

Ionizing radiation-induced modification of nialamide as an anti-inflammatory agent against lipopolysaccharide-induced RAW 264.7 and DH82 cells

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Abstract. Nialamide is a non-selective monoamine oxidase inhibitor that was widely used as an antidepressant. However, it has been prohibited for decades in the depressive medicine market due to the adverse hepatotoxic side effects. The re-use of drugs that have been withdrawn from the market represents a promising approach for the development of novel incrementally modified drugs and, in this context, ionizing radiation can serve as a powerful tool for producing new drug candidates. The present study exposed nialamide to γ radiation at 50 kGy to obtain the novel cyclized benzylamide, nialaminosin (compound 2), along with five known compounds, 3-amino-*N*-benzylpropanamide (compound 3), 3-methoxy-*N*-benzylpropanamide (compound 4), 3-hydroxy-*N*-benzylpropanamide (HBPA; compound 5), *N*-benzylpropanamide (compound 6) and isonicotinamide (compound 7). Among the isolated compounds, HBPA was established to inhibit the lipopolysaccharide-induced overproduction of pro-inflammatory mediators, including nitric oxide

(NO) and prostaglandin E₂ and cytokines including TNF- α , IL-6 and IL-10, without causing cytotoxicity to both RAW 264.7 and DH82 cells. Furthermore, HBPA was found to reduce the protein expression of inducible NO synthase and cyclooxygenase-2 in macrophages and compared with nialamide, it was established to have more potent radical scavenging activity. The present study therefore suggested the application of HBPA for the improvement of anti-inflammatory properties using ionizing radiation technology on the withdrawn drug nialamide.

Introduction

Radiation modification technology is an eco-friendly chemical technique that can be applied to transform the chemical structures of biological materials, thereby contributing to an enhancement of their biological efficacy (1,2). Several research groups have previously reported the use of ionizing radiation for the semi-synthesis of novel compounds and the development of anti-obesity materials from natural products, such as rotenone, curcumin and rosmarinic acid using ionizing irradiation (3,4). It has been widely suggested that γ irradiation can enhance the anti-inflammatory effect of natural products (5,6). Furthermore, we have previously demonstrated that γ irradiation combined with the anti-depressant nomifensine (Merital) can contribute to the suppression of the proliferation of breast cancer cells (7). Given its diverse properties, ionizing radiation may thus represent an innovative technique for the development of incrementally modified drugs. To the best of the authors' knowledge, no previous studies have evaluated the structural and immunity enhancement effects of γ irradiation on hydrazine-based compounds.

With the growing awareness of companion animal health, there has been an increasing necessity for methods that can be used to discover novel drugs for these animals. In this regard, DH82 cells, a canine macrophage cell line, can serve as a valuable tool for evaluating the anti-inflammatory activity

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of candidate drugs for companion animals (8,9). Due to their striking resemblance to *in vivo* macrophages, these cells are excellent for screening natural extracts, chemical compounds, or other functional materials with potential therapeutic benefits for canine and feline inflammatory diseases. Studies assessing anti-inflammatory activity in DH82 canine macrophages cells for screening functional companion animal materials are rapidly emerging. Inflammation is a localized response to injury or infection that involves the accumulation of body fluids, plasma proteins and white blood cells that can trigger the excessive production of inflammatory mediators such as pro-inflammatory nitric oxide (NO), prostaglandin E₂ (PGE₂), TNF- α and IL-6 (10,11). The formation of inflammatory mediators, such as prostaglandins, plays a key role in inflammation. These mediators result from the interaction of arachidonic acid with cyclooxygenase (COX)-2 (12) and the NO production is known to be regulated by the enzyme inducible NO synthase (iNOS) (11). Excessive inflammation can contribute to the development of a range of chronic inflammation-related diseases and disorders, including diabetes, cancer, arthritis and cardiovascular disease, both in humans and companion animals.

Nialamide, a non-selective, irreversible monoamine oxidase inhibitor, has in the past been used clinically to treat depression in both humans and animals (13,14). However, it was withdrawn as an antidepressant in the 1970s owing to the adverse hepatotoxic effects (15). However, despite this detrimental activity, scientific interest in nialamide as a novel drug candidates has persisted and several studies in this regard have been reported (16). For examples, Lougheed *et al* (17) demonstrated that nialamide is a potent anti-tuberculosis agent that can effectively inhibits *Mycobacterium tuberculosis* at low concentrations. As part of an ongoing search for new drugs for humans and companion animals, the present study assessed the γ -ray-induced modification of nialamide and examined the enhancement of its biological properties. The present study detailed the isolation and characterization of the novel cyclized compound 2 and degradation compounds 3-7 from irradiated nialamide. These compounds, when compared with the nialamide parent (compound 1) exhibited significantly enhanced anti-inflammatory properties in lipopolysaccharide (LPS)-stimulated both RAW 264.7 and DH82 macrophages.

Materials and methods

Materials. Nialamide (purity >99%), acetonitrile, methanol, formic acid [high-performance liquid chromatography (HPLC) grade], dimethyl sulfoxide (DMSO)-*d*₆, LPS and Griess reagent were purchased from MilliporeSigma. All other chemicals used were of analytical grade. Compound analyses were conducted using an Agilent HPLC 1200 system (Agilent Technologies, Inc.) equipped with a photodiode array detector (1200 Infinity series; Agilent Technologies, Inc.) and a series of YMC-Pack ODS A-302 columns (4.6 mm i.d.x150 mm, particle size 5 μ m; YMC Co., Ltd.) were used to analysis the compounds. HPLC was performed using an ODS column (YMC-Pack A-302; YMC Co., Ltd.) using an elution gradient of 5-100% MeCN in 0.1% HCOOH (detection: 280 nm; flow rate: 1.0 ml/min; oven temperature: 40°C). ¹H and ¹³C nuclear magnetic resonance (NMR) spectra were measured using an

Avance NEO-600 instrument (Bruker Corporation) operated at 600 and 150 MHz, respectively. Chemical shifts are reported as δ (parts per million) values relative to those of the solvent DMSO-*d*₆ (δ _H 2.50; δ _C 39.5) on a tetramethylsilane scale. High-resolution electrospray ionization (ESI) mass spectra were obtained using a Vanquish UPLS System (Thermo Fisher Scientific, Inc.) and an Infinite F200 microplate reader (Tecan Austria GmbH) was used to measure absorbances.

