CH_2CH_3	CH_2CH_3	H	H	62 ± 38	5.5 ± 3.9
E4	CH_3	Cyclobutane	CH_3	H	H	662 ± 214	6.5 ± 3.0
E5	CH_2CH_3	OCH_3	CH_2CH_3	H	H	461 ± 237	>10
E6	CH_2CH_3	SCH_3	CH_2CH_3	H	H	637 ± 171	>10
E7	CH_2CH_3	NH_2	CH_2CH_3	H	H	847 ± 52	>10
E8	CH_3	CH_2CH_3	CH_3	Cl	Cl	402 ± 209	7.9 ± 3.5
E9	CH_2CH_3	CH_2CH_3	CH_2CH_3	Cl	Cl	741 ± 241	9.0 ± 2.3
E10	$(CH_2)_3SO_3H$	NH_2	$(CH_2)_3SO_3H$	H	H	51,873 ± 1797	>10
E11	CH_2CH_2OH	CH_3	CH_2CH_2OH	H	H	20,245 ± 2287	>10
E12	CH_2CH_3	CH_2CH_3	$(CH_2)_3SO_3H$	H	H	5781 ± 360	>10

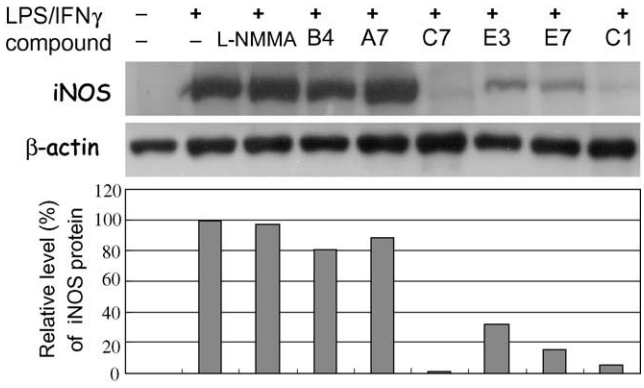


Figure 1. Western Analysis of iNOS protein expressed in RAW264.7 cells stimulated with LPS/IFNγ or plus compound treatments, respectively, as indicated at the concentration of 10 μM. Western analysis of β-actin was used for internal loading control and the relative iNOS protein levels were normalized with the respective amounts of β-actin. Results shown here are representative of three independent experiments.

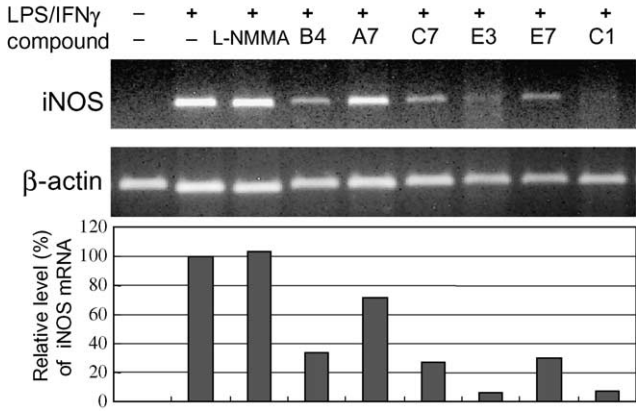


Figure 2. RT-PCR analysis for iNOS mRNA expression levels in RAW264.7 cells stimulated with LPS/IFNγ or plus compound treatments, respectively, as indicated at the concentration of 10 μM. RT-PCR products of β-actin were used for house keeping gene control and the relative iNOS mRNA levels were normalized with the respective amounts of β-actin. Results shown here are representative of three independent experiments.

compounds in NO production of LPS/IFNγ stimulated RAW264.7 cells were related through decreased iNOS protein synthesis or mRNA expression. Thus, the active benzothiazolium compounds, at least in part, alleviate the pathway(s) of stimulation by LPS/IFNγ to iNOS gene or protein expression, and thereby suppress the production of NO.

