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Melampomagnolide B: A new antileukemic sesquiterpene

Shama Nasim [a,†], ShanShan Pei [b,†], Fred K. Hagen [c], Craig T. Jordan [b,d], Peter A Crooks [a,*]

[a] Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536, USA
[b] Department of Biomedical Genetics, University of Rochester, Rochester, NY 14642, USA
[c] Department of Biochemistry and Biophysics, University of Rochester, Rochester, NY 14642, USA
[d] James P. Wilmot Cancer Center, University of Rochester, Rochester, NY 14642, USA

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ABSTRACT

Melampomagnolide B has been identified as a new antileukemic sesquiterpene. A biotin-conjugated derivative of melampomagnolide B was designed and synthesized in order to elucidate its mechanism of action. A study of the biochemical interactions of the biotin probe suggests that melampomagnolide B derives its remarkable selectivity for leukemic cells over normal hematopoietic cells from its unique ability to exploit biochemical differences between the two cell types.

1. Introduction

The past several years have seen a surge of interest in the anticancer properties of sesquiterpene lactones. A germacrenolide, parthenolide (PTL, **1**, Fig. 1) has been noted for its remarkable antileukemic properties.[1] Initial efforts pertaining to the biomechanistic study of parthenolide and its analogs revealed that they seem to promote apoptosis by inhibiting the activity of the NF-κB transcription factor complex, and thereby down-regulating anti-apoptotic genes under NF-κB control.[2–7] We have recently demonstrated that parthenolide induces robust apoptosis of primary acute myeloid leukemic (AML) cells.[8,9] In particular, parthenolide causes cell death in AML stem and progenitor cells in vitro, with minimal toxicity towards normal hematopoietic cells. The apoptosis induced by parthenolide is not solely due to NF-κB inhibition, but rather arises from a broad set of biological responses, which likely include activation of p53 and an increase in reactive oxygen species. Parthenolide has also been the source of several novel antileukemic compounds arising from our program over the past decade. We successfully overcame the poor water-solubility of parthenolide by adding amines to the exocyclic olefin of the enone function of **1**, thereby rendering the resulting compounds water-soluble.[10,11] Such adducts showed retention of antileukemic properties of parthenolide; in particular, the dimethylamine-adduct of parthenolide (DMAPT, LC-1, **2**, Fig. 1), which has progressed to phase-I clinical trials in the United Kingdom for the treatment of AML, ALL, and CLL.[10]

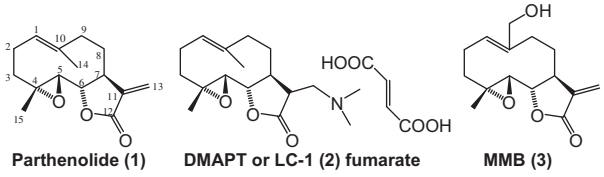

Figure 1. Structures of PTL (**1**), DMAPT (**2**), and MMB (**3**).

We now report on the identification of melampomagnolide B (MMB, **3**, Fig. 1), a melampolide originally isolated from *Magnolia grandiflora*,[12] as a new antileukemic sesquiterpene with properties similar to parthenolide. MMB was synthesized utilizing a modification of the method of Macias et al.[13] via selenium oxide oxidation of the C10 methyl group of PTL, which also results in concomitant conversion of the geometry of the C9–C10 double bond from *trans* to *cis*. This compound is of great interest to us for two reasons. First, the anti-leukemia activity of MMB is excellent, and indistinguishable from PTL. Second, as a functionalized analog of PTL, the MMB molecule allows the synthesis of conjugated analogs that retain biological activity. For example, as a laboratory tool, we created a biotinylated analog of MMB via conjugation at the allylic hydroxyl group, and used this reagent to identify MMB target proteins in AML cells. This approach has proven to be extremely useful in better understanding the underlying mechanisms by which anti-leukemia activity is achieved. Thus, as a basis for the further development of this

* Corresponding author. Tel.: +1 859 257 1718; fax: +1 859 257 7585.
E-mail address: pcrooks@email.uky.edu (P.A Crooks).
† These authors contributed equally to this work.

