Structure—Activity Relationships for Withanolides as Inducers of the Cellular Heat-Shock Response


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ABSTRACT: To understand the relationship between the structure and the remarkably diverse bioactivities reported for withanolides, we obtained withaferin A (WA; 1) and 36 analogues (2–37) and compared their cytotoxicity to cytoprotective heat-shock-inducing activity (HSA). By analyzing structure—activity relationships for the series, we found that the ring A enone is essential for both bioactivities. Acetylation of 27-OH of 4-epi-WA (28) to 33 enhanced both activities, whereas introduction of β-OH to WA at C-12 (29) and C-15 (30) decreased both activities. Introduction of β-OAc to 4,27-diacetyl-WA (16) at C-15 (37) decreased HSA without affecting cytotoxicity, but at C-12 (36), it had minimal effect. Importantly, acetylation of 27-OH, yielding 15 from 1, 16 from 14, and 35 from 34, enhanced HSA without increasing cytotoxicity. Our findings demonstrate that the withanolide scaffold can be modified to enhance HSA selectively, thereby assisting development of natural product-inspired drugs to combat protein aggregation-associated diseases by stimulating cellular defense mechanisms.

INTRODUCTION

Withanolides, a class of steroidal lactones structurally based on an ergostane skeleton, are abundant in plants of the family Solanaceae.1 Plants of this family belonging to genera Withania, Acnistus, and Physalis have been extensively investigated, which, in large part, is because they are used in many of the traditional systems of medicine practiced throughout Asia and South America.2 The beneficial effects of many of these plants have been attributed to the presence of withanolides. One of the best studied of these withanolides is withaferin A (1, WA), a major constituent of the plant Withania somnifera (L.) Dunal.3,4 Popularly known as Ashwagandha or Indian ginseng, it has been used in Indian Ayurvedic medicine for over 3000 years.5 Various preparations of Ashwagandha are available as herbal dietary supplements worldwide. The National Center for Complementary and Alternative Medicine (NCCAM) of the U.S. National Institutes of Health has recently recognized Ashwagandha as a high-priority topic for mechanistic research.6 Numerous reports describe anticancer,7 neuroprotective,8–10 anti-inflammatory,11 immunomodulatory,12,13 and antioxidant5 activities for medicinal preparations of W. somnifera and its constituent withanolides. Among these, the most extensively studied has been the anticancer activity of WA.14–16 For example, the Developmental Therapeutics Program (DTP) of the U.S. National Cancer Institute has tested WA (NSC 101088) against its panel of 60 human cancer cell lines and found a mean 50% growth inhibitory concentration (GI50) of 620 nM.17 Other studies have demonstrated significant activity for WA against human brain,18 prostate,19 pancreatic,20 and breast cancer21 xenografts in mice.

In previous work, we used the heat-shock response (HSR) as a biosensor to discover potential anticancer compounds that target protein homeostasis. We found that WA and other thiol-reactive natural products activate the heat shock factor 1 (HSF1)-dependent stress response as a prominent component of their anticancer activity.18,22 Others have reported inhibition of cell motility and angiogenesis,23,24 inhibition of NF-κB activation,25–29 protein kinase C30 and Notch-1.31 Reports also describe induction of Par-4-dependent apoptosis,19 FOXO3a- and Bim-dependent apoptosis, and sensitization to TRAIL-induced apoptosis.32 Despite the great diversity of biological effects reported for natural and semisynthetic withanolides,
structure–activity relationship studies have relied almost exclusively on cytotoxicity as their end point for activity.