Two potent compounds, **C1** and **E3**, were subjected to SD mouse paw edema assay for in vivo anti-inflammatory efficacy measurement.[24] The results (Fig. 3) showed compounds **C1** and **E3**, when compared to ibuprofen or blank (vehicle) controls, exerted potent anti-inflammatory effect at the dose of 10 mg/kg. The in vivo efficacy of compound **E3** was greater than that of **C1**, which was correlated to their in vitro inhibitory activity of NO production. In addition, **C1** compound was also examined for its effect in activation of iNOS promoter and NF-κB in RAW264.7 cells stimulated by LPS/IFNγ. Neither inhibition effects were found significantly exerted by compound **C1** (data not shown), which suggest the effective mechanism of the compound **C1** was distinct from dexamethasone,[25–27] another iNOS inhibitor reportedly inhibiting the expression of iNOS mRNA and protein through inhibiting the activation of iNOS promoter and NF-κB.

In conclusion, we have identified a series of benzothiazolium compounds as novel potent inhibitors of NO production using a cell-based system and correlated the in vitro and in vivo efficacy. The broad spectrum in structure and effective mechanisms of these active compounds highly warrants further study for their therapeutic potential. More studies for in vivo activity and effective mechanisms of these compounds, for exam-

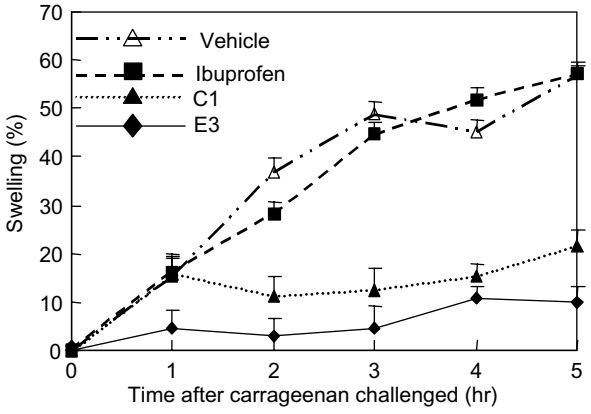


Figure 3. In vivo anti-inflammatory activities of compounds **C1** and **E3** using carrageenan-induced hind paw edema test in rats. Vehicle (DMSO) and ibuprofen were used as blank and reference compound controls. Compounds **E3**, **C1**, and ibuprofen were administrated at the dose of 10 mg/kg.

ple, C and D classes, for suppressing NO production and anti-inflammation/anti-arthritis are being carried out in our laboratory.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.02.063.

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18. Cell culture and chemicals—RAW 264.7 cells were maintained in high glucose DMEM (Hyclone) with 4 mM glutamine, 4500 mg/L glucose, 1% non-essential amino acids (Biological industries, Israel), and 10% bovine serum (FetaClone III, HyClone) but without sodium pyruvate. RAW264.7 cells were scrapped off the culture plates for passage without any trypsin or EDTA treatment. All cells were grown in an incubator at 37 °C and 5% CO_2. Lipopolysaccharide of *E. coli* O111:B4 was purchased from Chemicon International (California, USA,), IFN-γ recombinant protein from R&D systems Inc. (US, Minneapolis), and L-NMMA from Sigma–Aldrich (US). The assayed benzothiazolium compounds were purchased from ChemDiv Inc. (US, San Diego) unless indicated specifically. The compounds D1 and F2 were synthesized by Dr. Wei-Tong Jiaang at DBPR/NHRI. Compounds A2 and D2 were purchased from Sigma–Aldrich and E2 and F2 from Acros Organics. Compound C1 was purchased from ChemDiv Inc. and also synthesized by Dr. Wei-Tong Jianng for in vivo efficacy measurement. NMR spectra data of these compounds were examined for purity. Determination of nitric oxide production—RAW 264.7 cells were seeded 70,000 cells /well and cultured in 96-well culture plate. After 24-h incubation, the medium was replaced with complete medium containing stimuli of LPS (5 μg/mL)/IFNγ (20 ng/mL) and the tested compounds were added. After 18 h, the supernatants were subjected to measurement of nitric oxide production using Nitrate/Nitrite assay kit (Cayman Chemical). Nitric oxide was measured as the accumulation of nitrite and nitrate in the incubation medium. Nitrate was reduced to nitrite with nitrate reductase and determined spectrophotometrically with Griess reagent at OD_{405}. The attached cells were subjected to growth inhibition measurement using MTS assay (Promega, Madison, WI, USA). The cells were treated with at least five different concentrations of test compounds. The results of these assays were used to obtain the dose–response curves from which IC_{50} of nitric oxide production and cytotoxicity values were determined.
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22. Western Blot analysis—iNOS and β-actin protein were analyzed by immunoblotting with anti-iNOS (Biomol) and anti-β-actin (Chemicon) antibodies respectively. The cell