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drug, we sought to perform comprehensive analysis of drug mechanism. To this end, we initiated a program that focused on delineating the cellular proteins and signaling cascades influenced by **3** or its analogs. One branch of this program is to synthesize chemical probes based on **3** that would retain its antileukemic potential, but contain a 'reporter' or a marker that could serve to highlight the subcellular localization or biochemical interactions of the probe. This report describes the first of such efforts, which sought to utilize the potential of a biotin moiety in highlighting the interactions of **3** with cellular proteins, as well as to study its localization into organelles through microscopy. In the present study, we describe the chemical synthesis of a biotinylated analog of **3** and demonstrate that this compound is a robust agent for the identification of protein binding events.

2. Design rationale and antileukemic activity studies

A biotin analog of **3** that involved the conjugation of the allylic hydroxyl group with a suitable biotinylated moiety to afford **4** (Fig. 2), was synthesized. The utilization of this probe presented an opportunity to identify all proteins directly modified by **3**, and thereby reveal other mechanisms contributing to the antileukemic activity of MMB.

3. Chemistry

The synthesis of **4** utilized PTL as starting material, via selenium oxide oxidation of the allylic methyl group applying a modification of the method of Macias et al. (Scheme 1).[13] The allylic methyl group of PTL was subjected to SeO_2/*t*-BuOOH oxidation, yielding a mixture of MMB (**3**) and aldehyde **5**. This reaction proved to be particularly fickle, with the aldehyde **5** being the major side-product that formed in significant quantities and impaired chromatographic isolation of **3**. There was a significant variation in the quantity of **5** formed in relation to **3**. For optimal oxidation of **1** to MMB (**3**), the literature procedure prescribes a combination of SeO_2 and *t*-butyl hydroperoxide, which in our hands afforded a mixture of the required alcohol **3** and the aldehyde **5** in approximately a 1:1 ratio. While the quality of *t*-butyl hydroperoxide was inconsequential to the ratio, the quality (purity) of SeO_2 was found to be very important. Samples that possessed the characteristic pink color of selenium were found to afford higher quantities of **5**, at the expense of **3**. In addition, alcohol **3** could only be separated from **5** with difficulty by silica gel chromatography. NMR spectroscopic analysis was consistent with the structural assignments reported by Macias et al.,[13] and an X-ray crystal structure obtained in our hands was identical to that previously reported by Gonzalez et al.[14] A Mitsunobu reaction on **3** with Fmoc-protected 12-aminododecanoic acid afforded **6**. While the use of morpholine/piperidine is common for Fmoc deprotections, this transformation had to be carried out with TBAF instead, due to the possibility of morpholine/piperidine adding to the enone function of **6**. The amine **7**, formed in situ, was treated directly with the pentafluorophenyl ester of biotin to afford the target compound **4**. Purification of this reaction mixture proved to be challenging, with the best conditions being the elution of the evaporated reaction mixture from an Et_3N-treated silica column with a gradient of *i*-PrOH in CH_2Cl_2. It should also be noted that the germacrenolide ring of **3** seemed to be susceptible to the basicity of F^- from TBAF, with numerous by-products being formed that lacked the Fmoc-dodecanoic acid–biotin appendage, according to NMR spectral data. The identity of these by-products was not determined. The use of other coupling reagents, that is, HATU and EDC, afforded reaction mixtures that could not be resolved on silica so as to afford **4** in an analytically pure form.

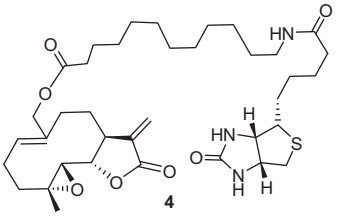
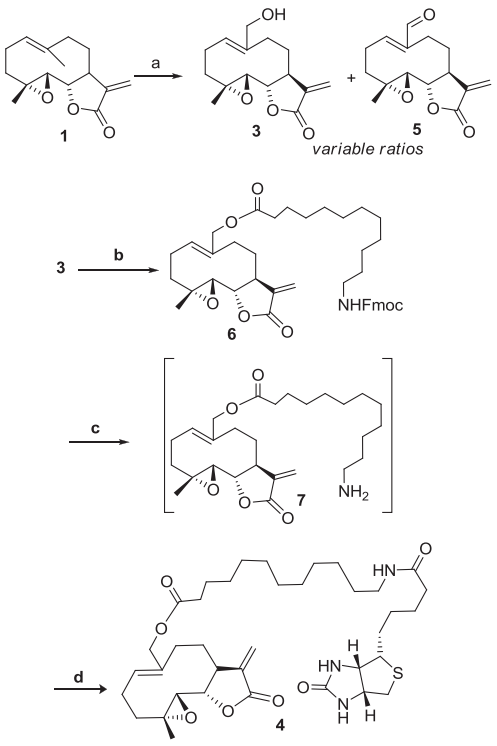


