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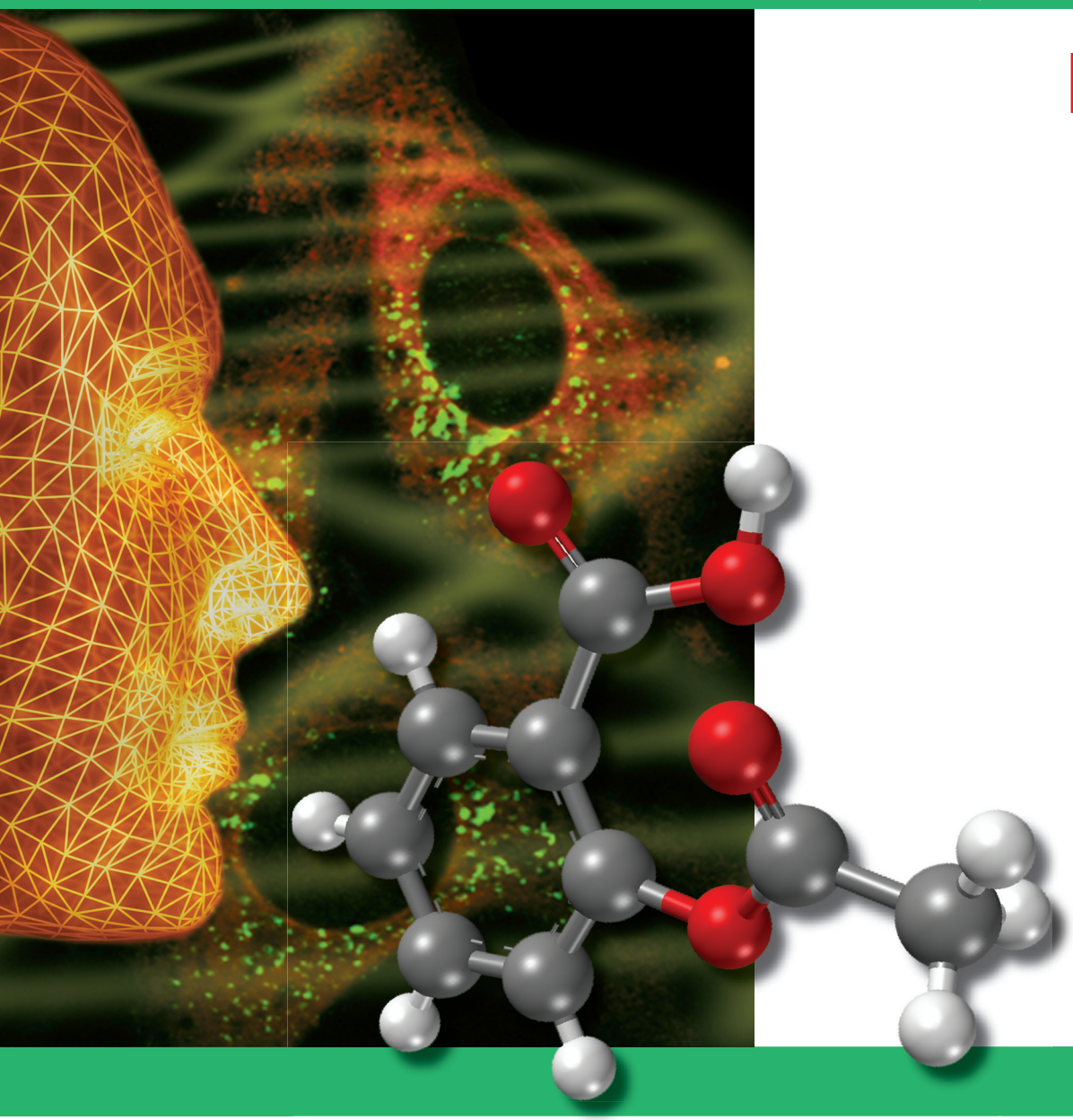

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Full Paper

Synthesis, Molecular Mechanism and Pharmacokinetic Studies of New Epoxy Lignan-Based Derivatives