To begin probing how cytoprotective heat-shock-inducing activity relates to the cytotoxic activity of withanolides, we isolated 1 and natural withanolides 2–13 using the biomass derived from aeroponically cultivated *Withania somnifera* and prepared 24 structurally related analogues, 14–37, by performing chemical and microbial transformations of 1 that had been isolated from the same material, providing a total of 36 analogues. We evaluated these compounds for their ability to activate the heat-shock response using cell-based reporter systems, whereas antiproliferative activity was measured in a reporter cell line as well as two other cancer cell lines. This approach allowed us to identify relatively modest structural modifications that alter the chemical reactivity of analogues toward thiols and selectively enhance heat-shock-inducing activity over cytotoxicity and vice versa. Importantly, we confirmed these reporter-based cell culture results through exploratory pharmacodynamic studies in mice. Our findings suggest that reporter assay-guided tuning of the withanolide scaffold provides a useful approach to improving the therapeutic potential of this class and perhaps other thiol-reactive natural products as anticancer or neuroprotective agents.
RESULTS AND DISCUSSION
Isolation and Semisynthesis of Withanolides 1−37.

We investigated the effects of various substituents, their position and stereochemistry, and their lipophilicity on antiproliferative versus heat-shock-induction activity for 37 withanolides. The panel of compounds used in this study included natural withanolides 1−13 obtained from aeroponically grown W. somnifera and their derivatives 14−26 (Figure 1). Analogues 27−31 were obtained from WA by chemical and microbial transformations. These compounds were further derivatized to yield compounds 32−37 (Figure 2).

Semisynthesis of 4-epi-withaferin A (28) was efficiently achieved by the MnO2 oxidation of 1 to 4-dehydrowithaferin A (27) followed by regio- and stereoselective reduction of its C-4 carbonyl group with NaBH4/CeCl3. The use of lanthanoid cations (such as Ce3+) in reactions of enones with NaBH4 is known to cause 1,2-reduction of the carbonyl group with high selectivity compared to 1,4-reduction caused by NaBH4 in the absence of these cations, with the ratio of epimeric alcohols formed being determined by steric factors. Conversion of 27 to 28, however, constitutes the first report of a stereoselective reduction of only one carbonyl group of an ene-dione with NaBH4/CeCl3/MeOH/THF. The high degree of regio- and stereoselectivity observed for 27 yielding 28 may be explained as being due to the chelation of the boron atom of the reducing species [NaBH4−(OMe)n]3+ to the oxygen atom of the ring-B oxirane of 27, delivering the hydride from the β-phase. The structure of 28 was elucidated by the analysis of its 1H, 13C, and 2D NMR spectroscopic data including HMBC. The α-orientation of the 4-OH was confirmed by NOE experiments (see Supporting Information Figure S17). The two O-sulfated analogues of 1, withaferin A-27-sulfate (18) and withaferin A-4,27-disulfate (19), were prepared by the reaction of WA with SO3-pyridine. 3-Azido-2,3-dihydrowithaferin A (31) was obtained by treating 1 with NaN3/Et3N. Microbial biotransformation of 1 with the fungus Cunninghamella echinulata afforded 12β-hydroxywithaferin A (29) and 15β-hydroxywithaferin A (30). Controlled acetylation (Ac2O/pyridine) of 1 yielded 27-acetylwithaferin A (15) and 4,27-diacetylwithaferin A (16). Acetyl analogues 21−26, 32, and 35−37 were obtained by the standard acetylation of their corresponding alcohols using Ac2O/pyridine. In contrast, preparation of the 4-acetyl analogues, 4-acetylwithaferin A (14) and 4-acetyl-4-epi-withaferin A (34), of 1 and 28, respectively, required protection of their more reactive 27-OH groups as tert-butyldimethylsilyl (TBDMS) ethers (Scheme 1) and (Scheme 2). Treatment of 39 and 42 with Ac2O/pyridine followed by deprotection (HCl/THF/MeOH) afforded 14 and 34, respectively.

Scheme 1. Conversion of 1 to 14

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1  a  39  b  40  c  14
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Reagents and conditions: (a) TBDMS-Cl, 4-PP, DMF, 60 °C; (b) Ac2O, pyridine, 25 °C; (c) 2 N HCl, THF, MeOH, 0 °C.