γ -irradiation of nialamide. Nialamide irradiation was performed at the Advanced Radiation Technology Institute (ARTI; Jeongseup, Republic of Korea) using a cobalt-60 irradiator (point source AELC; IR-79; MDS Nordion International Co. Ltd.) with a source strength of ~320 kCi and a dose of 10 kGy/h. Pure nialamide (1 g) was initially dissolved in MeOH (1:1), placed in chapped glass bottles and then directly irradiated with a dose of 50 kGy. After irradiation, the sample solution was promptly concentrated using a rotary vacuum evaporator to eliminate the methanol and was subsequently lyophilized.

Isolation of the newly modified products. The irradiated reactant (965.6 mg) irradiated at a dose of 50 kGy was promptly subjected to open column chromatography on a YMC gel ODS AQ HG (2.5 cm i.d.x40 cm) column (YMC Co., Ltd.) with aqueous MeOH, to obtain fractions N1-N6. Subfraction N4 (225.4 mg), which was flowed with 50% MeOH, was isolated using preparative HPLC (YMC-prep column, 20 mm i.d.x250 mm; flow rate: 9 ml/min; solvent systems 78:22=H₂O:MeOH; YMC Co., Ltd.) to produce compounds 3 (2.7 mg, *t*_R 10.6 min), 5 (6.7 mg, *t*_R 10.0 min) and 7 (56.7 mg, *t*_R 15.5 min). Fraction N5 (67.0 mg) was separated using preparative-HPLC (YMC-prep column, 20 mm i.d.x250 mm; solvent, flow rate: 9 ml/min; solvent systems 75:25=H₂O:MeOH; YMC Co., Ltd.) to yield compounds 2 (2.0 mg, *t*_R 21.6 min), 4 (2.8 mg, *t*_R 21.6 min) and 6 (1.4 mg, *t*_R 21.6 min). Fractions N1-N3 were subjected to recrystallization and identified as HBPA (compound 5; 500 mg).

Cell culture. Canine DH82 macrophages were purchased from the American Type Culture Collection and murine RAW 264.7 macrophages were purchased from the Korean Cell Line Bank. The DH82 and RAW 264.7 macrophages cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and were incubated at 37°C in an incubator with a humidified atmosphere of 5% CO₂.

Cell viability. Cell viability was measured using the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Macrophage cells were seeded in 24-well plates at a density of 1x10⁵ cells/well, cultured for 24 h and treated with various concentrations of nialamide and isolated compounds 2-7 for an additional 24 h. Thereafter, the MTT reagent was added to each well. The formazan crystals thus produced were dissolved in DMSO and the absorbance of the well contents was measured at 570 nm using a scanning a microplate reader. The cell viability was determined from the proportions of viable and dead cells (18).

Measurement of NO, PGE₂ and cytokine productions. RAW 264.7 and DH82 macrophage cells were cultured in 24-well plates at a density of 1×10^5 cells/well for 24 h. Following this initial incubation, the cells were pre-treated with nialamide and derived compounds 2-7 for 1 h and subsequently treated with LPS (RAW 264.7 cells: $0.1 \mu\text{g/ml}$; DH82 cells: $2 \mu\text{g/ml}$) for an additional 24 h. NO production in the macrophage culture medium was assessed using the Griess reagent method with a standard curve for quantification (19). In addition, the macrophage supernatant was used to assess for levels of PGE₂ and cytokines using the respective enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences), according to the manufacturer's instructions.

Western blot analysis. RAW 264.7 and DH82 cells were cultured in six-well plates at a density of 2×10^5 cells/well for 24 h. Following incubation at 37°C , the cells were pre-treated with compounds 1 and 5 for 1 h and were thereafter treated with LPS (RAW 264.7 cell: $0.1 \mu\text{g/ml}$; DH82 cell: $2 \mu\text{g/ml}$) for an additional 24 h. Total proteins were extracted using RIPA buffer (Rockland Immunochemicals, Inc.) and the concentrations of the isolated proteins were determined using a bicinchoninic acid protein assay kit. Proteins ($40 \mu\text{g}$) were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10%) and subsequently transferred to polyvinylidene difluoride membranes (Merck KGaA). The membranes were then blocked with 5% non-fat milk in Tris-buffered saline containing 10% Tween-20 (TBST) for 1 h at room temperature and subsequently probed overnight at 4°C with the following primary antibodies at dilution of 1:1,000: Anti-COX-2 (cat. no. 4842), anti-iNOS (cat. no. 2977), anti-p65 (cat. no. 8242), anti-p-p65 (cat. no. 3033), anti-I κ B (cat. no. 4812), anti-p-I κ B (cat. no. 2859) and anti-GAPDH (cat. no. 2118; all obtained from Cell Signaling Technology, Inc.). For canine macrophages, the following primary antibodies were used at a dilution of 1:1,000: anti-COX-2 (cat. no. PA1-9032), anti-iNOS (cat. no. PA1-036) and anti-GAPDH (cat. no. PA1-987; Invitrogen; Thermo Fisher Scientific, Inc.). Following incubation with the primary antibodies, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 2 h at room temperature. Protein bands were detected using a chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Inc.) and exposure to an X-ray film. The blots were scanned and densitometric analysis was performed using ImageJ software (version 1.51k; National Institutes of Health).

Measurement of reactive oxygen species (ROS). The production of intracellular ROS was measured using ROS detection reagents. In brief, RAW 264.7 macrophage cells were seeded in 96-well plates at a density of 1×10^4 cells/well and treated with $0.1 \mu\text{g/ml}$ LPS in combination with varying concentrations of compound 5 (50 to $200 \mu\text{M}$) at 37°C for 2 h. On day 1, the cells were washed with phosphate-buffered saline (PBS) and incubated with 7'-dichlorofluorescein diacetate (DCFH-DA) at 37°C for 45 min. DCF fluorescence was measured at 0, 10 and 30 min using a microplate reader at excitation and emission wavelengths of 492 and 520 nm, respectively (20).

The scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical of the derived compounds was assessed using a modified Blois method (21). Briefly, a solution of DPPH ($200 \mu\text{M}$) was prepared in ethanol (EtOH), $60 \mu\text{l}$ of which was mixed with $120 \mu\text{l}$ of each of the assessed compounds at concentrations from 50 to $200 \mu\text{M}$ in 70% EtOH. After incubation at 30°C in the dark for 15 min, the reduction in absorbance at 517 nm was recorded using an ELISA reader. For the determination of 3-ethylbenzothiazoline-6-sulphonic acid (ABTS⁺) radical scavenging activity, an ABTS⁺ solution was prepared by mixing 5 ml of a 7 mM ABTS⁺ solution in ethanol with 5 ml of a 2.4 mM potassium persulfate solution and incubating the mixture in the dark at 25°C for 24 h prior to use. The ABTS⁺ solution ($100 \mu\text{l}$) was thereafter added to the wells of 96-well plates containing different concentrations of compound 5 and mixed for 30 sec, followed by incubation for 30 min at 25°C (22). The DPPH and ABTS⁺ radical-scavenging activities were calculated using the following formula:

$$\text{radical scavenging activity (\%)} = [1 - (A_2/A_1)] \times 100,$$

where A_1 is the absorbance of reaction mixtures without samples and A_2 is the absorbance of reaction mixtures containing the assessed compounds.

