Bioorganic & Medicinal Chemistry 17 (2009) 2210–2214

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

2,3-Dihydrowithaferin A-3β-*O*-sulfate, a new potential prodrug of withaferin A from aeroponically grown *Withania somnifera*

Ya-ming Xu [a], Marilyn T. Marron [a], Emily Seddon [a], Steven P. McLaughlin [a], Dennis T. Ray [b], Luke Whitesell [c], A. A. Leslie Gunatilaka [a,d,*]

[a] *Southwest Center for Natural Products Research and Commercialization, Office of Arid Lands Studies, College of Agriculture and Life Sciences, The University of Arizona, 250 E. Valencia Road, Tucson, AZ 85706, USA*
[b] *Department of Plant Science, College of Agriculture and Life Sciences, The University of Arizona, Tucson, AZ 85721, USA*
[c] *Whitehead Institute, 9 Cambridge Center, Cambridge, MA 02142, USA*
[d] *BIO5 Institute, The University of Arizona,1657 E. Helen Street, Tucson, AZ 85721, USA*

ARTICLE INFO

Article history:
Received 14 May 2008
Revised 9 August 2008
Accepted 31 October 2008
Available online 12 November 2008

Keywords:
Aeroponic culture
2,3-Dihydrowithaferin A-3β-*O*-sulfate
Prodrug
Anticancer activity
Cytoskeletal reorganization
Heat-shock induction

ABSTRACT

Preparations of the roots of the medicinal plant *Withania somnifera* (L.) Dunal commonly called ashwagandha have been used for millennia in the Ayurvedic medical tradition of India as a general tonic to relieve stress and enhance health, especially in the elderly. In modern times, ashwagandha has been shown to possess intriguing antiangiogenic and anticancer activity, largely attributable to the presence of the steroidal lactone withaferin A as the major constituent. When cultured using the aeroponic technique, however, this plant was found to produce a new natural product, 2,3-dihydrowithaferin A-3β-*O*-sulfate (**1**), as the predominant constituent of methanolic extracts prepared from aerial tissues. The characteristic bioactivities exhibited by **1** including inhibition of cancer cell proliferation/survival, disruption of cytoskeletal organization and induction of the cellular heat-shock response paralleled those displayed by withaferin A (**2**). The delayed onset of action and reduced potency of **1** in cell culture along with previous observations demonstrating the requirement of the 2(3)-double bond in withanolides for bioactivity suggested that **1** might be converted to **2** in cell culture media and this was confirmed by HPLC analysis. The abundant yield of **1** from aeroponically cultivated plants, its good aqueous solubility and spontaneous conversion to **2** under cell culture conditions, suggest that **1** could prove useful as a readily formulated prodrug of withaferin A that merits further evaluation in animal models.

1. Introduction

Withania somnifera (L.) Dunal has been used for over 3000 years in the Ayurvedic medical tradition of India where preparations of dried root powder called 'ashwagandha' have been administered as a general tonic to increase energy, improve overall health and longevity, and to prevent disease in the elderly. Recent cell culture and animal model experiments have shown that ashwagandha possesses anti-inflammatory,[1] immuno-modulatory,[2,3] cardioprotective,[4] antioxidant,[5] and antitumor[6] activities. Much of this information has been summarized in recent reviews.[7–9] Although no controlled clinical trials of ashwagandha have been reported for any indication, it appears to have a relatively low toxicity profile based on a single human study[10] and toxicological studies in mice.[11] In Ayurvedic medicine ashwagandha has been claimed to be effective against arthritis, anxiety, insomnia, and stress.[9] Ashwagandha is currently regulated in the U.S. and Europe as a dietary supplement. The primary bioactive constituents of ashwagandha include tetracyclic terpenoids, alkaloids, and an array of steroidal lactones known collectively as withanolides. Laboratory investigation of the anticancer activity of withanolides dates back to the 1960's when Kupchan and coworkers reported the isolation, structure elucidation, and tumor inhibitory activity of withaferin A (**2**).[12] Subsequent work by Shohat et al. confirmed the anticancer activity of **2** at well tolerated doses against several transplantable mouse tumors grown either as ascites or subcutaneous xenografts.[13] They also found that low doses of **2** which had only modest antitumor activity were much more effective in combination with low dose radiotherapy. This finding was confirmed by Devi et al. who performed dose-response studies in mice bearing Ehrlich Ascites carcinomas.[14] Shohat et al. further showed that mice cured by **2** were refractory to re-challenge with the same tumor due to induction of an antitumor immune response.[15] The Developmental Therapeutics Program of the U.S. National Cancer Institute has also evaluated **2** (NSC 101088) against its panel of 60 human cancer cell lines and has reported a mean 50% Growth Inhibitory Concentration (GI_{50}) of 0.62 μM. More recently the activity of **2** against human prostate cancer xenografts at systemically well-tolerated drug exposures has been confirmed in mice, but organic solvents