Figure 2. Structure of the biotinylated MMB probe (**4**).



a: SeO_2, tert.BuOOH, CH_2Cl_2, 1.5 h, 40 °C, 50 %; b: Fmoc-protected dodecanoic acid, Ph_3P, DIEAD, rt, 18 h, 68 %; c: TBAF, DMF, 30 min, d: Biotin pentaflurophenol, 18 h, rt, 45 %.

Scheme 1. Synthesis of **3** from **1**, and conjugation of biotin to **3**.

4. Antileukemic properties of 4

To validate the retention of functional properties, the activity of **3** and **4** were compared to **1** using assays that measure inhibition of NF-κB activity. Primary leukemia cells were treated with varying concentrations of **1**, **3** or **4** for 6 h, followed by lysis and analysis by immunoblot. Inhibition of NF-κB activity was assessed by measuring phosphorylation of the NF-κB p65 subunit at Ser-536. As shown in Figure 3A, a significant loss of phosphorylation is observed for **3** at 7.5 μM concentration, and for **4** at 15 μM concentration. To further evaluate the cytotoxicity of **3** and **4**, cell viability was measured following 24 h exposure to each compound. As shown in Figure 3C, efficient induction of cell death is achieved at 7.5 μM **3**. To achieve a similar degree of cell death with **4**, treatment at 20–30 μM was required, in good agreement with the NF-κB phosphorylation analysis shown in Figure 3A. Thus, while **3** and **4** retain the biological properties of **1**, the activity of **4** is reduced approximately two-fold. We attribute this minor loss in activity to steric hindrance resulting from the addition of the bulky biotin moiety. We compared the activities of **1** and **3**. Allylic alcohol **3** was found to possess potency and selectivity similar to **1**. Thus, MMB is active in spite of being a melampolide and not a germacrenolide, and further studies into the newly found antileukemic activity of **3** are warranted.

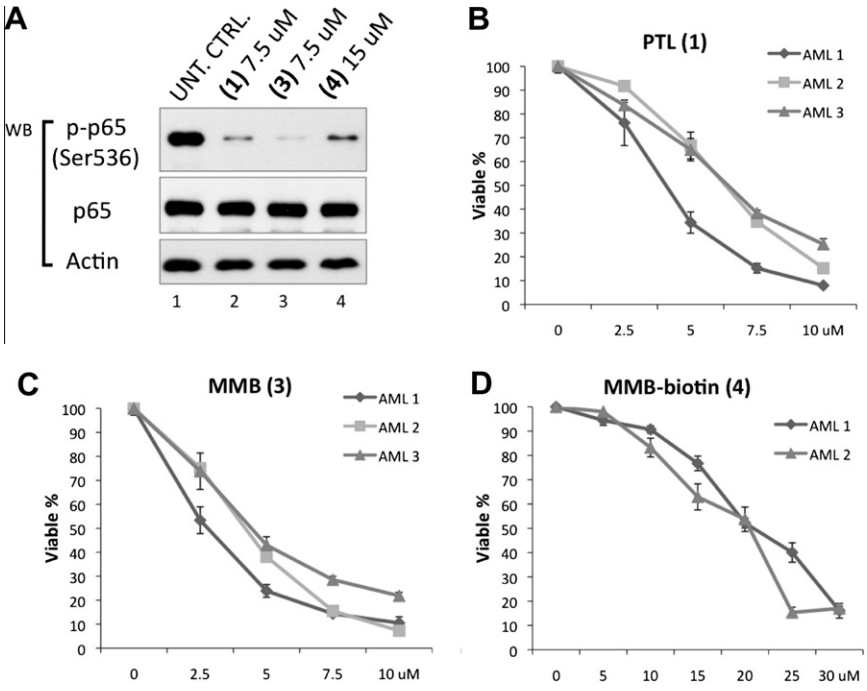


Figure 3. Biological activities of **4** compared to **1** and **3**. (A) Immunoblot of phosphorylated p65 subunit of the NF-κB complex. Also shown are total p65 and actin. (B–D) Viability of primary leukemia cells after overnight culture at varying concentrations.

