Abstract: The previous discoveries of butyl fenbufen amide analogs with antitumor effects were further examined. The amide analogs with 1, 3, 4 and 8 carbons chains were prepared in 70-80% yield. Fenbufen had no cytotoxic effects at concentrations ranging from 10 to 100 μM. Methyl fenbufen amide had significant cytotoxic effects at a concentration of 100 μM. As the length of the alkyl amide side chain increased, the cytotoxic effects increased, and the octyl fenbufen amide had the greatest cytotoxic effect. After treatment with 30 μM octyl fenbufen amide, nearly seventy percent of the cells lost their viability. At the concentration of 10 μM, fenbufen amide analogs did not show cytotoxicity according to the MTT assay results. The NO scavenging activities of the fenbufen amide analogs were not significantly different from those of fenbufen.

Keywords: macrophage RAW 264.7; nitric oxide; anti-inflammatory; fenbufen amide analog
1. Introduction

The library and in-situ enzymatic screening protocol was firstly developed by Wong et al. [1-4]. Our work has successfully extended this concept to in-situ screening at a cellular level. The previous study on the library and in-situ screening indicated that fenbufen, a non-steroidal anti-inflammatory drug, could be modified with a butyl group through an amide formation reaction [5]. The derived butyl amide analogs of fenbufen were found to display significant anti-tumor effects. These findings encouraged a further study of the relationship between chemical structure and bioactivity. For example, whether the effect was mediated through cyclooxygenase, a transmembrane protein responsible for inflammatory signaling. In this paper we describe our attempts to synthesize alkyl substituted fenbufen analogs with 1, 3, 4 and 8 carbon chains and the evaluation of their cell toxicities and NO suppression effects on RAW 264.7 cells.

Anti-inflammatory compounds have been investigated in many studies for their potential in vitro inhibitory effects using lipopolysaccharide (LPS)-stimulated macrophages [6]. In this system, bacterial LPS is one of the best-characterized stimuli used to induce upregulation of pro-inflammatory proteins such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [7]. Inducible COX-2 is responsible for the high prostaglandin levels observed in many inflammatory pathologies [8]. Similarly, iNOS produces large amounts of nitric oxide (NO) and is thought to play a central role in inflammatory disease [9]. Numerous studies have reported that NO and prostaglandin (PGE2) participate in inflammatory and nociceptive events [10].

2. Results and Discussion

2.1. Synthesis of the fenbufen amide analogs

Preparation of the fenbufen amide analogs 1-4 (Figure 1) was accomplished in a good yield (70-80%), according to the usual coupling condition as described before. A chromatographic purification on silica gel was employed and spectroscopic data, including 1H-NMR and ESI-MS, were consistent with the structures and fully confirmed the identity of these analogs.

Figure 1. Fenbufen amide analogs: methyl fenbufen (1); propyl fenbufen (2); butyl fenbufen (3); octyl fenbufen (4).
2.2. Effects of fenbufen amide analogs on cell viability

As a first step towards determining the effects of fenbufen derivatives on NO production, we measured the cell number in RAW 264.7 cells. Cells treated with various concentrations (10-100 μM) of the fenbufen amide analogs were estimated using the mitochondria MTT reduction assay. These results demonstrated that fenbufen had no cytotoxic effect at concentrations ranging from 10 to 100 μM (Figure 2-a). According to the results from Figure 2, we found that the methyl fenbufen amide had the significant cytotoxic effect at the concentrations of 100 μM. As the length of the alkyl substituted chain increased, the cytotoxic effects increased, and the octyl fenbufen amide analogue had the greatest cytotoxic effect. After treatment with 30 μM octyl fenbufen amide, nearly seventy percent of the cells lost their viability (Figures 2-b, c, d, e).

**Figure 2.** Effects of fenbufen and its amide analogs on cell viabilities in RAW 264.7 cells. Cell viability was estimated using mitochondria MTT assay: (a) fenbufen; (b) methyl fenbufen amide; (c) propyl fenbufen amide; (d) butyl fenbufen amide; (e) octyl fenbufen amide. *** p < 0.001 indicate statistically significant differences.
2.3. Effects of fenbufen amide analogs on NO production in LPS-activated RAW 264.7 cells

NO in LPS-activated RAW 264.7 cells was measured by the accumulation of nitrite, the stable metabolite of NO, in the culture broth. At the concentration of 10 μM used in the study, the fenbufen amide analogs did not show cytotoxicity according to the MTT assay results. The NO scavenging activities of the fenbufen amide analogs were not significant different from that of fenbufen (Figure 3).

Figure 3. Effect of fenbufen amide analogs on LPS-activated NO production in RAW 264.7 cells. Nitrite was measured using Griess reaction at 24 h after treatment with LPS (100 ng/ml) in the presence or absence fenbufen and its amide analogs (10 μM). All data were presented as the mean ± S.D. of four independent experiments. CTL, control; F, fenbufen; F1, methyl fenbufen amide; F2, ethyl fenbufen amide; F3, propyl fenbufen amide; F8, octyl fenbufen amide.