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The oxidative demethylation procedure for a new epoxy lignan isolated from *Piper nigrum* was applied to the synthesis of 3′-methoxy-3″,4″-(methylenedioxy)-2,5-epoxylignan-4′-ol-6′-one. This compound inhibited the mRNA expression of the protein patched homolog (Ptch) in human pancreatic cancer cells (PANC1) and therefore might be valuable as a probe for tumor-related disease. The pharmacokinetic profile of 3′-methoxy-3″,4″-(methylenedioxy)-2,5-epoxylignan-4′-ol-6′-one was rapidly determined using ultra-fast liquid chromatography. The compound was rapidly absorbed in blood.

Keywords: Epoxy lignan / Glioma / Pharmacokinetics profile / Ptch mRNA expression / UFLC

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Introduction

Gli is a transcriptional target of the hedgehog (Hh) pathway. Recent studies have reported that overexpression of Gli contributes to the progression of pancreatic [1] and prostate [2] cancer due to the direct association of Gli with a specific binding site (5′-GACCACCCA-3′) in the promoter region of the target genes [3]. Several Gli inhibitors were previously isolated from plants [4–7], including a new epoxy lignin: 3′,6-dimethoxy-3″,4″-(methylenedioxy)-2,5-epoxylignan-4′-ol (**1**) [8].

Compound **1** was isolated from *Piper nigrum* using the immobilization of Gli-GST on carboxylic acid magnetic Dynabeads [8, 9] and was confirmed to have Hh signaling inhibitory activity and to be selectively cytotoxic against PANC1 cells [10]. At a concentration of 4.1 μM, the compound downregulated the protein level of nuclear Gli1 in a pancreatic cancer cell line (PANC1) by blocking the translocation of Gli transcription factors into the nucleus in PANC1 cells [10]. The structure is unique due to the capacity of a methoxy group attached to C-6 between benzene and five-membered rings to freely rotate. This position was chemically modified using the procedure developed by McOmie et al. [11] and Sowa and Thomas [12].

In the presence of BBr_3 in CH_2Cl_2 at −80°C, treatment of **1** for 20 h at room temperature gave compound 3′-methoxy-3″,4″-(methylenedioxy)-2,5-epoxylignan-4′,6-diol (**2**). The synthesis of 3′-methoxy-3″,4″-(methylenedioxy)-2,5-epoxylignan-4′-ol-6′-one (**3**) was accomplished in the presence of dimethyl sulfoxide in acetic anhydrate reagents at 35–40°C, followed by organic layer separation. Because a similar preparative method applied for obtaining modified structures of the epoxy lignan derivative has not yet been reported, we herein report the first example.

Structural modification of natural compounds should be environmentally free of metal traces. In organic chemistry, oxidative demethylation can be performed using nitric acid [13], silver(II) oxide (AgO)-mineral acid [14], cerium(IV) ammonium nitrate (CAN) [15], NBS-H_2SO_4 [16] and cobalt(III) fluoride (COF_3) [17]. Tohma et al. [18] reported that polymer-supported hypervalent iodine reagents replaced highly toxic metal oxidants due to their low toxicity and high yield. In the present study, we performed the standard procedure without metal waste interference. The oxidative demethylation reaction was completed after 7 h at room temperature by mixing reagents in inert solvent which produced **3** in 65% yield (Scheme 1).

The majority of lignan analogs and epoxy lignan derivatives had inhibitory activities against various cancer cell