Scheme 2. Conversion of 1 to 28 and Its Derivatives

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1  a  27  b  28  c  35
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d

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32  b  33
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c  

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1  a  27  b  28  c  35  e  34
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d  

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41  b  42  c  43
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ej  

Reagents and conditions: (a) MnO2, CHCl3, EtOAc, 25 °C; (b) NaBH4, CeCl3·7H2O, MeOH, THF, 0 °C; (c) Ac2O, pyridine, 25 °C; (d) TBDMSCl, 4-PP, DMF, 60 °C; (e) 2 N HCl, THF, MeOH, 0 °C.
Cytotoxicity. As an initial screen, we measured the acute cytotoxicity of 1 and its analogues 2–37 at a single concentration (4.0 μM) using the human Ewing’s sarcoma cell line CHP-100 (Supporting Information, Figure S29). We chose this very rapidly proliferating cell line to maximize sensitivity over a short period of compound exposure. Cells were incubated for 24 h with compounds, and the relative viable cell number was measured by a standard dye-reduction assay. Doxorubicin and DMSO were used as positive and negative controls, respectively. WA and analogues 2, 5, 14–17, 21, 22, 24, 28, and 31–37 inhibited the overall proliferation and survival of CHP-100 cells over this short time interval by >80%. In a follow-up experiment, we determined the IC_{50} for each of these cytotoxic compounds using the same methodology (Table 1). Intriguingly, the most potent analogues, namely, 15 and 33, contained a 27-OAc in addition to the 4β-hydroxy-2(3)-en-1-one moiety in ring A. These data are consistent with previous reports that the 2(3)-en-1-one moiety in ring A of withanolides is essential for their cytotoxic activity.40–47

Our data indicate that the nature of the substituent at C-4 has a major effect on the antiproliferative activity of withanolides, at least the ones we examined. An electron-withdrawing carbonyl group at C-4, as in 27, reduced activity when compared to 1, supporting our previous observation that the reactivity of the 2(3)-en-1-one moiety of withanolides determines their ability to adduct thiols and their consequent cytotoxicity.48 4-Dehydrowithaferin A (27) has been previously reported to be more cytotoxic than 1,49 but different conditions and cell lines used for cytotoxicity assays could easily account for such discrepancy.

Comparing the potencies of 1 and 28 indicates that the orientation of the 4-OH has very little effect on cytotoxicity toward CHP-100 cells. In our previous study of the antiproliferative activities of 28 and 1 against pancreatic cancer cell lines MIA PaCa-2 and BxPC-3, both withanolides also had similar activities. In pancreatic cancer cell line Panc-1, however, 1 demonstrated ca. 4-fold higher potency than 28 for reasons that are unclear, but this could be due to their differences in cellular uptake and/or metabolism.48 Several SAR studies have noted the importance of the 5β,6β-epoxy group in ring B for the cytotoxicity of withanolides.41,46 When this group is replaced with a double bond, as in 24, acute cytotoxicity was retained against CHP-100 cells (Table 1 and Supporting Information, Figure S29). This finding indicates that the 5β,6β-epoxy group is not required but can enhance the cytotoxicity of withanolides. Our findings with 2 and 15 agree with previous reports that the presence of an OH group at C-27 of withanolides leads to a reduction in their antiproliferative activity.46,47 This finding together with the enhancement of activity observed for acetyl derivatives 15, 16, 21, 22, 24, 32, 34, and 35–37 as compared to their parent alcohols suggests that increased lipophilicity for substituents at C-4, C-12, C-15, or C-27 tends to enhance the cytotoxicity of withanolides. However, our results using an alternative cell line (H929 myeloma cells) indicated that acetylation of the OH at C-27 of 1, 14, and 34 to 15, 16, and 35, respectively, had very little effect on cytotoxicity in a more typical 3 day drug-exposure design, but C-27 acetylation of 28 to provide 33 yielded over a 5-fold enhancement of cytotoxic activity under these conditions (Table 2).