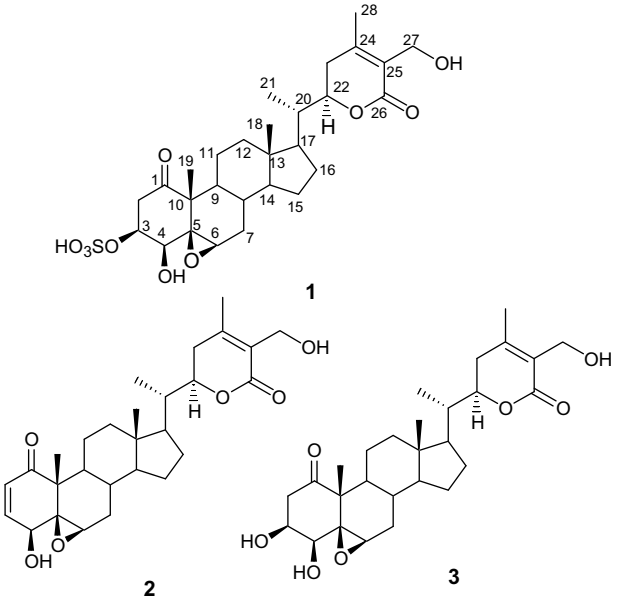
* Corresponding author. Tel.: +1 520 741 1691; fax: +1 520 741 0113.
E-mail address: leslieg@ag.arizona.edu (A.A. Leslie Gunatilaka).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.10.091

and complex emulsifying vehicles were required for the formulation for parenteral administration due to its poor solubility.[16]

The mode of action of withaferin A (**2**) remains controversial and may be cell-type specific. We and others have previously reported the actin bundling protein annexin II,[17] the 20S proteasome,[16] and the intermediate filament protein vimentin[18] as potential direct protein targets of **2** in cells. In addition to cytoskeletal disruption and inhibition of cell motility, other reported biological effects of **2** include inhibition of NFκB activation,[19] inhibition of protein kinase C,[20] and induction of Par-4-dependent apoptosis.[21] While the proximal molecular target(s) responsible for the anticancer activity of withaferin A (**2**) remains elusive, limited structure-activity studies have suggested the requirement of the C-2(3) unsaturation for its cytotoxic activity and that the double bond at C-24(25) was not essential for this activity;[22] the lactone moiety in the side chain and the C-5(6)-epoxide functionality also appear to be important for the anticancer activity of **2** and the other withanolides reported to date.

Commercial cultivation of *W. somnifera* is known primarily in various regions of India and to a lesser extent in the U.S. The constituent metabolite profiles of various chemotypes of *W. somnifera* growing in different regions of the world have been investigated by TLC analysis of their extracts and were found to have significant variability, but all chemotypes were shown to produce withaferin A (**2**).[23] In an attempt to generate bulk quantities of *W. somnifera* biomass in an efficient and ecologically friendly manner and to isolate sufficient amounts of **2** for detailed biological and structure–activity relationship studies, we have investigated its cultivation under aeroponic conditions.[24] This technique utilizes a soil-less method of growing plants in chambers under a controlled environment while spraying the roots intermittently with nutrients of defined chemical composition. It has important advantages over traditional plant production techniques because it provides opportunities to optimize yield and consistency of metabolite production under well controlled conditions, thereby facilitating commercial-scale production of bioactive compounds. When grown aeroponically, the aerial parts of *W. somnifera* were found to yield large quantities of a new withanolide identified as 2,3-dihydrowithaferin A-3β-*O*-sulfate (**1**). More importantly, we have demonstrated that this compound is water-soluble (making it amenable to formulation for in vivo studies) and undergoes ready conversion to **2** in cell culture media, thereby demonstrating the same spectrum of bioactivities reported for withaferin A.[17]