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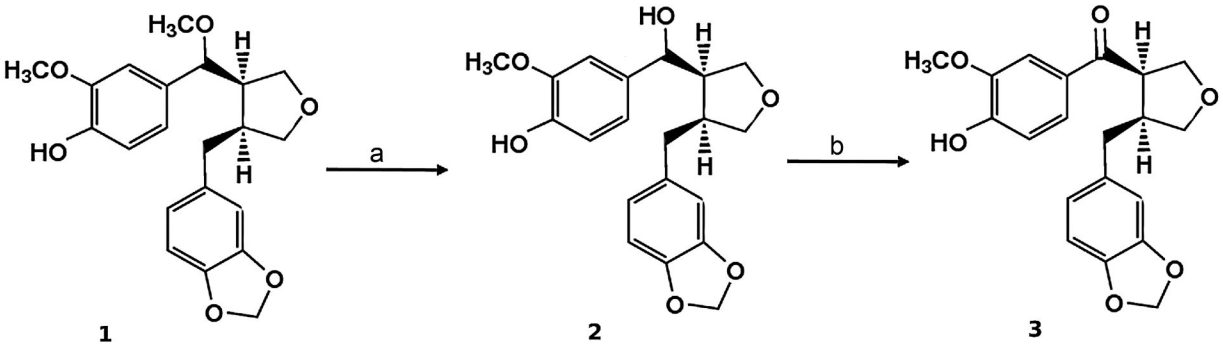

Scheme 1. Synthetic routes of the compounds: (a) BBr_3, CH_2Cl_2, −80°C, 20 h, 86%; (b) DMSO, Ac_2O, 35–40°C, 65%.

lines [19–21]. Huang et al. [22] found that lignans not only modulate transduction signal pathways and hormone metabolism but also trigger apoptosis by cell cycle arresting. As described in our previous report, DMEO, a lignan precursor of MMEO, is a potential Gli inhibitor in the nuclei site of PANC-1 without Smo interference [10].

To further understand the molecular mechanism of new epoxy lignan-based derivatives, we examined the inhibition capability of compounds **1**, **2**, and **3** on Ptch mRNA expression of PANC1 cells using real-time PCR analysis. Ptch is known as a repressive sonic Hh receptor [23]. The elevated expression of this gene results in the release of Gli leading to tumor formation and progression [23, 24]. Because compound **3** represents a promising approach in the search for antitumor agents, it is also important to study the pharmacokinetics of **3** in the plasma of rabbits.

Results and discussion

Chemistry

The target compounds were synthetized through two steps (Scheme 1). In the first step, compound **1** was demethylated using boron tribromide to obtain compound **2** [11]. In the last step, compound **2** was converted into compound **3** in the presence of a mixture of DMSO and acetic anhydride [3]. The HRFAB mass spectrometry of **2** and **3** run in the postive mode showed a molecular protonated ion $[M+Na]^+$ peak at *m/z* 381.072 and *m/z* 379.125, respectively. The IR spectrum of compound **3** but not compound **2** confirmed the presence of a carbonyl group (1725 cm^{-1}) and a hydroxyl group (3317 cm^{-1}, br).

Molecular mechanism

In cancer cells, the binding of the Hh protein ligand to Ptch receptor allows proto-oncogene Smoothened (Smo) to activate the Gli family of transcription factors [25]. Searching for compounds that deactivate Gli by inhibiting Ptch mRNA expression in PANC1 cells thus may be a good protein target for the development of anticancer drugs. In this research, we used cyclopamine, one of the naturally occurring Hh inhibitors, as a positive control. As depicted in Fig. 1, treatment with 0.8 μM cyclopamine downregulated Ptch mRNA expression in PANC1 cells. Likewise, treatment with compound **3** but not compounds **1** and **2** at 0.8 μM reduced the mRNA expression of Ptch in PANC1 cells compared to a negative control.

Pharmacokinetic studies

The pharmacokinetic parameters of compound **3** were determined for each of the assay data using Microsoft Excel 2010. Pharmacokinetics data were fitted to a two-compartment pharmacokinetic model in which the mean AUC were approximately proportional to dose. Cpmax values were determined as model-estimated concentrations immediately after bolus administration, and AUC values were calculated from estimated plasma concentration profiles using the trapezoidal rule and extrapolation to infinity by standard