Statistical analysis. Each experiment was performed at least three times and the results were expressed as the mean \pm standard deviation. For multiple comparisons, one-way analysis of variance (ANOVA) was used followed by Tukey's multiple comparisons test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Structural determination of the nialamide degradation products. Nialamide irradiated at a 50 kGy dose was established to be characterized by the most significantly enhanced inhibitory effects with respect to the production of NO and PGE₂ in LPS-stimulated RAW 264.7 cells, with IC₅₀ values of 98.7 ± 0.9 and $88.2 \pm 0.9 \mu\text{g/ml}$, respectively. Comparatively, an IC₅₀ value $> 200 \mu\text{g/ml}$ was obtained for the parent compound, nialamide. DH82 canine macrophages treated with irradiated nialamide displayed notable inhibitions in both NO and PGE₂ production (Table I). The molecular changes in irradiated nialamide were assessed using reverse-phase HPLC. Newly generated peaks in the HPLC chromatograms corresponding to the modified products of nialamide were accordingly detected (Fig. S1). Successive chromatographic separation of this sample resulted in the purification of a new cyclized product, designated compound 2, along with five known degradation products (compounds 3-7).

Compound 2 was obtained as a colorless oil. The pseudomolecular ion in the positive high-resolution electrospray ion mass spectrum (HRESIMS) at m/z 191.1176 ($\text{M} + \text{H}$)⁺ had the molecular formula $\text{C}_{12}\text{H}_{15}\text{N}_2\text{O}$. The ¹H NMR spectrum of compound 2 (Fig. S2) showed characteristic resonances of a benzene ring system at δ_{H} 7.34 (2H; t; $J = 1.8$ Hz; H-3; 5), 7.26 (2H; dd; $J = 8.4$; 1.8 Hz; H-2; 6) and 7.24 (1H; dd; $J = 8.4$; 1.8 Hz; H-4) and a nitrogenated methylene proton at δ_{H} 4.44 (2H; s; H-7), indicating the presence of a benzylamide moiety

Table I. Inhibitory activities of modification products 2-7 on LPS-stimulated NO and PGE₂ production in macrophages¹.

Compounds	RAW 264.7 cells			DH82 cells		
	Cell viability (%) ²	IC ₅₀ of NO, μ M	IC ₅₀ of PGE ₂ , μ M	Cell viability, % ²	IC ₅₀ of NO, μ M	IC ₅₀ of PGE ₂ , μ M
Nialamide (1)	102.3 \pm 2.3	>200 ^a	>200 ^a	99.9 \pm 1.2	>200 ^a	>200 ^a
irradiated nialamide ³	101.0 \pm 1.3	98.5 \pm 0.9	88.2 \pm 0.9	99.9 \pm 1.0	89.0 \pm 1.0	67.9 \pm 0.8
2	99.5 \pm 1.9	186.2 \pm 2.1 ^a	150.7 \pm 2.0 ^b	98.3 \pm 1.3	101.6 \pm 1.5 ^b	98.5 \pm 1.0 ^b
3	101.5 \pm 2.0	170.5 \pm 1.9 ^a	155.1 \pm 1.9 ^b	99.7 \pm 1.2	158.2 \pm 1.7 ^b	122.2 \pm 1.3 ^b
4	102.0 \pm 2.2	177.6 \pm 2.0 ^a	160.9 \pm 2.0 ^a	97.6 \pm 1.1	171.1 \pm 2.1 ^a	125.5 \pm 1.3 ^b
5	99.9 \pm 2.2	63.5 \pm 0.8 ^c	55.4 \pm 0.7 ^c	97.2 \pm 1.0	57.5 \pm 0.6 ^c	49.4 \pm 0.4 ^c
6	100.7 \pm 1.8	>200 ^a	>200 ^a	98.6 \pm 0.9	194.4 \pm 2.3 ^a	176.4 \pm 1.7 ^a
7	100.8 \pm 1.9	>200 ^a	>200 ^a	98.4 \pm 1.2	>200 ^a	>200 ^a

¹IC₅₀ value was defined as the concentration that resulted in a 50% decrease in NO and PGE₂ production. Activity data are expressed as mean \pm standard deviation from triplicate experiments. Different letters (a-c) within the same column indicate significant differences (P<0.05).

²Cell viability was measured at 200 μ M. ³Results of irradiated nialamide are expressed as the IC₅₀ value in μ g/ml. 1, nialamide; 2, nialamionsin; 3, 3-amino-*N*-benzylpropanamide; 4, 3-methoxy-*N*-benzylpropanamide; 5, 3-hydroxy-*N*-benzylpropanamide; 6, *N*-benzylpropanamide; 7, isonicotinamide.

in the molecule of compound 2. Additionally, the low-field region of the ¹H NMR spectrum displayed signals for doubly nitrogenated methylene protons at δ_{H} 4.03 (2H; d; *J*=6.0 Hz; H-13) and two cyclized methylene protons at δ_{H} 2.92 (2H; br d; *J*=6.0 Hz; H-11) and 2.25 (2H; t; *J*=6.0 Hz; H-10), which is consistent with a tetrahydropyrimidione skeleton (23).

In addition, ¹³C NMR combined with heteronuclear single quantum coherence (HSQC) analysis of compound 2 (Figs. S3 and S4) revealed 11 carbon signals, attributable to one carbonyl carbon signal at δ_{C} 167.5 (C-9), five benzylamide signals at δ_{C} 137.9 (C-1), 128.6 (C-3; 5), 127.6 (C-2; 6), 127.1 (C-4) and 46.3 (C-7) and three methylene carbons at δ_{C} 63.0 (C-13), 42.0 (C-11) and 32.9 (C-10). Collectively, the aforementioned informative results provide evidence to indicate that compound 2 is a tetrahydropyrimidione-substituted benzylamide. A detailed interpretation of the combination of ¹H-¹H correlated spectroscopy, heteronuclear multiple bond correlation (HMBC) and nuclear Overhauser effect spectroscopy data for compound 2 further indicated that it features a characteristic cyclized product (Figs. S5-S7). The proposed linkage between the benzylamide and tetrahydropyrimidione moieties is suggested to occur at the C-7 position, as supported by the HMBC correlations (Fig. 1). On the basis of the aforementioned analyses, the planar structure of compound 2 was assigned to nialamionsin, a novel benzylamide analog with a tetrahydropyrimidione unit (Fig. 1).