2. Results and discussion

Air-dried and powdered aerial tissues of aeroponically- grown *W. somnifera* were extracted with MeOH. The crude extract containing a major polar constituent by TLC was subjected to reversed-phase chromatography eluting with a gradient of 50–100% aqueous MeOH. The fraction eluted with 50% aqueous MeOH was further fractionated by reversed-phase chromatography to obtain the polar constituent **1** as a colorless crystalline solid. The ^{1}H NMR (500 MHz, C_5D_5N) spectrum of **1** showed four methyl signals typical of a withanolide including three singlets at δ 2.07 (CH_3-28), 1.63 (CH_3-19), and 0.50 (CH_3-18), and a doublet at δ 0.95 (J = 6.5 Hz, CH_3-21).[25] The proton signals at δ 4.84 and 4.74 (J = 12.0 Hz) suggested the presence of C-27-CH_2OH. The broad singlet at δ 3.40 was assigned to the proton at C-6 bearing the 5,6-epoxide. The ^{13}C NMR (125 MHz, C_5D_5N) spectrum of **1** was similar to that of viscosalactone B (**3**)[25] except for the signal due to C-3. Complete assignment of the ^{13}C NMR spectrum of **1** was made with the help of its DEPT spectrum and the data reported for known withanolides.[26] The low-field shift ($\Delta\delta$ + 5.3) of the C-3 signal compared to that of **3** indicated that the 3-OH group of **1** has undergone derivatization leading to an ester. The negative ESI-MS of **1** gave the pseudomolecular ion peak at m/z 567 $[M-H]^-$, which is 80 mass units higher than that of **3** suggesting that **1** was a sulfate or a phosphate ester of **3**. The negative HRESI-MS provided the accurate mass of the pseudomolecular ion $[M-H]^-$ at m/z 567.2248 (calcd for $C_{28}H_{39}O_{10}S$, 567.2264) and confirmed that **1** is a sulfate ester of **3**. The appearance of the protons at C-3 and C-4 as broad singlets in the ^{1}H NMR spectrum of **1** suggested that these two protons have α-orientation. Based on the foregoing evidence, the structure of **1** was determined as 2,3-dihydrowithaferin A-3β-*O*-sulfate. Treatment with K_2CO_3 in pyridine at 90 °C led to quantitative conversion of **1** to **2**, further supporting the structure proposed for this natural product. Although sulfated withanolides are known to occur in soil-grown *W. somnifera* as minor metabolites,[27] this constitutes the first report of the occurrence of 2,3-dihydrowithaferin A-3β-*O*-sulfate (**1**) and its production by *W. somnifera* as a major metabolite.

To begin characterizing the bioactivity of 2,3-dihydrowithaferin A-3β-*O*-sulfate (**1**), we first compared its concentration-dependent inhibition of cell proliferation/survival to that of withaferin A (**2**). As a representative human cancer cell line, MCF-7 breast adenocarcinoma was cultured in the presence of serial dilutions of the two compounds and relative viable cell number determined by standard dye reduction (MTT) assay at either 24 or 72 h post drug treatment (Fig. 1). Interestingly, at the earlier time point, **1** demonstrated significantly less activity than **2** with only ~50% inhibition achieved at a concentration that resulted in near complete inhibition by compound **2**. In contrast, by 72 h, both compounds appeared nearly equipotent in this assay. The same experimental design was repeated using two other cancer cell lines [NCI-H460 (non small cell lung) and PC-3M (metastatic prostate cancer)] with similar results (data not shown), consistent with an intrinsic difference between the compounds rather than an idiosyncrasy of the particular cell line tested.

Although the precise molecular mechanism responsible for the anticancer activity of **2** remains controversial, a striking hallmark of drug action is the disruption of cytoskeletal organization with the appearance of focal aggregates of filamentous actin (F-actin).[17] To evaluate the ability of 2,3-dihydrowithaferin A-3β-*O*-sulfate (**1**) to induce this unusual phenotype, human diploid fibroblast (WI-38) cells were cultured in the presence of the compound and cells after treatment were fixed and stained at various intervals to visualize F-actin (Fig. 2). For comparison, parallel cultures were incubated with withaferin A (**2**) applied at the same concentration.

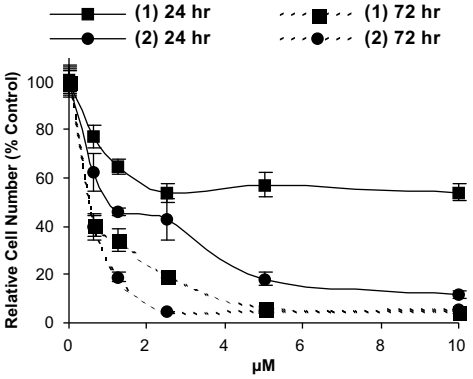


Figure 1. Concentration- and time-dependent cell proliferation/survival inhibition of compounds **1** and **2**. MCF-7 breast cancer cells were exposed to the indicated concentrations of compounds and relative viable cell number determined by dye reduction assay after the time intervals noted. Data are representative of three independent experiments. Error bars: SD.

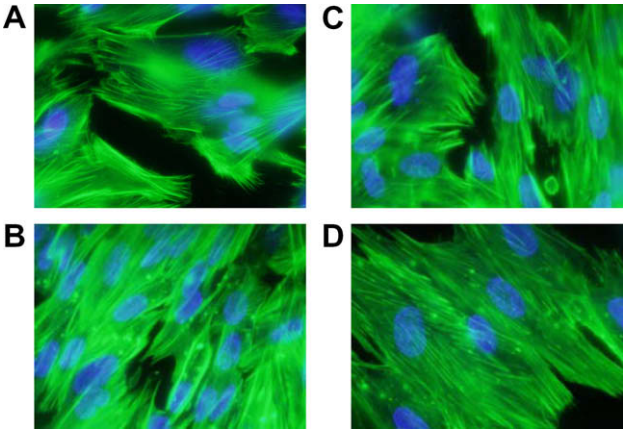


Figure 2. Induction of F-actin aggregation. Following incubation with test compounds for the indicated intervals, WI-38 fibroblasts were fixed and stained with fluorescently labeled phalloidin (green signal) to visualize the actin cytoskeleton. Cells were counterstained with DAPI (blue signal) to identify their nuclei. (A) DMSO, 24 h incubation. (B) Compound **2**, 4 h; (C) Compound **1**, 4 h; (D) Compound **1**, 24 h. All images were acquired using the same magnification and exposure conditions. Data are representative of two independent experiments.