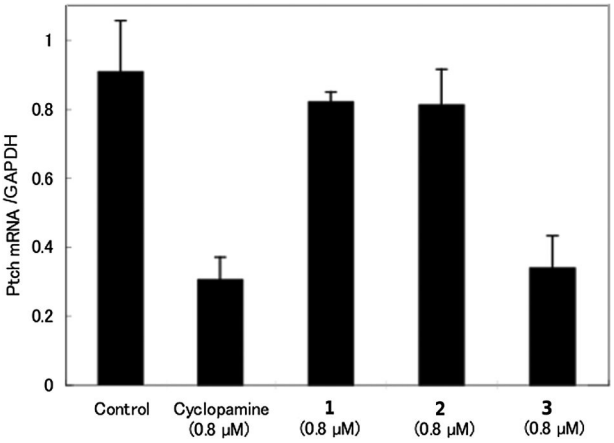

Figure 1. Inhibition of glioma-mediated mRNA expression of Ptch by compounds **1**, **2**, and **3** in PANC1 cells. GAPDH was used as an internal control. The assays were performed at 0.05% DMSO ($n = 3$). Error bars represent the SD.

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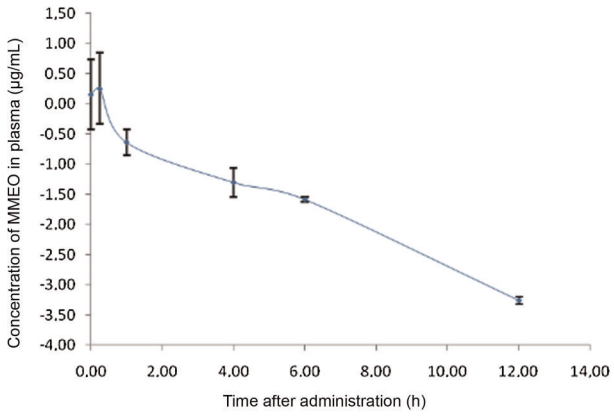

Figure 2. Concentration-versus-time plots after single dosing with 0.8 mg/kg of body weight. Each point plots the mean concentration ± standard error of the mean (error bars) for three rabbits each at that time.

techniques. β is the first-order rate constant for the elimination and distribution of **3**. *T*/2 (α) and *T*/2 (β) are the plasma half-lives of the drug during the distribution and elimination phases, respectively. *T*/2 (α) was calculated by extrapolation as described by Greenblatt and Koch-Woesser [26]. V_1 is the total drug distribution volume of **3** in the central compartement, and the V_{darea} is the volume of distribution by area. Mean plasma concentration-versus-time profiles and the parameters of **3** are summarized in Fig. 2 and Table 1, respectively. The administration of **3** at single doses at 0.8 µg/kg of body weight resulted in the reduction of reducing peak levels in plasma that ranges from 1.17 ± 0.58 to 0.04 ± 0.06 mg/mL (means ± standard errors of the means). The mean of the area under the concentration-time curve was 5.06 ± 0.50 (µg.h/mL) and the renal clearance was 0.29 ± 0.02 mL/min. The mean distribution and elimination half-life were 0.37 ± 0.12 and 7.13 ± 4.53 h, respectively. No significant changes in pharmacokinetic parameters and a little accumulation were noted upon repeated administration.

Conclusion

In conclusion, new epoxy lignan-based derivatives were synthetized and their effect on Ptch mRNA expression on PANC1 cell lines was examined. Compound **3** exhibited linear pharmacokinetics in plasma that fits into a two-compartment open pharmacokinetic model. A little accumulation in plasma was observed after a single dose and the compound was well tolerated after the single dose. Pharmacokinetic studies in rabbits will contribute to selecting appropriate dosing regiments in tumor models and may be useful for translating the findings of the models into clinical studies.

Experimental

Chemistry

General

Melting points were determined on a Thomas–Hoover melting point apparatus and were corrected. IR and mass spectra were obtained on a PerkinElmer 257 and Hitachi PerkinElmer RMU-6E, respectively. ^{1}H NMR and ^{13}C NMR spectra were recorded in CD_3OD unless otherwise indicated with Bruker AC-400P spectrometer, using TMS as an internal standard. Optical rotations were measured with a PerkinElmer Model 141 polarimeter. All reactions were monitored using thin-layer chromatography (silica gel GF, Analtech, Newark, DE). HRFABMS were recorded on a JEOL HX-110 spectrometer.