Subsequent studies employed **4** as a reagent to identify potential leukemic proteins modified by MMB. Primary human cells were treated with 20 μM **4** for 6 h, followed by lysis and immunoblot analysis using a streptavidin probe. As shown in Figure 4, multiple protein targets were identified in the whole cell lysates. Notably, the analysis of normal bone marrow (NBM) cells (lane 2) in comparison to acute myeloid leukemia (AML) cells (lane 3) indicates distinct differences in the spectrum of cellular targets, even though the identical protein–compound **4** ratio is used for normal and leukemic preparations (*marks a non-specific band recognized by SA-HRP probe). Thus, while the chemistry of **4** should be essentially the same in any cell type, there must be intrinsic differences between normal and leukemic cells that lead to the interaction with unique targets. We propose that this differential binding contributes to the leukemia-specific cell death previously described for **1**. To further validate the utility of **4** for analysis of protein targets, we performed biochemical pull-down studies. Previous studies have demonstrated that biotinylated **1** binds to the NF-κB regulatory protein IKK-β.[15] Therefore, we tested whether **4** would also bind this protein. In Figure 5, primary leukemia cells were pre-treated for 2 h with **1** (lanes 2 and 5) or biotin control (b.c.) (lanes 3 and 6), prior to a 4 h incubation with **4**. Cells were then lysed and immuno-precipitated by streptavidin beads. Pull-down products were analyzed by immunoblot to identify specific proteins. As shown in Figure 5, one product identified was IKK-β, the known target of **1**,[15,16] thereby validating the specificity of the reagent for targets relevant to the anti-leukemia mechanism of action. In addition, the NF-κB p65 subunit was identified as a direct target in the pull-down, suggesting interactions with multiple components of the NF-κB signaling pathway.

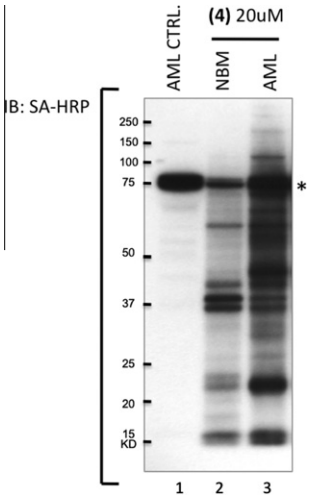


Figure 4. Immunoblot analysis of whole cell protein lysates. Lane 1 shows control using AML protein probed with biotin to detect non-specific interactions. Lanes 2 and 3 are normal bone marrow (NBM) lysate and AML, respectively, probed with analog **4**.

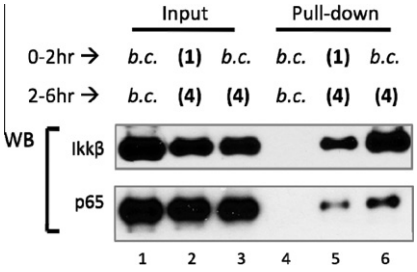


Figure 5. Identification of MMB binding proteins. Analog **4** was used for pull-down studies to identify specific target proteins. As indicated above each lane, specimens were subjected to a 2-h pre-incubation with either parthenolide (**1**) or a biotin control (b.c.) to test specificity of subsequent interactions. Next, each specimen was incubated for 4 h with either analog **4** or biotin control. Lanes 1–3 show total input lysate for the three independent conditions. Lanes 4–6 show pull-downs for the same conditions shown in lanes 1–3. Input or pull-down products were probed with antibodies to the NF-κB regulator IKK-β or the NF-κB subunit p65. As shown in lane 6, both IKK-β and p65 were successfully detected by pull-down. Lane 5 indicates that pre-incubation with **1** successfully competes for binding with **4**, thereby indicating specificity of the pull-down for **4**.

Notably, pre-incubation with **1** potently reduced binding of IKK-β or p65 to **4** (lane 5), indicating such binding between IKK-β or p65 and **4** is through the same mechanism between IKK-β or p65 and **1**.

5. Summary

Taken together, these data show that melampomagnolide B is a new antileukemic agent with remarkable selectivity for leukemic cells over normal hematopoietic cells. This selectivity is derived from its unique ability to exploit biochemical differences between the two cell types. The study also demonstrates the utility of the biotin conjugate **4** for the identification of protein binding targets of both **3** and **1**. Additional studies are ongoing to identify all the proteins that interact with **4**.