As expected, punctate aggregates of F-actin were visible within 4 h post addition of **2**. An equal concentration of **1** induced a comparable pattern of aggregation, however, it was not detectable until 24 h post drug addition, a time at which cells exposed to **2** had completely lost integrity and could no longer be evaluated. Similar results were obtained with the epithelial cell line MCF-7 (data not shown).

Following exposure to proteotoxic insults, cells dramatically increase the production of a relatively small group of proteins that are collectively known as 'heat-shock' or 'stress' proteins and induction of their expression has become a key hallmark of the heat-shock response.[28] Work by our group and by many others over the last three decades has revealed that these heat-shock proteins and their close, constitutively expressed relatives are actually molecular chaperones, which guard against 'illicit or promiscuous interactions' between other proteins. Their basal levels facilitate normal protein folding and guard the proteome from the dangers of misfolding and aggregation. In the face of proteotoxic stressors including heat, hypoxia/ischemia, free radicals, ATP depletion, acidosis, and mutation (conditions especially relevant to cancer and neurodegeneration), heat-shock proteins also assume the roles of disaggregating, refolding, and, in case of failure, diverting aggregated and misfolded proteins to the proteasome machinery for final destruction. Perhaps as a consequence of the aggregation phenomenon demonstrated in Figure 2, we find that exposure of cells to **2** results in profound activation of the cellular heat-shock response (Fig. 3a and b). Although induction of the heat-shock response has been described for the related withanolide, withangulatin A,[29] it has not been previously reported for **2**. The increased heat-shock protein expression stimulated by **2** requires Heat-Shock Factor 1 (HSF1), the dominant transcriptional regulator of the classical response to heat[30] (Fig. 3a). Exposing a heat-shock reporter cell line to serial concentrations of **2** demonstrated that the response can be induced at compound exposures compatible with cell survival and robust expression of the fluorescent reporter protein (Fig. 3b). These findings suggest potential applications for this class of compounds as therapeutic inducers of the heat-shock response that could ameliorate neurodegenerative disorders associated with protein aggregation and highlight the need to identify analogs that can be more readily formulated for in vivo administration.[31] In this regard, we found that freely water-soluble 2,3-dihydrowithaferin A-3β-*O*-sulfate (**1**) induces a robust heat-shock response after overnight treatment comparable to that of withaferin A (**2**) (Fig. 3b), but consistent with findings in Figures 1 and 2, at a significantly higher concentration.

Considering the structural relationship between **1** and **2** and the reported requirement of C-2(3) unsaturation in withanolides for bioactivity, we hypothesized that conversion of **1** to **2** occurs spontaneously in cell culture media and that such transformation is responsible for the observed biological activities of **1**. To test this hypothesis, **1** was incubated in cell culture medium consisting of

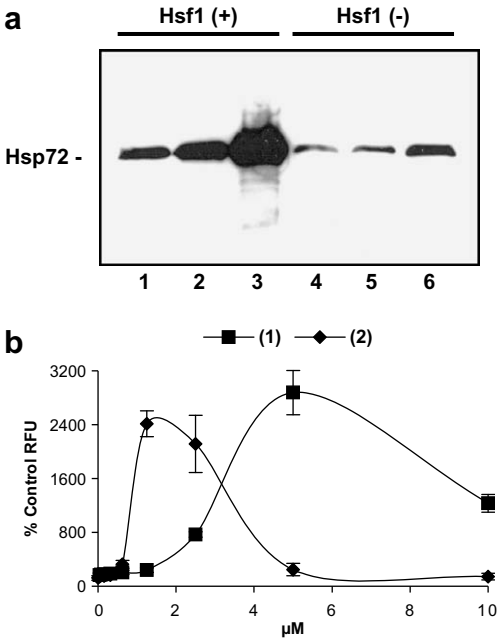


Figure 3. (a) HSF1-dependent induction of the heat-shock response. Immortalized mouse embryo fibroblasts derived from mice in which *Hsf1* was knocked out [*Hsf1*(−)] or their wild-type littermates; [*Hsf1*(+)] were exposed overnight to DMSO (0.2%) (lanes 1 and 4), geldanamycin (GA, 0.5 μM) (lanes 2 and 5) or withaferin A (**2**) (2 μM) (lanes 3 and 6). Equal amounts of total cellular protein were immunoblotted for relative levels of a highly inducible heat-shock protein (Hsp72). (b) Heat-shock reporter induction measured by micro plate fluorometer. Reporter cells stably transduced with a plasmid encoding enhanced green fluorescent protein (EGFP) under the control of a minimal heat-shock response element were exposed to **1** or **2** at the indicated concentrations overnight. Relative fluorescence units (RFU) per well was determined as a measure of reporter activation. Each point represents the mean of nine determinations from three independent experiments. Error bars: SD.