Heat-Shock Induction. The heat-shock response plays a critical role in maintaining protein homeostasis and helps cells cope with a wide range of proteotoxic insults.50 As a result, the ability of WA and other electrophilic natural products to activate this response could provide a valuable approach to combating protein aggregation-associated neurodegenerative disorders such as Parkinson’s disease and Alzheimer’s disease.51,52 Previous SAR studies on the withanolide scaffold, however, have focused on cytotoxicity as their end point, not the heat-shock response. To begin defining structural features contributing to heat-shock induction, we measured concentration-dependent activation of the response by withanolides 1–37. We applied serial dilutions of each compound in a 384-

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“Concentration required to inhibit cell proliferation/survival by 50% after 24 h of compound exposure, with each measured in octuplicate. IC_{50} values were determined from dose–response curves using Microsoft Excel software; ± refers to standard deviation.”
well format to transduced reporter cells stably expressing a green fluorescent protein—firefly luciferase fusion protein under transcriptional control of classical heat-shock promoter elements.\(^a\) After overnight incubation, luciferase activity was determined as a quantitative measure of relative heat-shock activation. As evident in Figure 3, for 1 and its five most active analogues (15, 16, 33, 35, and 36), reporter response was not a monotonic function but rather peaked over a limited concentration range for each active compound and then declined, presumably as toxicity compromised the ability of cells to respond. To quantitate this type of concentration dependence in a way that would capture overall heat-shock-inducing potential, we defined a heat-shock index (HSI) for each compound calculated as \(\log_2\) of the maximal response (fold induction) divided by the concentration required to stimulate this response (Table 2). Interestingly, all five withanolide analogues (15, 16, 33, 35, and 36) that demonstrated a HSI more than 2-fold of that of 1 contained acetyl substituents at C-12 and/or C-27 in addition to the ring-B \(\beta\)-oxirane moieties. Greater heat-shock-induction activity for analogues 33 and 35 as compared to WA was confirmed using an alternate heat-shock reporter cell line, as previously described.\(^b\) Using this reporter system in nontransformed cells instead of cancer cells and fluorescence instead of luciferase activity as an end point, heat-shock indices for 1, 33, and 35 were calculated as 2.1, 4.7, and 5.2, respectively (data not shown). Conservation of the rank order for these compounds under different assay conditions indicates that their heat-shock induction activity is an intrinsic property, not an artifact of the particular system used to measure it.

**Heat-Shock Induction vs Cytotoxicity.** Next, to determine whether the heat-shock-inducing activity and cytotoxicity of withanolides 1–37 could be dissected on the basis of structural features, we examined the correlation of these two activities across all 37 compounds (Figure 4). To monitor cytotoxicity in the most sensitive manner possible, we incubated H929 myeloma cells for 3 days with serial dilutions of each compound in a 384-well format. This human cell line, as with most myeloma cell lines, is particularly sensitive to agents that disrupt protein homeostasis, especially proteasome inhibitors such as MG-132.\(^c\) If heat-shock induction was solely a consequence of cytotoxicity arising from impairment of the proteasome and HSP90 (previously reported targets for WA\(^d\)) or other mediators of protein homeostasis, then we would have expected to see a consistent correlation between these activities across all analogues tested. Instead, we found a relatively poor correlation (\(r^2 = 0.62\)), primarily because of a group of outlying analogues (15, 16, 35, and 36) that displayed greater heat-shock induction than 1 at approximately the same level of toxicity. In contrast, withanolide 33 was both more cytotoxic and more heat-shock active than 1, and the proteasome inhibitor MG-132 used as a positive control showed greater