6. Experimental

Biotin was purchased from AK Scientific, Inc, Mountain View, CA. All other reagents and chemicals were purchased from Aldrich Chemical Co., Milwaukee, WI. THF and diethyl ether were distilled over sodium-benzophenone ketyl and stored under argon. All other solvents and chemicals were used as received. TLC analyses were run on Analtech Silica Gel GF® plates. Melting points were determined on a Fisher Scientific melting point apparatus and are uncorrected. NMR spectra were run on a Varian 300 MHz NMR spectrometer in $CDCl_3$ and chemical shifts are reported in ppm relative to TMS as internal standard. Mass spectra were recorded on a JEO: JMS-700T MStation or on a Bruker Autoflex MALDI-TOF MS. DMF was either distilled over P_2O_5 immediately before use or the anhydrous grade from Aldrich® was used. Parthenolide was purchased from Aldrich (St. Louis, MO). CHN analysis was performed by Atlantic Micro Labs, and are within ±0.4% of theoretical values. The synthesis of compound **3** has been reported elsewhere,[13] and its characterization data are in good agreement with reported values.

6.1. Synthetic procedures for the synthesis of compounds 3, 4, and 6

6.1.1. Melampomagnolide B (3)

A solution of **1** (250.0 mg, 1.0 mmol) in CH_2Cl_2 was treated with SeO_2 (111.0 mg, 1.0 mmol) and *tert*-butyl hydroperoxide (1 M in dodecane, 1 mL) and the mixture was refluxed gently for an hour, after which it was evaporated. The resulting semisolid was subjected to silica gel chromatography to afford **3** (132.0 mg, 50%). Hexane/acetone = 85:15); white crystals; mp 172–176 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.25 (app., J = 10.0 Hz, 1H), 5.66 (app. t, J = 6 Hz, 1H), 6.56 (app. d, J = 10.0 Hz, 1H), 4.12 (q, J = 12.6 Hz, 2H), 3.86 (t, J = 9.6 Hz, 1H), 2.83 (m, 2H), 2.49–1.13 (m, 5H), 1.72–092 (m, 2H), 1.56 (s, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.5, 139.6, 138.9, 127.7, 120.3, 81.3, 66.1, 63.6, 60.3, 43.1, 37.1, 25.9, 24.2, 24.0, 18.3 ppm; EI-MS *m/z*; 264.

6.1.2. 14-(*N*-Fmoc-12-aminododecanoxy)melampomagnolide B (6)

A solution of **3** (264 mg, 1 mmol), Fmoc-dodecanoic acid (437 mg, 1 mmol) and Ph_3P (262 mg, 1 mmol) in THF (3 mL) was treated drop-wise with diethyl azodicarboxylate until a yellow color persisted. The resulting solution was stirred for 12 h at room temperature and then evaporated to afford a semisolid that was subjected to silica gel chromatography (hexane/acetone, 9:1) to afford **6** (463.0 mg, 68%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 (d, J = 7.5 Hz, 2H), 7.59 (d, J = 7.5 Hz, 2H), 7.42–7.25 (m, 4H), 6.25 (d, J = 3.6 Hz, 1H), 5.67 (app. t, J = 8.1 Hz, 1H), 5.53 (d, J = 3.0 Hz, 1H), 4.78 (br d, s, 1H), 4.65 (d, J = 12.3 Hz, 1H), 4.46–4.19 (m, 5H), 3.84 (t, J = 9.3 Hz, 1H), 3.19 (m, 2H), 2.93–2.83 (m, 2H), 2.45–2.11 (m, 6H), 1.43–0.87 (m, 21 H), 1.53 (s, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.5, 169.4, 156.5, 144.1, 141.4, 138.8, 135.0, 130.7, 127.7, 127.1, 125.1, 120.4, 120.1, 81.2, 66.77, 66. 71, 63.5, 60.21, 47.5, 42.9, 41.3, 36.9, 34.5, 30.2, 29.8, 29.7, 29.5, 29.4, 27.0, 26.0, 25.2, 24.8, 24.1, 18.3 ppm. ESI-MS *m/z*: 683 $(M+H)^+$.