3. Experimental

3.1. General

DMF was dried and distilled over CaH₂. The distillate was collected and stored over 4 Å MS until use. The eluents for chromatography, including EtOAc, acetone, and n-hexane, were industrial grade and distilled before use. CHCl₃ was reagent grade and used without further purification. NMR spectroscopy including ¹H-NMR (500 MHz) and ¹³C-NMR (125 MHz, DEPT-135) was performed either at the Department of Chemistry of National Tsing-Hua University (NTHU) or the Department of Applied Chemistry of National Chiao-Tung University (NCTU), both employing Varin Unity Inova 500NMR. Deuterated solvents employed for NMR spectroscopy including CD₃OD, CDCl₃ and C₆D₆ were purchased from Cambridge Isotope Laboratories, Inc. ESI-MS spectrometry employing a Micromass Q-Tof liquid chromatography tandem mass spectrometer was performed at the Department of Applied Chemistry of National Chiao-Tung University (NCTU). TLC was performed with Machery-Nagel silica gel 60 F254 precoated plates. The starting materials and products were visualized by UV (254 nm). Further confirmation was carried out by using staining with 5% p-anisaldehyde, ninhydrin or ceric ammonium molybdate [Ce(NH₄)₂(NO₃)₆, (NH₄)₆Mo₇O₂₄·H₂O] under heating. Flash chromatography was performed using Geduran Si 60 silica gel (230-400 mesh).
3.2. Typical procedure for the coupling

The chemical synthesis for the amide products followed a similar procedure to that described before [3].

*N-methyl-4-(biphenyl-4-yl)-4-oxobutanamide* (1). Anal. C_{17}H_{17}NO_{2}, M (calcd.) = 267.2 (m/z), ESI+Q-TOF: M = 267.2 (m/z), [M+H]^+ = 268.2 (99%), 269.2 (18%), [M+Na]^+ = 290.2 (100%), 291.2 (15%), [2M+H]^+ = 535.3 (6%), [2M+Na]^+ = 557.2 (53%), 558.2 (19%), 559.3 (3%), equivalent to the calculated isotopic ratio 100:38:8 for [2M+Na]^+; 1H-NMR (CD_6D_6): δ 1.11 (t, 3H, CH_3), 2.26 (t, J = 6.5 Hz, 2H, CH_2), 3.08 (t, J = 6.5 Hz, 2H, CH_2), 4.52 (bs, CONH), 7.16-7.22 (m, 3H, aromatic), 7.33-7.37 (m, 4H, aromatic).

*N-propyl-4-(biphenyl-4-yl)-4-oxobutanamide* (2). Anal. C_{19}H_{21}NO_{2}, M (calcd.) = 295.2 (m/z), ESI+Q-TOF: M = 295.2 (m/z), [M+H]^+ = 296.3 (100%), 297.2 (38%), [M+Na]^+ = 318.2 (67%), 319.2 (10%), [2M+H]^+ = 591.3 (3%), [2M+Na]^+ = 613.3 (28%), 614.3 (6%), equivalent to the calculated isotopic ratio 100:42:9 for [2M+Na]^+; 1H-NMR (CD_3OD): δ 0.93 (t, J = 7.0 Hz, 3H, CH_3), 1.52 (sextet, J = 6.5 Hz, 2H, CH_2), 2.61 (t, J = 7.0 Hz, 2H, CH_2), 3.14 (t, J = 7.0 Hz, 2H, CH_2), 7.37-7.40 (m, 1H, aromatic), 7.45-7.48 (m, 2H, aromatic), 7.66-7.68 (m, 2H, aromatic), 7.74-7.75 (m, 2H, aromatic).