Nialamionsin (compound 2): Colorless oil: UV λ_{max} (MeOH): 207 (3.14), 220 (sh) and 249 (2.26) nm; ¹H NMR (DMSO-*d*₆; 600 MHz): δ 7.34 (2H; t; *J*=1.8 Hz; H-3; 5), 7.26 (2H; dd; *J*=8.4; 1.8 Hz; H-2; 6), 7.24 (1H; dd; *J*=8.4; 1.8 Hz; H-4), 4.44 (2H; s; H-7), 4.03 (2H; d; *J*=6.0 Hz; H-13), 2.92 (2H; br d; *J*=6.0 Hz; H-11), 2.25 (2H; t; *J*=6.0 Hz; H-10); ¹³C NMR (DMSO-*d*₆; 150 MHz): δ 167.5 (C-9), 137.9 (C-1), 128.6 (C-3; 5), 127.6 (C-2; 6), 127.1 (C-4), 63.0 (C-13), 46.3 (C-7), 42.0 (C-11), 32.9 (C-10); ESIMS *m/z* 191 (M+H)⁺; HRESIMS *m/z* 191.1176 (M+H)⁺ (calculated for C₁₁H₁₅N₂O, 191.1178; Fig. S8).

The five known isolated compounds were characterized as 3-amino-*N*-benzylpropanamide (compound 3; ABPA), 3-methoxy-*N*-benzylpropanamide (compound 4; MBPA), 3-hydroxy-*N*-benzylpropanamide (compound 5; HBPA), *N*-benzylpropanamide (compound 6; BPA) and isonicotinamide (compound 7; INA), based on comparisons of the respective spectroscopic data (NMR and MS) with reports from the literature (24-28).

ABPA (compound 3): Colorless oil: ¹H NMR (DMSO-*d*₆; 600 MHz): δ 7.33 (2H; t; *J*=1.2 Hz; H-3; 5), 7.27 (2H; dd; *J*=7.8; 1.2 Hz; H-2; 6), 7.23 (1H; dd; *J*=7.8; 1.2 Hz; H-4), 4.45 (2H; s; H-7), 2.93 (2H; br d; *J*=6.0 Hz; H-11), 2.26 (2H; t; *J*=6.0 Hz; H-10); ¹³C NMR (DMSO-*d*₆; 150 MHz): δ 167.7 (C-9), 137.0 (C-1), 127.2 (C-3; 5), 126.4 (C-2; 6), 126.0 (C-4), 46.2 (C-7), 42.2 (C-11), 32.3 (C-10); ESIMS *m/z* 179 (M+H)⁺ (24,25).

3-Methoxy-*N*-benzylpropanamide (compound 4): Colorless oil: ¹H NMR (DMSO-*d*₆; 600 MHz): δ 7.33 (2H; t; *J*=1.8 Hz; H-3; 5), 7.24 (2H; dd; *J*=7.8; 1.8 Hz; H-2; 6), 7.22 (1H; dd; *J*=7.8; 1.8 Hz; H-4), 4.26 (2H; d; *J*=6.0 Hz; H-7), 3.55 (2H; t; *J*=6.0 Hz; H-11), 3.22 (3H; s; 11-OCH₃), 2.38 (2H; t; *J*=6.0 Hz; H-10); ¹³C NMR (DMSO-*d*₆; 150 MHz): δ 170.2 (C-9), 139.6 (C-1), 128.4 (C-3; 5), 127.3 (C-2; 6), 125.8 (C-4), 65.5 (C-11), 58.0 (11-OCH₃), 42.1 (C-7), 35.1 (C-10), ESIMS *m/z* 194 (M+H)⁺ (26).

3-Hydroxy-*N*-benzylpropanamide (compound 5): Colorless oil: ¹H NMR (DMSO-*d*₆; 600 MHz): δ 7.32 (2H; t; *J*=1.8 Hz; H-3; 5), 7.26 (2H; dd; *J*=8.4; 1.8 Hz; H-2; 6), 7.22 (1H; dd; *J*=8.4; 1.8 Hz; H-4), 4.27 (2H; d; *J*=6.0 Hz; H-7), 3.65 (2H; t; *J*=6.0 Hz; H-11), 2.31 (2H; t; *J*=6.0 Hz; H-10); ¹³C NMR (DMSO-*d*₆; 150 MHz): δ 170.9 (C-9), 139.9 (C-1), 128.5 (C-3; 5), 127.5 (C-2; 6), 126.9 (C-4), 57.9 (C-11), 42.2 (C-7), 39.0 (C-10); ESIMS *m/z* 180 (M+H)⁺ (25).

N-Benzylpropanamide (compound 6): Colorless oil: ¹H NMR (DMSO-*d*₆; 600 MHz): δ 7.32 (2H; d; *J*=7.8 Hz; H-2; 6), 7.26 (2H; t; *J*=1.8 Hz; H-3; 5), 7.23 (1H; dd; *J*=8.4; 1.8 Hz; H-4), 4.27 (2H; d; *J*=6.0 Hz; H-7), 2.15 (2H; q; *J*=7.8 Hz; H-10),