lysates were subject to SDS-PAGE and the separated proteins were electrophoretically transferred to nitrocellulose membrane. The resultant membranes were incubated with blocking solution, first antibody, and second antibody for 2, 2 and 1 h, respectively and wash procedures were carried out accordingly.Antigen-antibody complexes were detected using ECL detection reagents (Perkin–Elmer, Western Blot Chemiluminescence Reagent Plus) according to the manufacturer instruction.

23. Reverse transcription–polymerase chain reaction (RT-PCR) analysis of iNOS mRNA expression—RAW264.7 cells (880,000 cells/well in 6-well plates) were incubated overnight before LPS (5 μg/mL)/INFγ (20 ng/mL) and/or compound treatment for18 h. After wash with PBS twice, total RNA was isolated from the cell pellet, using RNA isolation kit (Trizole, Invitrogen, San Diego, USA). The total amount of RNA was determined by the absorbance at 260 nM. Five micrograms (5 μg) of RNA was reverse transcribed into cDNA using SuperScript™ II Reverse transcriptase and olig(dT) primers (Invitrogen, co.). The PCR samples, contained in 50 μL of the reaction mixture, comprised of 5 μL 10X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 2.5 units of VioTaq™ DNA polymerase (Viogene-Biotek Corp.) and 0.5 μM of sense and anti-sense primers each and 1 μL of RT product aforementioned. The sense and anti-sense primers used for iNOS PCR were 5′-GTGGTGACAAGCACATTTGG-3′ and 5′-GGCTG-GACTTTTCACTCTGC-3′, respectively and the sense and anti-sense primers for β-actin were 5′-TGTTACCAACTGGGACGACA-3′ and 5′-TTTGA-TGTCACGCACGATTT-3′, respectively. The PCR was carried out with variable cycle numbers to obtain the results within the exponential range. The PCR amplification was performed under the following conditions: 20–28 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, using a thermal cycler (Eppendorf). The amplified PCR products, in length of 487 bp for iNOS and 415 bp for β-actin, respectively, were separated on a 2% agarose gel and visualized by CYBR Green staining. The bands in the agarose-gel were photographed and the fluorescence intensities were analyzed using an Alpha Imager and Gel-Pro (Media Cybernetics).
24. Determination of anti-inflammatory activities using carrageenan-induced hind paw edema test in rats—Male albino Wistar rats (250–285 g) were housed and cared for under the guidelines of the Institutional Animal Care and Use Committee at the National Defense Medical Center, Taiwan. Rats were divided into six groups, one of them being the control. In order to produce inflammation, 100 μL of 1% carrageenan solution in normal saline was injected into right hind paw subplantar tissue, according to the modified method of Winter et al.[28] The development of paw edema was measured plethysmographically (UGO Basile 7140 Plethysmometer, Varese, Italy) and recorded prior to this administration. One hour before carrageenan challenged, the sample preparations (10 mg/kg) were injected i.p. to the divided groups. Normal saline was injected in the same way to the control group. After carrageenan challenged, paw volumes (ml) were measured hourly up to 5 h. The percentage of paw edema and the inhibition of inflammation were calculated by the previously reported protocol. Experimental data were expressed as the mean ± SEM. A value of $p < 0.05$ was considered statistically significant. Experimental data versus control were evaluated by the one-way analysis of variance (ANOVA), followed by Turkey's *t*-test compared to control group.[29]
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