The NMR spectra and InChI codes of compounds **2** and **3** are provided as Supporting Information.

*Synthesis of 3′-methoxy-3″,4″-(methylenedioxy)-2,5-epoxylignan-4′,6-diol (**2**)*

BBr_3 (0.5 mL) in CH_2Cl_2 (10 mL) was added to compound **1** (0.43 g) in CH_2Cl_2 (970 mL) at −80°C. The mixture was kept at room temperature for 20 h and then hydrolyzed by the addition of water. The phenolic product, collected by extraction with ether, gave 3′-methoxy-3″,4″-(methylenedioxy)-2,5-epoxylignan-4′,6-diol (**2**) as a powder.

Table 1. Pharmacokinetics data on MMEO.

	Kinetic parameter							
Rabbit no.	Cp (µg/mL)	β (h^{-1})	AUC (µg . h/mL)	Total clearance (mL/min)	V_1 (mL)	V_{darea} (mL)	T/2 (α) (h)	T/2 (β) (h)
1	2.12	0.12	5.20	0.29	0.59	3.16*	0.37	7.40*
2	1.85	0.13	5.08	0.29	0.64	2.70	0.36	6.99
3	2.01	0.12	4.95	0.28	0.68	2.91	0.39	7.03
Mean ± SD	1.99 ± 0.60	0.12 ± 0.06	5.06 ± 0.50	0.29 ± 0.02	0.63 ± 0.24	2.92 ± 1.68	0.37 ± 0.12	7.13 ± 4.53

SD, standard deviation.
*$p < 0.05$.

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*Synthesis of 3′-methoxy-3″,4″-(methylenedioxy)-2,5-epoxylignan-4′-ol-6-one (**3**)*

Compound **2** (0.37 g) was dissolved in a mixture of DMSO (10 mL) and acetic anhydride (5 mL). The mixture was distilled in a 35–40°C bath, which was then extracted with water (50 mL) and ethyl acetate (3 × 50 mL). Sodium chloride (2 mg) was added to promote separation of the aqueous and organic layers. The organic layer was evaporated and dried over anhydrous sodium sulfate. Five drops of water were added and then each mixture was partitioned with EtOAc three times. The EtOAc layer was washed with saturated aqueous $NaHCO_3$ and H_2O, dried over Na_2SO_4 and concentrated to yield compound **3** (0.24 g).

*3′-Methoxy-3″,4″-(methylenedioxy)-2,5-epoxylignan-4′,6-diol (**2**)*

Product **2** was obtained as a white solid; m.p: 65–70°C; ^{1}H NMR (400 MHz, CD_3OD, TMS): δ 8.42 (1H, s, OH), 6.40–6.81 (6H, m, Ar-H), 5.93 (2H, S, OCH_2O), 5.57 (1H, s, OH), 4.19 (2H, d, $J = 3.0$ Hz, H-6), 3.95 (3H, s, OCH_3), 3.86 (2H, dd, $J = 3.0$ Hz, 13.0 Hz, H-5), 3.69 (2H, dd, $J = 3.0$ Hz, 13.0 Hz, H-2), 2.98 (1H, dd, $J = 3.0$ Hz, 13.0 Hz, H-7), 2.82 (1H, m, H-7), 2.35 (1H, dd, $J = 3.0$ Hz, 13.0 Hz, H-4), 2.01 (1H, $J = 3.0$ Hz, 13.0 Hz, H-3); ^{13}C NMR (400 MHz, CD_3OD, TMS): δ 150.5, 149.0, 148.0, 146.6, 135.9, 134.5, 124.0, 122.2, 114.0, 113.5, 110.0, 108.2, 103.1, 89.8, 74.0, 73.0, 56.4, 54.5, 43.6, 36.5. $[\alpha]^{22}_D$ −139 (*c* 0.1, MeOH), UV (MeOH) λ_{max} (log ε) 248 nm (3.6) and 355 nm (3.0); IR (ATR) ν_{max} 3310 (br), 2901, 2825, 1437, 1022 cm^{-1}; HR-FABMS *m/z* 381.072 $[M+Na]^+$ (calcd. for $C_{20}H_{22}O_6N_a$, 381.117).