Dulbecco/Vogt Modified Eagle's Minimal Essential Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and its conversion to **2** was monitored by HPLC. Over the course of 24 h at 37 °C, nearly all of **1** was lost from the culture medium in association with the corresponding de novo appearance of **2** (Fig. 4A). Incubation of **1** in PBS supplemented with 10% FBS instead of DMEM markedly slowed the loss of **1** and the appearance of **2** suggesting that buffering conditions or certain small molecule components in DMEM drive the conversion of **1** to **2** and not metabolism by serum enzymes (data not shown). In this regard, omission of free L-cysteine/L-methionine from the DMEM formulation used for drug incubation slowed the disappearance of **1** and enhanced the appearance of **2** over time (Fig. 4B). L-cysteine has been reported to react readily via its thiol group with **2** leading to its bio-inactivation.[22] This reactivity may explain the lack of quantitative conversion in DMEM of **1** to **2** evident in Figure 4A. In summary, the abundant yield of 2,3-dihydrowithaferin A-3β-*O*-sulfate (**1**) from aeroponically cultivated plants, its good aqueous solubility and spontaneous conversion to withaferin A (**2**) under cell culture conditions suggest that the compound could prove useful as a readily formulated withaferin A prodrug that merits further evaluation in animal models.

3. Experimental

3.1. General experimental procedures

Reagents and solvents for extraction and chemical reactions were purchased from Aldrich Chemical Co. Bakerbond C_{18} (40 μm) was a product of J.T. Baker Inc., Kromasil C_{18} reversed phase column (250 × 4.6 mm, 5 μm) for HPLC was obtained from SUPELCO Inc. Melting point was determined on an electrothermal melting point apparatus and is not corrected. Optical rotation was measured with JASCO Dip-370 polarimeter. IR spectrum was for KBr disk recorded on a Shimadzu FTIR-8300 spectrometer. UV was recorded with a Shimadzu UV-1601 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were measured on Bruker DRX-500. Mass spectra were recorded on a Shimadzu LCMS QP8000α and an IonSpec FT mass spectrometer (for HRMS).

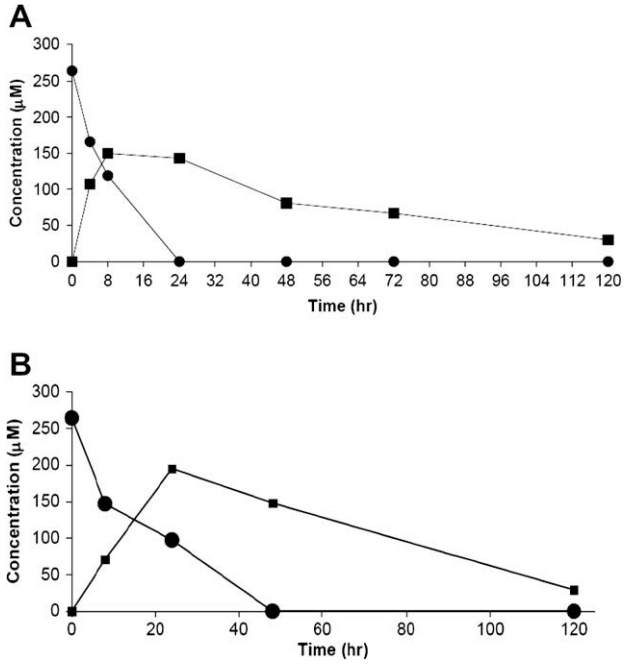


Figure 4. Conversion of compound **1** to **2** in cell culture medium. (A) Incubation of compound **1** in DMEM supplemented with 10% fetal bovine serum. (B) Incubation in cysteine/methionine-free DMEM supplemented with 10% fetal bovine serum. The concentration of **1** (solid circles) and its conversion to **2** (solid squares) was measured over time by HPLC using an external standard curve method.

3.2. Aeroponic culture of *W. somnifera*

Chambers for aeroponic cultivation of plants measured 1.0 m × 1.0 m × 1.5 m ($W \times L \times H$), and were equipped with 6 nozzles powered by an external pump to spray nutrient solution every 4 min for a period of 1 min. A reservoir of 450 L of nutrient solution was maintained at the bottom of the chamber. The nutrient solution was prepared according to a general hydroponic recipe with a pH of 6.0.[32] Each box accommodated 20 plants. The mature plants were harvested and aerial parts (leaves and stems) and roots were collected separately. Roots were freeze-dried, while the aerial parts were air-dried.