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\(^a\)Measured in the 293T reporter line and calculated as \([\log_2(\text{treated/control})/\text{concentration} (\mu M)]\). \(^b\) Concentration resulting in 50% reduction in relative viable myeloma (H929) cell number after 72 h exposure based on a nonlinear curve fit of the dose–response data in Prism 5.0 software (95% confidence interval).
toxicity than heat-shock induction compared to 1. As noted earlier, analogues with the greatest heat-shock activity all contained acetyl substituents at C-4 and/or C-27 in addition to the ring-A 2(3)-en-1-one moiety as the end point (Supporting Information, Figure S31). The ratio of the starting material to reaction of NAC with acetylated at only C-27, whereas 35 carried acetyl substituents at both C-4 and C-27. 

Given the surprising nature of these findings, we verified the results by repeat testing of analogues 33 and 35 in a low-throughput format. 1 and MG-132 were included for reference (Figure 5). Although the absolute magnitude of effects changed with the alternate format, relative relationships were preserved. Again, 35 demonstrated greater heat-shock-inducing activity (Figure 5a) with less cytotoxicity. This was the case both in the cell line used for the reporter assays (293T cells, Figure 5b) and in H929 cells, the line used for our initial correlation analysis (Figure 5c). MG-132 was more cytotoxic for H929 cells than 293T cells, consistent with the known hypersensitivity of myeloma cells to proteasome inhibition. It is noteworthy that the more potent cytotoxicity of 33 in these assays was consistent with results from the acute toxicity assay we used to generate the data summarized in Table 1. Here, using CHP-100 cells, 24 h compound exposure, and MTT dye reduction as the end point, 33 (IC_{50} = 0.22 ± 0.02) was also significantly more potent than withanolide 35 (IC_{50} = 0.35 ± 0.01). 

Toxicity and Biological Activity. Compound 1 contains three electrophilic sites that could play important roles in its biological activity, including HSA and cytotoxicity. These are C-3 of the ring-A 2(3)-en-1-one moiety, C-5/C-6 of the ring-B 5\beta,6\beta-oxirane moiety, and C-24 of the ring-E unsaturated lactone moiety. We have previously shown that coincubation of our heat-shock reporter cells with N-acetylcysteine (NAC) and 1 leads to a near complete suppression of heat-shock activation caused by 1.\(^\text{18}\) Thus, it was of interest to assess the chemical reactivity of 1 compared to informative analogues using NAC as a representative thiol nucleophile. Treatment of 1 with NAC afforded the product of Michael addition at C-3 (38) in 66% yield. No addition was observed at C-5, C-6, or C-24 by 1H NMR and HPLC. As expected, 2,3-dihydrowithaferin A (20) was unreactive under these conditions. Because 20 is devoid of both HSA and cytotoxic activity, the presence of the ring-A 2(3)-en-1-one moiety appears to be essential for many, if not all, of WA’s biological activities. To probe the relationship between the thiol reactivity of this enone moiety and HSA further, we monitored reaction of 1 with 1 equiv of NAC in DMSO-d6 and, in parallel, reaction of NAC with 35, our analogue with the highest heat-shock index. The progress of these reactions was assessed by 1H NMR using the disappearance of the signal from H-2 of the ring-A 2(3)-en-1-one moiety as the end point (Supporting Information, Figure S31). The ratio of the starting material to product was used to determine the Gibbs’s free energy (\(\Delta G\)) of the thiol addition/elimination processes.\(^\text{55}\) Reactions of 1 and

Figure 4. Correlation of heat-shock induction with cytotoxicity for compounds 1–37. The proteasome inhibitor MG-132 is included as a mechanistically and structurally distinct control compound. The solid line depicts a linear curve fit for all data points (r² = 0.62), as performed in Microsoft Excel software. The heat-shock-active analogues lying furthest off the curve fit are circled in red. Heat-shock index (determined in 293T reporter cells) was calculated as \[\log_{2}((treated/control)) / concentration.\] Cytotoxicity (determined in H929 myeloma cells) was calculated as Log IC_{50}, where IC_{50} is concentration (\(\mu M\)) resulting in 50% reduction in the relative viable cell number.