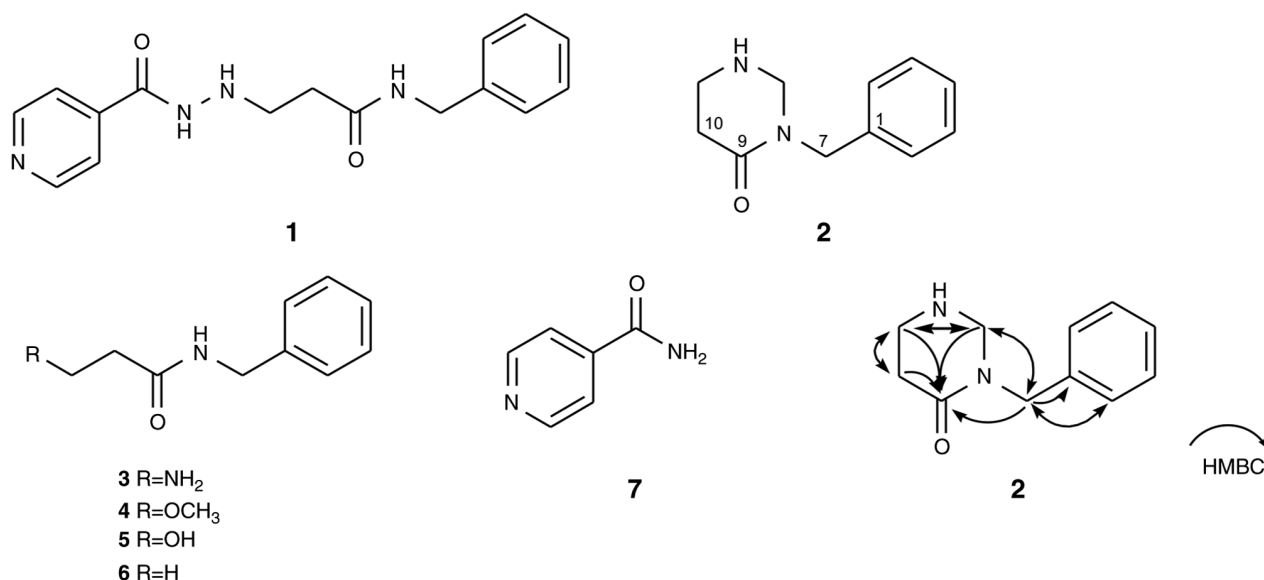


Figure 1. Chemical structures of newly modified products 2-7 of nialamide (1) and key HMBC correlations of the novel cyclized compound 2. 1, nialamide; 2, nialaminosin; 3,3-amino-*N*-benzylpropanamide; 4,3-methoxy-*N*-benzylpropanamide; 5,3-hydroxy-*N*-benzylpropanamide; 6, *N*-benzylpropanamide; 7, isonicotinamide; HMBC, heteronuclear multiple bond correlation.

1.03 (3H; t; $J=7.8$ Hz; H-11); ^{13}C NMR (DMSO- d_6 ; 150 MHz): δ 173.3 (C-9), 140.2 (C-1), 128.7 (C-2; 6), 127.6 (C-3; 5), 127.1 (C-4), 42.4 (C-7), 18.9 (C-10), 10.4 (C-11); ESIMS m/z 164 ($\text{M}+\text{H}$) $^+$ (27).

Isonicotinamide (compound 7): White amorphous powder; ^1H NMR (DMSO- d_6 ; 600 MHz): δ 8.73 (2H; d; $J=8.4$ Hz; H-3; 5), 7.72 (2H; d; $J=8.4$ Hz; H-2; 6); ESIMS m/z 123 ($\text{M}+\text{H}$) $^+$ (28).

Effects of the nialamide degradation products on NO and PGE₂ production. Inflammatory responses promote the excessive production of pro-inflammatory mediators, such as NO and PGE₂ (11), the abnormal overproduction of which is implicated in the development of a diverse range of disorders, including circulatory shock, cancer and atherosclerosis (29,30). Consequently, regulating the production of pro-inflammatory agents is a desirable pharmacological property when seeking to develop new drugs. To investigate the anti-inflammatory activities of isolated products 2-7, the present study treated both RAW 264.7 and DH82 macrophages with different concentrations of these compounds. Using the MTT assay, the cytotoxicity of these compounds was initially assessed at concentrations ranging from 10-200 μM and it was accordingly established that all assessed compounds showed negligible cytotoxicity toward these two cell types at concentrations of ≥ 200 μM (Table I).

In the current study, compounds were evaluated for their inhibitory activity on the production of NO and PGE₂ in LPS-stimulated macrophages. Among these compounds, compared with the parent nialamide (1) the novel cyclized product nialaminosin (2) showed enhanced inhibitory effects against the production of NO and PGE₂ in LPS-stimulated RAW 264.7 cells with respective IC₅₀ values of 86.2 ± 1.1 and 80.7 ± 1.0 μM (Table I). Similarly, enhanced anti-inflammatory effects were detected for benzylpropanamide derivatives 3-6, with IC₅₀ values ranging from 63.5-194.4 μM for NO production

and from 55.4-176.4 μM for PGE₂ production (Table I). Among these derivatives, it was found that hydroxylation at the C-11 position of HBPA conferred the most potent inhibitory activity against the activation of pro-inflammatory mediators (Table I). Comparatively, the simple pyridine amide isonicotinamide (7) was characterized by relatively lower inhibitory activity against the production of both NO and PGE₂ in RAW 264.7 macrophages (Table I). Consistently, in canine DH82 macrophages, the hydroxyl-substituted benzylpropanamide derivative was established have the most potent inhibitory activity against the production of NO and PGE₂, with IC₅₀ values of 57.5 ± 0.6 and 49.4 ± 0.4 μM , respectively (Table I). On the basis of these observations, the potent compound 5 was accordingly selected for further evaluation of its inhibitory effects on the release of NO and PGE₂ using western blot analysis.

Effects of compound 5 on iNOS and COX-2 protein expression. Within cells, iNOS and COX-2 are induced in response to a range of stimuli, including pro-inflammatory mediators, growth factors, oncogenes, carcinogens and tumor promoters, thereby indicating that these two proteins play roles in both inflammation and the control of cell growth (31,32). Accordingly, for the development of new anti-inflammatory drugs, it would be desirable to identify compounds that can inhibit both the activity and expression of iNOS and COX-2 (33,34). The present study found that, compared with nialamide (1), compound 5 (identified in this study as the most potent inhibitor of NO and PGE₂ production) promoted a significant dose-dependent suppression of the expression of iNOS and COX-2 in both LPS-stimulated RAW 264. (Fig. 2A-C) and canine DH82 (Fig. 2D-F) cells, thereby resulting in an inhibition of the pro-inflammatory mediators NO and PGE₂ (Fig. S9).

Effects of compound 5 on cytokine production. Pro-inflammatory cytokines serve as key signaling