3.3. Extraction and isolation

Dry powder (100 g) obtained from the aerial tissue of *W. somnifera* was extracted three times with MeOH (3 × 250 mL) at room temperature. After evaporation under reduced pressure, 19.8 g of the crude extract was obtained. A portion (1.98 g) of this extract was applied to a column of C-18 (30.0 g) and eluted successively with a gradient of 50–100% aqueous MeOH. The fraction eluted with 50% MeOH was further fractionated on a column of C-18 (30 g) with 40% aqueous MeOH as the eluant. Fractions were collected and combined based on their TLC profiles. Final purification was carried out on a column of silica gel and elution with $CHCl_3$/MeOH (8:2). Crystallization from MeOH yielded **1** (40.4 mg, 0.4%) as colorless crystals.

3.4. 2,3-Dihydrowithaferin A-3β-*O*-sulfate (1)

Mp dec >167 °C; $[\alpha]_D^{25}$+14.5 (*c* 0.21, MeOH); UV (MeOH) λ_{max} 214 nm; ^{1}H NMR (C_5D_5N, 500 MHz) δ 5.66 (1H, br s, H-3), δ 4.84 (1H, d, J = 12.0 Hz, H-27a), 4.74 (1H, d, J = 12.0 Hz, H-27b), 4.43 (1H, br s, H-4), 4.37 (1H, br d, J = 13.0 Hz, H-22), 3.62 (1H, br dd, J = 8.5, 16.0 Hz, H-2), 3.40 (1H, br s, H-6), 3.25 (1H, d, J = 16 Hz, H-2), 2.07 (3H, s, CH_3-28), 1.63 (3H, s, CH_3-19), 0.95 (3H, d, J = 6.5 Hz, CH_3-21), 0.50 (3H, s, CH_3-18); ^{13}C NMR (C_5D_5N, 125 MHz) δ 208.7 (qC, C-1), 166.4 (qC, C-26), 155.9 (qC, C-24), 127.3 (qC, C-25), 78.4 (CH, C-22), 75.5 (CH, C-4), 73.8 (CH, C-3), 65.0 (qC, C-5), 57.0 (CH, C-6), 56.2 (CH_2, C-27), 56.0 (CH, C-14), 52.0 (CH, C-17), 49.7 (qC, C-10), 42.8 (qC, C-13), 42.5 (CH, C-9), 41.6 (CH_2, C-2), 39.2 (CH_2, C-16), 39.0 (CH, C-20), 31.3 (CH_2, C-23), 30.0 (CH_2, C-7), 29.9 (CH, C-8), 27.3 (CH_2, C-12), 24.5 (CH_2, C-15), 21.4 (CH_2, C-11), 20.1 (CH_3, C-28), 15.5 (CH_3, C-19), 13.6 (CH_3, C-21), 11.5 (CH_3, C-18); Negative HRESIMS *m/z* 567.2248 (calcd for $C_{28}H_{39}O_{10}S$, 567.2264).

3.5. Conversion of 2,3-dihydrowithaferin A-3β-*O*-sulfate (1) to withaferin A (2)

A solution of 2,3-dihydrowithaferin A-3β-*O*-sulfate (**1**) (1 mg) and anhydrous K_2CO_3 was heated in pyridine (0.2 mL) at 90 °C for 1 h. Standard work up followed by purification on PTLC (silica gel, $CHCl_3$:MeOH, 9:1) afforded withaferin A (**2**) (0.8 mg, 93%).

3.6. Cytotoxicity assay

A standard tetrazolium dye [3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT]-based colorimetric assay was used to measure the proliferation/survival of cells in triplicate wells using a 96-well plate-based format. Compounds **1** and **2** were formulated in DMSO and applied to cells such that final DMSO con-

centration did not exceed 0.2%. Cells were exposed continuously to test compounds for 24 or 72 h at which times relative viable cell number per well was determined as previously described.[33]

3.7. Detection of actin aggregation

Cells were seeded in 8-well chamber slides at a density of 2×10^4 cells per well and allowed to adhere for 48 h. Compounds **1** and **2** were freshly prepared as 5 mM stock solutions in DMSO and applied to cells at a final concentration of 4 μM in RPMI culture medium supplemented with 10% fetal bovine serum, Glutamax™, and penicillin/streptomycin. Control wells were treated with an equal volume of DMSO, not exceeding 0.2% in culture media. Cells were incubated for 4 or 24 h in the continuous presence of the indicated compounds, then washed twice with PBS, fixed with 4% paraformaldehyde/PBS (pH 7.6), and permeabilized with 0.1% Triton-X 100/PBS. Slides were blocked for 30 min at room temperature with 10% (v/v) goat serum and 1% bovine serum albumin (w/v) in PBS, then incubated with AlexaFluor 488-conjugated phalloidin to stain F-actin (Molecular Probes). To visualize nuclei, cells were counterstained with DAPI (1 μg/mL) in PBS for 3 min. After extensive washing, cells were visualized using an Olympus IX71 microscope with 100× objective and identical exposure conditions.