Figure 5. Verification of WA analogues found as outliers for their heat-shock-inducing activity by correlation analysis: 33 was most cytotoxic, whereas 35 was least toxic. In each panel, the mean percent compared to vehicle-treated control cells is plotted, with all determinations performed in quadruplicate wells. Error bars, SD. (a) Heat-shock reporter induction in confluent 293T cells after overnight exposure to compounds, (b) inhibition of 293T cell growth and survival as measured by resazurin dye reduction after 72 h, and (c) inhibition of H929 cell growth and survival as measured by resazurin dye reduction after 72 h.
35 with NAC showed typical second-order reaction curves, with calculated $\Delta G$ values of $-7.0$ and $-9.3$ kcal/mol, respectively, indicating that analogue 35 reacts more readily with NAC than 1. Therefore, in regards to the group appended at C-4 of the withanolide scaffold, its identity (OH or OAc), orientation ($\alpha$ or $\beta$), or a combination of these factors can alter the chemical reactivity of this eneone system and HSA fully. We can conclude, however, that orientation of the group at C-4 appears to exert little or no effect on acute cytotoxicity, as the pairs of analogues 1 and 28, 15 and 33, 16 and 35, and 17 and 34 showed very similar IC$_{50}$ values in limiting the growth and survival of CHP-100 cells (Table 1).

Heat-Shock Induction In Vivo. To determine whether the heat-shock activity of withanolides demonstrated in cell culture would translate to whole animals, we performed exploratory pharmacodynamic studies in mice (Figure 6). Establishing a biocompatible cyclodextrin-based formulation for these very poorly water-soluble compounds, we compared the dose-dependent ability of 1 and 35 to activate a systemic heat-shock response after parenteral administration. Single doses up to 50 mg/kg were tolerated without overt acute toxicity. Choosing spleen as a sentinel organ representative of the hematopoietic compartment, we assayed lysates for upregulation of heat shock protein 72 (HSP72), the most highly heat-inducible isof orm of the HSP70 family of molecular chaperones. At a dose of 25 mg/kg, several mice receiving 35 responded with a robust increase in relative HSP72 level, leading to a highly significant increase in variance for this group. In contrast, 1 caused a much smaller, nonsignificant effect on variance, indicative of little treatment effect under these conditions. Increasing the dose of 35 to 40 mg/kg produced more uniform induction across the treatment group and a significant difference in the mean HSP72 level compared to the vehicle control group. Although much work beyond the scope of this initial report obviously remains, these exploratory findings confirm that withanolides can activate the heat-shock response in mice at systematically tolerable exposures. Whether activation of the heat-shock response per se will prove a key determinant of the therapeutic benefits ascribed to withanolides in diverse human diseases remains to be determined. Equally important may be their ability to form thiol adducts with a range of important electrophile sensors in cells that are among the first-line defenses for launching adaptive transcriptional and post-transcriptional responses $^{,56,57}$. Nevertheless, findings presented here indicate that heat-shock induction can serve as a useful biomarker for their activity in vivo.

CONCLUSIONS

To identify structural features responsible for the divergent biological activities ascribed to withaferin A (WA), we examined a total of 37 compounds ($1-37$) consisting of natural withanolides, chemical and microbial transformation products of 1, and their derivatives. We focused on heat-shock activation (HSA) and cytotoxicity as distinct activities because, contrary to common assumption, HSA is not a general defense for launching adaptive transcriptional and post-transcriptional responses $^{,56,57}$. Nevertheless, findings presented here indicate that heat-shock induction can serve as a useful biomarker for their activity in vivo.