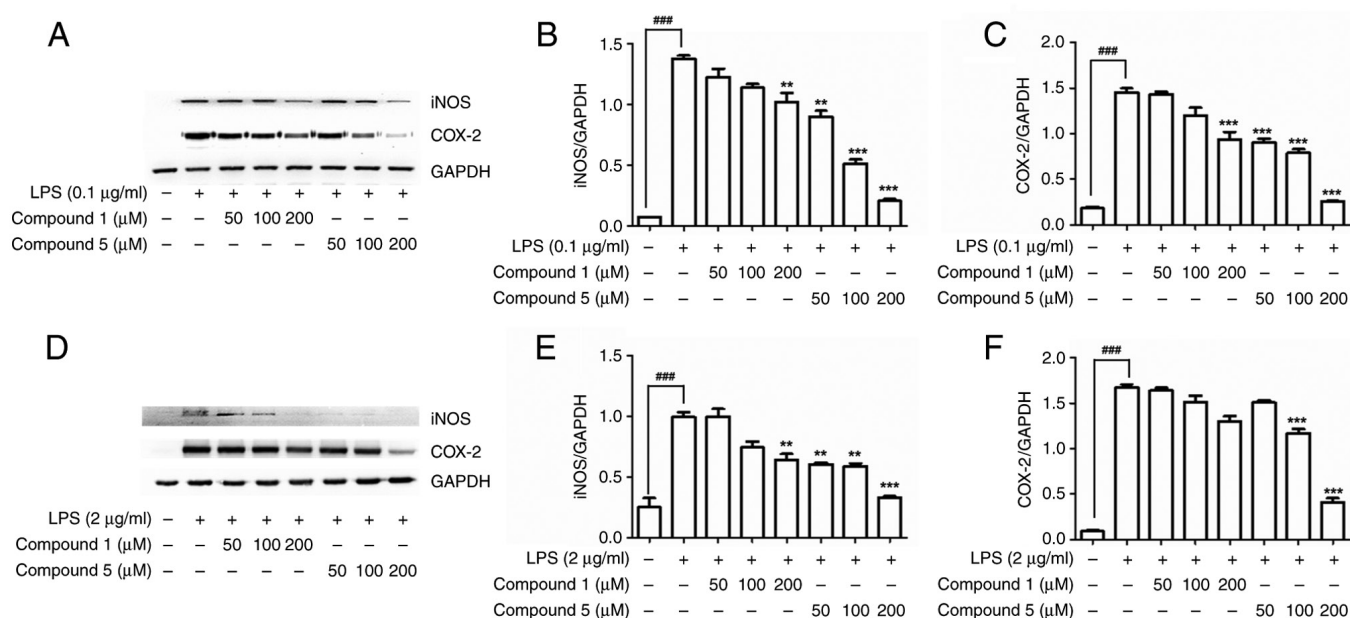


Figure 2. Effects of major product 5 derived from irradiation-modified nialamide on LPS-stimulated iNOS and COX-2 protein expression in macrophages. (A-C) RAW 264.7 and (D-F) DH82 cells were pretreated with compounds for 1 h prior to incubation with LPS (0.1 or 2 μg/ml) for 24 h. Cell lysates were then prepared and western blot analysis was performed using anti-iNOS and anti-COX2 antibodies. GAPDH was used as an internal control for western blot analysis. The results are expressed as the mean ± standard deviation (n=3). ***P<0.001 vs. the control group. **P<0.01 and ***P<0.001 vs. the LPS-induced group. 1, nialamide; 5, 3-hydroxy-N-benzylpropanamide; iNOS, inducible NO synthase; COX, cyclooxygenase.

molecules in the immune system and thereby play important roles in inflammatory responses (35). However, an excessive release of pro-inflammatory cytokines such as TNF-α and IL-6 can lead to damage to the epithelial barrier, initiate epithelial cell apoptosis and induce inflammation (36,37). Several studies have reported that the anti-inflammatory activities of benzylamide derivatives are associated with the control of the levels of diverse pro-inflammatory cytokines (35,38). In macrophages, exposure to LPS can induce the upregulation of IL-10, a well-established anti-inflammatory cytokine, by promoting the activation of the p38, JNK and ERK1/2 MAPK signaling pathways, which typically lead to the phosphorylation and activation of the Sp1 transcription factor and, in turn, expression of the IL-10 gene. The analysis in the present study of the inhibitory effects of compound 5 on the production of the cytokines TNF-α, IL-6 and IL-10 in LPS-induced macrophages revealed that, compared with the original nialamide, product 5 was characterized by a potent dose-dependent suppression of all three of these cytokines in LPS-stimulated RAW 264.7 cells, with similar effects being detected in LPS-stimulated DH82 macrophages (Fig. 3D-F).

Radical scavenging activity. ROS are key regulators of inflammatory responses, particularly the activation of the transcription factor NF-κB and its downstream signals (39). However, given their high reactivity, when generated in excess, ROS can have detrimental effects, particularly those associated with peroxidation and oxidative damage to normal DNA and proteins (40). Several studies have nevertheless reported that modified drugs can contribute to reductions in ROS production and enhance antioxidant activity (41,42). To investigate the inhibitory effects of major product 5 on ROS

generation, LPS-activated RAW 264.7 macrophages were stained with DCFH-DA and fluorescence microscopy observations performed. Treatment with compound 5 was found to effectively suppress ROS production in a dose-dependent manner (Fig. 4A and B). Previous study have demonstrated that LPS-stimulated ROS generation is associated with NF-κB activation (43) and, consistently, in the present study, significant increases in the phosphorylation levels of NF-κB and IκB in the treated RAW 264.7 cells were detected (Fig. S10). By contrast, treatment with compound 5 was found to promote a dose-dependent inhibition of the LPS-stimulated phosphorylation of NF-κB (Fig. 4C and D). Moreover, compared with cells treated with the parent compound nialamide, an enhancement of DPPH radical and ABTS⁺ scavenging activities were detected in those cells treated with compound 5 (Fig. 4E and F). Collectively, these findings thus provide evidence to indicate that the γ irradiation-mediated modification of nialamide contributes to enhancing the anti-inflammatory properties of this drug.

Discussion

Exposure to ionizing radiation, which generates a range of free radicals including hydroxymethyl ($\cdot\text{CH}_2\text{OH}$), hydroxyl ($\cdot\text{OH}$), methoxy ($\text{CH}_3\text{O}\cdot$) and peroxy ($\text{HOO}\cdot$) radicals, is an effective approach for the incremental modification of drugs that is more rapid and safer than conventional synthetic methods and can be used to obtain higher product yields (44). For example, studies have reported the enhanced anti-inflammatory capacities of natural flavonoids, both *in vitro* and *in vivo*, following modification using ionizing radiation, along with the isolation and characterization of the corresponding modified products (45,46). Similarly, radiolytic modification