3.8. Heat-shock induction

Immortalized mouse embryo fibroblasts derived from homozygous *Hsf1* knockout mice or their wild type littermates were generously provided by I. J. Benjamin.[34] Cells were exposed overnight to equitoxic concentrations of 1 or the known heat-shock inducing Hsp90 inhibitor geldanamycin. Whole cell lysates were prepared in non-ionic detergent buffer and immunoblotted for relative levels of Hsp72, a highly inducible member of the Hsp70 family of molecular chaperones, using monoclonal antibody C92F3A-5 (StressMarq Biosciences, Victoria, BC). Reactivity was detected using peroxidase-conjugated secondary antibody and chemiluminescent detection. To evaluate the relative ability of compounds to induce a heat-shock response at the transcriptional level, a reporter cell line was used as previously described.[35] These cells are stably transduced with a plasmid encoding enhanced green fluorescent protein (EGFP) under the control of a minimal heat-shock response element derived from the promoter region of the human *Hsp70B* gene. They demonstrate a robust, concentration-dependent fluorescent response to known heat-shock modulating drugs such as Hsp90 inhibitors and can be used as a sensitive and specific system to non-destructively monitor induction of the heat-shock response in live cells.

3.9. Conversion of 2,3-dihydrowithaferin A-3β-*O*-sulfate (1) to withaferin A (2) in cell culture media

Stock solutions of **1** in DMSO (50 μL) were diluted into cell culture medium (950 μL) to achieve the indicated starting concentration, mixed thoroughly and the solution incubated in a CO_2 incubator at 37 °C. Aliquots (100 μL) were withdrawn at 4, 16, and 24 h and subjected to HPLC analysis for **1** (RR_T = 18.5 min) and **2** (RR_T = 23.5 min) on a Kromasil C_{18} RP column (250 × 4.6 mm, 5 μm) with gradient elution using 40–100% aqueous MeOH and using an ELSD detector. An external standard curve method was used to calculate the concentration of each compound in the sampled aliquots.[36]

Acknowledgments

This work was supported by a grant from USDA-CSREES and this support is gratefully acknowledged. Ms. Betsy Lewis is thanked for her assistance with fabricating the aeroponic chambers.