6.1.3. 14-[*N*-(Biotinyl)-12-aminododecanoxy]melampomagnolide B (4)

To a solution of **6** (150 mg, 0.30 mmol) in DMF (2 mL), was added TBAF (1 M in THF, 0.30 mL), and the resulting solution stirred at ambient temperature for 30 min. In another flask, biotin–pentafluorphenol (0.435 mg, 0.22 mmol) was dissolved in DMF (2 mL) and this solution was added to the flask containing **6**. The resulting solution was stirred for 12 h at room temperature and then evaporated to afford a sticky brown-colored gum. This residue was purified by silica gel column chromatography to afford **4** (silica neutralized with Et_3N), CH_2Cl_2/*i*-PrOH, (67.0 mg), 45% yield; white solid; 130–132 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.27 (d, J = 3.3 Hz, 1H), 6.23 (s, 1H), 5.98 (t, J = 5.5 Hz, 1H), 5.69 (t, J = 8.4 Hz, 1H), 5.55 (d, J = 3.3 Hz, 1H), 5.40 (s, 1H), 4.65 (d, J = 12.6 Hz, 1H), 4.52 (m, 2H), 4.32 (m, 1H), 3.87 (t, J = 9.3 Hz, 1H), 3.23 (m, 3H), 2.92 (m, 3H), 2.75 (d, J = 15 Hz, 1H), 2.48–2.14 (m, 8H), 1.85–1.07 (m, 28H), 1.55 (s, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.5, 172.8, 169.4, 138.8, 135.0, 130.6, 120.4, 81.2, 66.7, 63.5, 61.9, 60.34, 60,2, 59.1, 55.5, 42.9, 40.8, 39.8, 36.8, 36.3, 34.5, 34.2, 32.2, 29.99, 29.90, 29.7, 29.6, 29.5, 29.4, 28.3, 27.2, 26.0, 25.8, 25.2, 24.8, 24.1, 18.3 ppm; ESI-MS *m/z*: 688 $(M+H)^+$. Anal. Calcd for $C_{37}H_{57}N_3O_7S$: C, 64.60; H, 8.35; N, 6.11. Found: C, 64.63; H, 8.24; N, 6.15.

6.2. Biological assays

6.2.1. Streptavidin (SA) beads pull-down assay

Treated cells were washed three times in cold PBS and lysed in Buffer F (10 mM Tris–HCl pH 7.5, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, 1% Triton X-100) with freshly added proteinase inhibitors (1 mM PMSF, 1× PIC, 0.1 mM Na_3OV_4). Lysates were cleared by 10 min of 12,000 rpm spinning at 4 °C and the supernatant was incubated with SA beads for 2 h on an end-to-end rotor at 4 °C. Beads were then washed sequentially with 1× PBS, high salt wash buffer (500 mM NaCl in 0.1 M pH 5.0 NaOAc), low pH wash buffer (0.1 M pH 2.8 glycine–HCl), and one last time in 1× PBS. After the wash steps, the SA beads were boiled for 10 min in 2× SDS–PAGE sample buffer to elute down all pull-down products.

6.2.2. Immunoblotting

Cell lysates or pull-down products were diluted in 5× SDS–PAGE sample buffer (10% w/v SDS, 10 mM DDT, 20% glycerol, 0.2 M Tris–HCl, pH 6.8, 0.05% w/v bromophenol blue), and run on 8–10% SDS–PAGE gels. Protein gels were then transferred to PVDF membrane and blocked with 5% milk in 0.1% TBST (20 mM Tris–HCl pH 7.5, 137 mM NaCl, 0.1% Tween 20), followed by incubation with antibodies against p-p65(ser-536) (Cell Signaling), IKK-β (Cell Signaling), p65 (Santa Cruz), β-actin (Sigma), or SA-HRP probe (Thermo).

6.2.3. Cell viability assays

Cells treated with different concentrations of **1**, **3** or **4** were washed with cold PBS and resuspended in 200 μL of Annexin binding buffer (10 mM HEPES/NaOH pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$). Annexin-V and 7-amino-actinomycin (7-AAD) were added and the tubes were incubated at ambient temperature in the dark for 15 min. Cells were then diluted with 200 μL of Annexin binding

buffer and analyzed immediately by flow cytometry. Viable cells were scored as Annexin-V negative/7-AAD negative. Percent viability data provided are normalized to untreated control specimens.

Acknowledgments

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