*N-butyl-4-(biphenyl-4-yl)-4-oxobutanamide* (3). A solution of fenbufen (214 mg, 0.8 mmol), HBTU (302 mg, 0.8 mmol), DIEA (1 mL, 0.8 mmol) and DMF (15 mL) was stirred. The corresponding carboxylic acid analog was added. TLC (acetone/n-hexane = 3/7) indicated the consumption of starting material (R_f = 0.39) and the formation of the product (R_f = 0.78). After stirring for 1 h, the mixture was concentrated under high vacuum with oil pump at 60 °C. The residue was purified by flash chromatography using acetone/n-hexane 3:7 as eluents to provide a snow-white solid in 77% yield (269 mg). Anal. C_{20}H_{23}NO_{2}, M (calcd.) = 309.2 (m/z), ESI+Q-TOF: M = 309.2 (m/z), [M+H]^+ = 310.3 (74%), 311.3 (13%), [M+Na]^+ = 332.2 (100%), 333.2 (42%), 643.3 (10%), equivalent to the calculated isotopic ratio 100:44:10 for [2M+Na]^+; 1H-NMR (CD_3OD): δ 0.78 (t, J = 6.5 Hz, 2H, CH_2), 1.28-1.40 (m, 2H, CH_2, n-butyl), 1.46-1.52 (m, 2H, CH_2, n-butyl), 2.60 (t, J = 6.5 Hz, 2H, CH_2), 3.18 (t, J = 7.0 Hz, 2H, CH_2), 3.35 (t, J = 6.5 Hz, 2H, CH_2), 7.37-7.42 (m, 1H, aromatic), 7.46-7.48 (m, 2H, aromatic), 7.67-7.68 (m, 2H, aromatic), 7.74-7.76 (m, 2H, aromatic), 8.07-8.08 (m, 2H, aromatic); 13C-NMR (CD_6D_6): δ 13.9 (CH_3), 20.2 (CH_2), 30.4 (CH_2), 32.0 (CH_2), 34.3 (CH_2), 39.3 (CH_2), 127.4 (CH), 127.5 (CH), 128.1 (CH), 129.1 (CH), 136.0 (C), 140.4 (C), 145.7 (C), 171.1 (C), 198.1 (C).

*N-octyl-4-(biphenyl-4-yl)-4-oxobutanamide* (4). Anal. C_{24}H_{31}NO_{2}, M (calcd.) = 365.2 (m/z), ESI+Q-TOF: M = 365.3 (m/z), [M+H]^+ = 366.3 (30%), 367.3 (7%), [M+Na]^+ = 388.3 (15%), 389.3 (4%), [2M+H]^+ = 731.5 (8%), 732.5 (4%), [2M+Na]^+ = 753.4 (13%), 754.4 (8%), equivalent to the calculated isotopic ratio 100:26 for [M+H]^+; 1H-NMR (CD_6D_6): δ 0.89 (t, J = 7.0 Hz, 3H, CH_3), 1.15-1.22 (m, 8H,
CH₃, 1.25-1.30 (m, 4H, CH₂), 2.36 (t, J = 7.0 Hz, 2H, CH₂), 3.10-3.14 (m, 4H, CH₂), 7.10-7.22 (m, 3H, aromatic), 7.33-7.38 (m, 4H, aromatic), 7.90-7.92 (m, 2H, aromatic).

3.3. Biological assay chemicals and reagents

Fetal bovine serum, dimethyl sulfoxide, lipopolysaccharide (LPS, _Escherichia coli_ serotype O55:B5), sulfanilamide, _N-_(1-naphthyl)ethylenediamine, phosphoric acid, sodium nitrite, α,α-diphenyl-2-picrylhydrazyl, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and fenbufen were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All samples were dissolved in dimethylsulfoxide (DMSO) and further diluted in culture medium. The final DMSO concentration in the medium was 0.1% and did not affect cellular function or the assay systems used in this study.

3.4. Cell culture

The murine macrophage cell line RAW 264.7 was purchased from Bioresource Collection and Research Center (BCRC, Taiwan). RAW264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B), and 10% heat-inactivated fetal bovine serum (FBS). The cells were incubated in a humidified incubator at 37 °C in 5% CO₂/air.

3.5. Cell viability assay

Cell viability was determined using MTT assay [11]. Briefly, cells were seeded at a density of 7 × 10⁴/mL on 96-well plates and cultured overnight as described above. The medium was then replaced with fresh medium containing fenbufen derivatives at various concentrations. After incubation for 24 h at 37 °C in 5% CO₂/air, MTT (final concentration, 0.5 mg/mL) was added, and the cells were then incubated at 37 °C for 2 h. Finally, the cells were lysed and absorbance was detected at 550 nm. For cell number determination, a standard correlation between the known cell numbers and the absorbance density values was constructed for measuring the cell number from various detected absorbance density values.

3.6. Determination of NO production

In order to measure NO production, macrophage RAW 264.7 cells were plated into 96-well plate (5 × 10⁵ cell/mL) and treated with 100 ng/mL of LPS in the presence or absence of fenbufen derivatives for 24 h. Nitrite, a soluble oxidation product of NO, in the culture supernatant, was determined using the Griess reaction [12]. The supernatant (50 μL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using a microplate reader. Sodium nitrite was used as a standard to calculate NO₂⁻ concentration.

3.7. Statistical analysis

Data are expressed as mean ± S.D. of the indicated number of separate experiments. A one-way analysis of variance was performed for multiple comparisons, and if there was significant variation
between treatment groups, the mean values were compared with the respective control using Student’s t-test. P values less than 0.05 were considered significant.

4. Conclusions

The methyl, propyl, butyl and octyl analogs of fenbufen were successfully synthesized in good yield. As the length of the alkyl amide side chain increased, the cytotoxic effects increased. The NO scavenging activities of the fenbufen amide analogs were not significantly different from those of fenbufen itself.

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*Sample Availability:* Samples of the compounds are available from the corresponding author C.-S.Y.

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