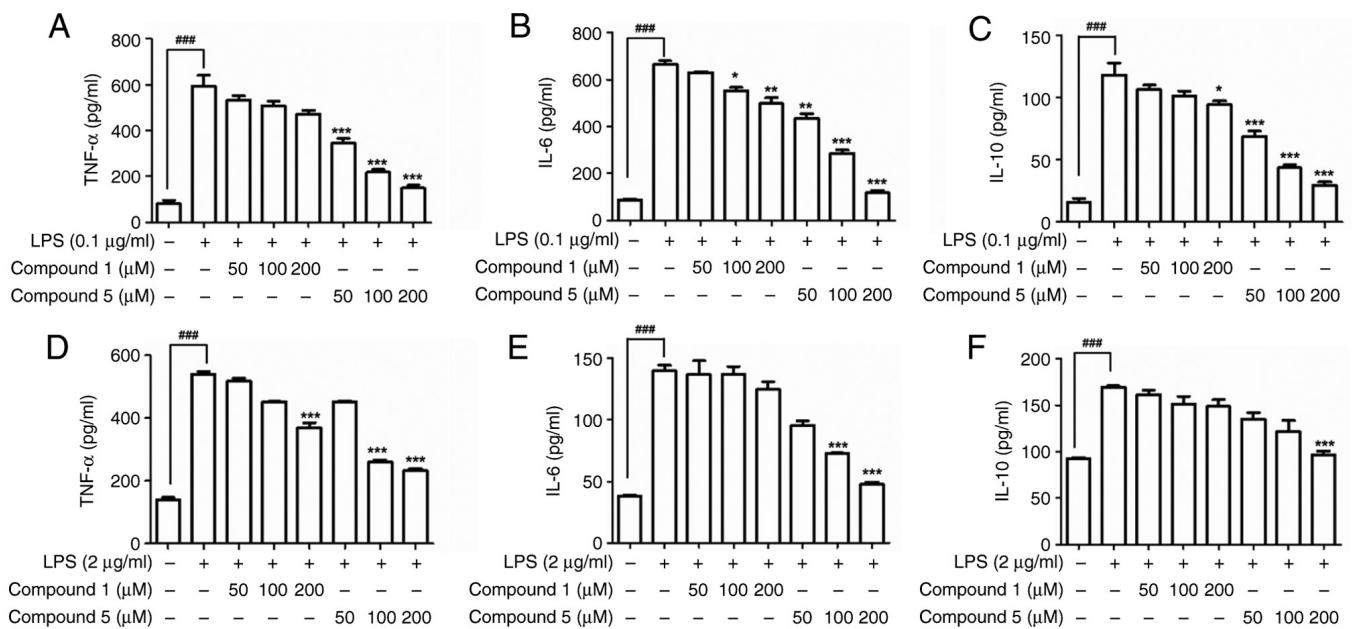


Figure 3. Effects of major product 5 derived from irradiation-modified nialamide on LPS-stimulated cytokine (TNF- α , IL-6 and IL-10) production in macrophages. (A-C) RAW 264.7 and (D-F) DH82 cells were pretreated with compounds for 1 h prior to incubation with LPS (0.1 or 2 μ g/ml) for 24 h. Cytokine levels (A and D) TNF- α , (B and E) IL-6, (C and F) IL-10 in culture media were determined using ELISA. The results are expressed as the mean \pm standard deviation (n=3). ###P<0.001 vs. the control group. *P<0.05, **P<0.01 and ***P<0.001 vs. the LPS-induced group. 1, nialamide; 5, 3-hydroxy-N-benzylpropanamide.

of hydrazine-based nomifensine has been demonstrated to enhance the anticancer effects of this drug in treated breast cancer cells (7). Radiolytic radicals are powerful modifying agents that can initiate a range of modification reactions in lead compounds during irradiation (47). The findings in the present study indicate that under methanolic conditions, γ irradiation-induced ROS and free radicals can contribute to the degradation, deamination, methoxylation and hydroxylation of nialamide, resulting in the generation of lower molecular weight amide derivatives, such as nialaminosin (2), 3-amino-N-benzylpropanamide (3), 3-methoxy-N-benzylpropanamide (4), HBP (5), N-benzylpropanamide (6) and isonicotinamide (7). Compared with the parent nialamide (1), the major irradiation-modified product HBP was found to be characterized by enhanced anti-inflammatory effects in LPS-stimulated RAW 264.7 and DH82 macrophages, involving marked suppression of the expression of NO, PGE₂, iNOS, COX-2 and the pro-inflammatory cytokines TNF- α , IL-6 and IL-10, with negligible cytotoxicity toward either of these cell lines. Treating RAW 264.7 macrophages with LPS induced ROS production, which was effectively inhibited by the γ -irradiated derivative of compound 5. This reduction in ROS probably contributed to the significant downregulation of NF- κ B and I κ B phosphorylation observed, suggesting newly generated product 5 from irradiated nialamide potential anti-inflammatory activity through suppressing the NF- κ B-mediated inflammatory signaling pathway. To further assess its antioxidant properties, the present study evaluated its DPPH and ABTS⁺ radical scavenging abilities, as scavenging these radicals mimics the removal of harmful free radicals found *in vivo*. While no control group was included for compound 5, (+)-catechin, a known radical scavenger, was utilized as a positive control to ensure accurate measurement.

Notably, (+)-catechin was excluded from the ROS and NF- κ B assays to avoid potential interference. Additionally, compound 5 markedly suppressed the expression of NF- κ B, the key regulator of pro-inflammatory mediators such as iNOS, COX-2, TNF- α and IL-6, in stimulated mouse macrophages. These synergistic anti-inflammatory effects suggested that compound 5 holds promising application potential not only in companion animal drugs but also in broader therapeutic development. Further research is needed to explore its full range of potential uses and optimize its production process.

Positive controls were not included in all cell experiments due to the focus on comparing the relative activity of modified compounds. The present study compared nialamide and modified products to both untreated and LPS-treated control groups. This internal comparison strategy provided sufficient information for assessing the effectiveness of the modifications without introducing the potential variability associated with an imperfectly matched positive control. While this approach does not provide absolute activity values, it offers a robust and relevant perspective within the context of our research goals. Additionally, in research on improving the functionality of natural products or abandoned pharmaceuticals, a number of researchers choose not to use positive controls, instead focusing on comparing the activity of the modified compound with the parent compound (6,7,44,45). The present study focused on unlocking the hidden potential of nialamide, a drug withdrawn from the antidepressant market. By delving into its unexplored anti-inflammatory capabilities, it is hoped to develop novel therapeutic options for inflammatory diseases. This could potentially lead to safer and more effective treatments with established safety profiles. However, despite the recent developments in research on the application