References and notes

1. Anbalagan, K.; Sadique, J. *Indian J. Exp. Biol.* **1981**, *19*, 245–249.
2. Ziauddin, M.; Phansalkar, N.; Patki, P.; Diwanay, S.; Patwardhan, B. *J. Ethnopharmacol.* **1996**, *50*, 69–76.
3. Dhuley, J. N. *J. Ethnopharmacol.* **1997**, *58*, 15–20.
4. Dhuley, J. N. *J. Ethnopharmacol.* **2000**, *70*, 57–63.
5. Dhuley, J. N. *J. Ethnopharmacol.* **1998**, *60*, 173–178.
6. Widodo, N.; Tagaki, Y.; Shrestha, B. G.; Ashii, T.; Kaul, S. C.; Wadha, R. *Cancer Lett.*, in press. (doi: 10.1016/j.canlet.2007.11.037).
7. Mishra, L. C.; Singh, B. B.; Dagenais, S. *Altern. Med. Rev.* **2000**, *5*, 334–346.
8. Gupta, G. L.; Rana, A. C. *Pharmacog. Rev.* **2007**, *1*, 129–136.
9. Kumar, P.; Kushwaha, R. A. *Asian J. Chem.* **2006**, *18*, 1401–1404.
10. Devi, P. U.; Sharada, A. C.; Solomon, F. E. *Indian J. Exp. Biol.* **1993**, *31*, 607–611.
11. Sharada, A. C.; Solomon, F. E.; Devi, P. U. *Int. J. Pharmacog.* **1993**, *31*, 205–212.
12. Kupchan, S. M.; Doskotch, R. W.; Bollinger, P.; McPhail, A. T.; Sim, G. A.; Renauld, J. A. S. *J. Am. Chem. Soc.* **1965**, *87*, 5805–5806.
13. Shohat, B.; Gitter, S.; Abraham, A.; Lavie, D. *Cancer Chemother. Rep.* **1967**, *51*, 271–276.
14. Devi, P. U.; Sharada, A. C.; Solomon, F. E. *Cancer Lett.* **1995**, *95*, 189–193.
15. Shohat, B.; Joshua, H. *Int. J. Cancer* **1971**, *8*, 487–496.
16. Yang, H.; Shi, G.; Dou, Q. P. *Mol. Pharmacol.* **2007**, *71*, 426–437.
17. Falsey, R. R.; Marron, M. T.; Gunaherath, G. M. K. B.; Shirahatti, N.; Mahadevan, D.; Gunatilaka, A. A. L.; Whitesell, L. *Nat. Chem. Biol.* **2006**, *2*, 33–38.
18. Bargagna-Mohan, P.; Hamza, A.; Kim, Y. E.; Ho, Y. K.; Mor-Vaknin, N.; Wendschlag, N.; Liu, J. J.; Evans, R. M.; Markovitz, D. M.; Zhan, C. G.; Kim, K. B.; Mohan, R. *Chem. Biol.* **2007**, *14*, 623–634.
19. Kaileh, M.; Vanden, B. W.; Heyerick, A.; Horion, J.; Piette, J.; Libert, C.; De Keukeleire, D.; Essawi, T.; Haegeman, G. *J. Biol. Chem.* **2007**, *282*, 4253–4264.
20. Sen, N.; Banerjee, B.; Das, B. B.; Ganguly, A.; Sen, T.; Pramanik, S.; Mukhopadhyay, S.; Majumder, H. K. *Cell Death Differ.* **2007**, *14*, 358–367.
21. Srinivasan, S.; Ranga, R. S.; Burikhanov, R.; Han, S.-S.; Chendil, D. *Cancer Res.* **2007**, *67*, 246–253.
22. Fuska, J.; Fuskova, A.; Rosazza, J. P.; Nicholas, A. W. *Neoplasma* **1984**, *31*, 31–36.
23. Dhar, R. S.; Verma, V.; Suri, K. A.; Sangwan, R. S.; Satti, N. K.; Kumar, A.; Tuli, R.; Qazi, G. N. *Phytochemistry* **2006**, *67*, 2269–2276.
24. Hayden, A. L. *HortScience* **2006**, *41*, 536–553.
25. Pelletier, S. W.; Gebeyehu, G.; Nowacki, J.; Mody, N. V. *Heterocycles* **1981**, *15*, 317–320.
26. Anjaneyulu, A. S. R.; Rao, D. S.; Lequesne, P. W.. In *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier Science: Amsterdam, 1998; vol. 20, pp 135–261.
27. Misra, L.; Lal, P.; Sangwan, R. S.; Sangwan, N. S.; Uniyal, G. C.; Tuli, R. *Phytochemistry* **2005**, *66*, 2702–2707.
28. Whitesell, L.; Lindquist, S. L. *Nat. Rev. Cancer* **2005**, *5*, 761–772.
29. Lee, W. C.; Lin, K. Y.; Chen, C. M.; Chen, Z. T.; Liu, H. J.; Lai, Y. K. *J. Cell Physiol.* **1991**, *149*, 66–76.
30. Dai, C.; Whitesell, L.; Rogers, A. B.; Lindquist, S. *Cell* **2007**, *130*, 1005–1018.
31. Balch, W. E.; Morimoto, R. I.; Dillin, A.; Kelley, J. W. *Science* **2008**, *319*, 916–919.
32. The aeroponic nutrient solution was made up by mixing solutions A and B prepared and mixed as follows: Solution A consisted of $Ca(NO_3)_2{\cdot}4H_2O$ (0.579 g/L), $CaCl_2{\cdot}6H_2O$ (0.278 g/L), 10% Fe-EDDHA (0.03 g/L) and water; Solution B consisted of KH_2PO_4 (0.24 g/L), K_2SO_4 (0.193 g/L), $MgSO_4{\cdot}7H_2O$ (0.6 g/L), H_3BO_3 (0.003 g/L), 20% $CuSO_4$ (0.003 g/L), 20% $MnSO_4$?H_2O (0.004 g/L), $Na_2MoO_4{\cdot}2H_2O$ (0.001 g/L), 20% $ZnSO_4{\cdot}7H_2O$ (0.004 g/L). Solution A (900 mL) and solution B (900 mL) were added to 140 L of water and mixed thoroughly and if necessary the pH of the solution adjusted to 5.6–6.0 with citric acid or KOH.
33. Wijeratne, E. M. K.; Turbyville, T. J.; Zhang, Z.; Bigelow, D.; Pierson, L. S., III; VanEtten, H. D.; Whitesell, L.; Canfield, L. M.; Gunatilaka, A. A. L. *J. Nat. Prod.* **2003**, *66*, 1567–1573.
34. McMillan, D. R.; Xiao, X.; Shao, L.; Graves, K.; Benjamin, I. J. *J. Biol. Chem.* **1998**, *273*, 7523–7528.
35. Turbyville, T. J.; Wijeratne, E. M. K.; Liu, M. X.; Burns, A. M.; Seliga, C. J.; Luevano, L. A.; David, C. L.; Faeth, S. H.; Whitesell, L.; Gunatilaka, A. A. L. *J. Nat. Prod.* **2006**, *69*, 178–184.
36. Khajuria, R. K.; Suri, K. A.; Gupta, R. K.; Satti, N. K.; Amina, M.; Suri, O. P.; Qazi, G. N. *J. Sep. Sci.* **2004**, *27*, 541–546.