*3′-Methoxy-3″,4″-(methylenedioxy)-2,5-epoxylignan-4′-ol-6-on (**3**)*

The product **3** was obtained as a white powder; m.p.: 67–80°C; ^{1}H NMR (400 MHz, CD_3OD, TMS): δ 6.39–6.78 (6H, m, Ar-H), 5.93 (2H, S, OCH_2O), 5.56 (1H, s, OH), 3.90 (3H, s, OCH_3), 3.86 (2H, dd, $J = 3.0$ Hz, 13.0 Hz, H-5), 3.69 (2H, dd, $J = 3.0$ Hz, 13.0 Hz, H-2), 2.98 (1H, dd, $J = 3.0$ Hz, 13.0 Hz, H-7), 2.80 (1H, m, H-7), 2.34 (1H, dd, $J = 3.0$ Hz, 13.0 Hz, H-4), 2.02 (1H, $J = 3.0$ Hz, 13.0 Hz, H-3); ^{13}C NMR (400 MHz, CD_3OD, TMS): δ 170.8, 150.5, 149.0, 148.2, 146.5, 135.8, 134.6, 124.1, 122.0, 114.2, 113.5, 110.2, 108.2, 103.0, 89.8, 74.0, 73.3, 56.4, 54.7, 43.5, 36.2. $[\alpha]^{20}_D$ −140 (*c* 0.1, MeOH), UV (MeOH) λ_{max} (log ε) 261 nm (3.8) and 355 nm (3.0); IR (ATR) ν_{max} 3317 (br), 2948, 2835, 1725, 1449, 1417, 1015 cm^{-1}; HR-FABMS *m/z* 379.125 $[M+Na]^+$ (calcd. for $C_{20}H_{20}O_6N_a$, 379.133).

Pharmacology

Single-dose studies

Healthy male rabbits weighting 1.5–2.5 kg were used in the experiments. They were individually housed and maintained with water and standard rabbit feed. Animals were divided into three groups and received compound **3** at 0.8 mg/kg of body weight as a single steady intravenous bolus over 1 min. Plasma samples were drawn immediately before administration (maximum concentration of drug in plasma [C_{max}]) and then at 0, 0.25, 1, 4, 6, and 12 h postdosing. Blood samples were collected in heparinized syringes. Plasma was immediately separated by centrifugation for 20 min (1200 rpm) and stored at −80°C. Drug levels in plasma were determined after solid-phase extraction and dilution in mobile phase by ultra fast liquid chromatography (UFLC). The mobile phase consisted of methanol 95% and acetonitrile 5% with a flow rate of 1 mL/min for 10 min. Separation was achieved using an ODS C18 column (4.6 mm inner diameter), particle size 5 μM; Shimadzu, at temperature 30°C. Compound **3** was detected at an excitation at wavelength of 254 nm.

Preparation of a stock solution and standards

A stock solution of **3** was prepared by dissolving the compound in 0.9% saline in sterile condition using an autoclave. Quality control (QC) samples were prepared by spiking 1000 μL of blank plasma with 10 μL solutions of **3**.

Plasma sample process

The blood samples (250 μL) were collected via the marginal vein of the rabbit ears at 0 (predose), 0.25, 1, 4, 6, and 12 h after administration. The blood was mixed with 0.1 mL sodium EDTA and collected in an Eppendorf tube. Samples centrifuged at a speed of 5000 rpm for 15 min. Plasma samples (50 mL) were transferred to a fresh Effendorf tube, and then mixed with 400 μL acetonitrile to precipitate the protein content in the sample. Centrifugation was performed at a speed of 12 × 100 rpm for 20 min. All plasma samples were stored at −80°C.

Statistical analyses

All statistical analyses were performed using SAS software (v 9.1; SAS Institute, Cary, NC, USA) and the level of significance was defined as $p < 0.05$. Data obtained from animal experiments were expressed as mean ± standard deviation (± SD). Statistical differences were tested by ANOVA and Student–Newman–Keuls posthoc tests.

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The authors have declared no conflict of interest.

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