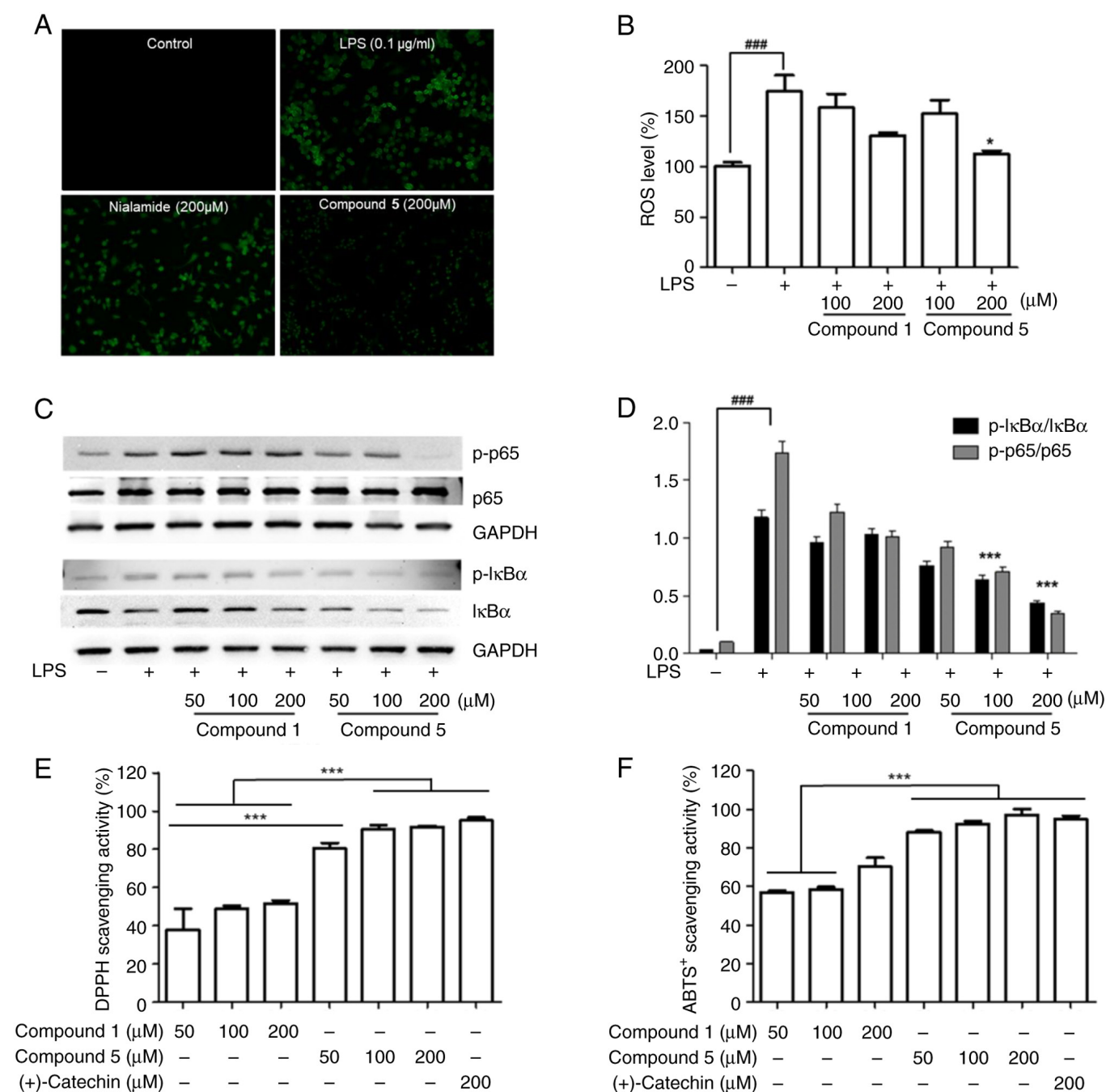


Figure 4. Effects of major product 5 on the radical scavenging activity and ROS production of LPS-activated RAW 264.7 cells. RAW 264.7 cells were pretreated with compounds for 1 h prior to incubation with LPS (0.1 $\mu\text{g/ml}$) for 24 h. (A) Cells were incubated for 24 h with 50 μM DCFH-DA, washed twice with PBS and incubated with LPS in the presence or absence of compound 5 (magnification, $\times 10$). (B) Cells were incubated with DCFH-DA for 45 min and the DCF fluorescence was measured at 492 nm (excitation) and 520 nm (emission) for 30 min. (C) The protein levels of p-IkB α , IkB α , p-p65 and p65 in cell lysates were analyzed by western blotting. GAPDH was used as a loading control. (D) Western blotting data are shown as representative values of three independent experiments. (E) DPPH and (F) ABTS⁺ radical scavenging activities of compound 5. The results are expressed as the mean \pm standard deviation ($n=3$). $^{***}P<0.001$ vs. the control group. $^{*}P<0.05$ and $^{***}P<0.001$ vs. the LPS-induced group. ROS, reactive oxygen species; LPS, lipopolysaccharide; DCFH-DA, 7'-dichlorofluorescein diacetate; PBS, phosphate-buffered saline; p-, phosphorylated; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS⁺, 3-ethylbenzothiazole-6-sulphonic acid; 1, nialamide; 5, 3-hydroxy-*N*-benzylpropanamide.

of γ irradiation technology for repurposing withdrawn pharmaceuticals, concerns still remain regarding the potential changes in toxicity and side effects of irradiation-modified products and data pertaining to the original drugs may not be strictly applicable to the modified versions. Accordingly, it is plan to conduct comprehensive animal studies to assess the safety profiles of modified drugs generated using γ radiation.

In conclusion, given its adverse side effects, the anti-depressant drug nialamide has been withdrawn from the pharmaceutical industry. The present study investigated the structural modification of nialamide using ionizing irradiation and characterized the anti-inflammatory properties of the irradiation-modified products. Nialamide can be readily modified to yield the new compounds nialaminosin,

3-amino-*N*-benzylpropanamide, 3-methoxy-*N*-benzylpropanamide, HBPA, *N*-benzylpropanamide, and isonicotinamide, the chemical structures of which were determined using one- and two-dimensional NMR analysis, as well as HRESIMS spectral data interpretation. Among these compounds, it was found that, compared with the parent nialamide, the hydroxyl-substituted benzylamide derivative was characterized by a more pronounced anti-inflammatory activity against the production of NO and PGE₂ in LPS-stimulated macrophages. Moreover, this compound was found to reduce the expression of iNOS and COX-2 proteins, suppress cytokine production and exhibit potent antioxidant activity. Collectively, the findings in the present study indicated that the modification of hydrazine-based benzylamide drugs using ionizing radiation could provide a unique approach to the semi-synthesis of hydroxylated benzylpropanamide compounds with enhanced anti-inflammatory capacity in both humans and companion animals. It was considered that by enhancing drug bioactivity and safety, the application of radiation technology for the modification of withdrawn drugs could provide a valuable novel approach for future drug discovery and development.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HL, SYW, and HJC were involved in conceptualization and design of the methodology. GHJ and BYC performed the experiments. HL, KBL and HWB contributed to data curation. HL, GHJ and KBL analyzed the data. HL wrote the original draft. GHJ, KBL and HWB reviewed and edited the manuscript. HL and GHJ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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