Figure 6. Induction of systemic heat-shock response by WA (1) and 4,27-diacetyl-4-epi-withaferin A (35). (A) Compounds were formulated in cyclodextrin vehicle and administered by subcutaneous injection (25 mg/kg, five mice per treatment group). Spleens were harvested the following day, and lysate from each animal was loaded in a separate lane for gel electrophoresis followed by immunoblotting for HSP72, a highly inducible isoform of the HSP70 family. Actin was blotted as a loading control. Integrated band intensity for HSP72 normalized to actin for each sample is plotted on the right. The median with interquartile range is indicated by horizontal bars for each treatment group. F test was used to compare variance between vehicle and withanolide 35, $p < 0.005$, and between vehicle and WA, not significant. (B) Withanolide 35 administered at higher dose (40 mg/kg, three mice per group) yields more consistent heat-shock induction. Immunoblotting was performed, and analysis is depicted as in panel A. Student’s $t$ test comparing vehicle to compound treatment, $p = 0.01$. 

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same relative level of cytotoxicity, it does appear possible to discriminate between these activities through specific structural modifications to the withanolide core. Conversely, other modifications increased bioactivity in general, both heat-shock activation and cytotoxicity, perhaps by enhancing cellular uptake or limiting metabolic inactivation.

From our results, we conclude that the withanolide scaffold can be modified to shift its spectrum of bioactivity while preserving potency. Enhancing heat-shock response while minimizing cytotoxicity could provide a better therapeutic index in pursuit of compounds that activate intrinsic cellular defense mechanisms to combat protein aggregation-associated neurodegenerative disorders. Conversely, minimizing activation of the cytoprotective heat-shock response while maintaining antiproliferative activity could provide more effective anticancer agents. Furthermore, reporter assay-guided tuning of the withanolide core appears to provide a practical route to realizing the full therapeutic potential of this versatile sca

MATERIALS AND METHODS

General Procedures. Melting points were determined in capillary tubes using a Mel-Temp apparatus and are uncorrected. Optical rotations were measured in MeOH or CHCl3 with a Jasco DIP-370 digital polarimeter. UV spectra were determined in MeOH on a Shimadzu UV-1601 spectrometer. One-dimensional and 2D NMR constants (δ) are given in parts per million (ppm), and the coupling values (J) are in Hz. LR-MS were recorded finally purified by C18 preparative TLC (8.5 mg) and 19.36 mg).

Isolation of Naturally Occurring Withanolides 1–13. Withaferin A (1), 27-deoxywithaferin A (2), viscosalactone B (3), 2,3-dihydrowithaferin A-3β-0-sulfate (4), 3α-(uracil-1-yl)-2,3-dihydrowithaferin A (5), 3β-(adenin-9-yl)-2,3-dihydrowithaferin A (6), 3β-O-butyryl-2,3-dihydrowithaferin A (7), withanolide A (8), 27-hydroxywithanolide B (9), 4β,27-dihydroxy-1-oxo-22R-witha-2,5,24-trienolide (10), 2,3-dihydroxysenniferin (11), jaborosalactone D (12), and pubesnelide (13) were obtained from aeroponically grown W. somnifera as described previously.

General Procedure for Acetylation of Withanolides. To a solution of the withanolide (2.0 mg) in anhydrous pyridine (0.5 mL) was added Ac2O (0.5 mL), and the mixture was stirred at 25 °C until the reaction was complete (judged by the disappearance of the starting material by TLC). The reaction mixture was poured into ice/water (10.0 mL), and the resulting solution was passed through a short column of RP (C18) silica gel (0.2 g). The column was washed with water (3.0 mL) followed by elution with MeOH (10.0 mL). The MeOH fraction after evaporation was subjected to preparative TLC (silica gel) to yield the corresponding acetyl derivative.

Preparation of 27-Acetylwithaferin A (15) and 4,27-Diacetylwithaferin A (16). To a solution of (1.0 mg) in pyridine (0.1 mL) was added Ac2O (2.4 μL), and the mixture was stirred at 25 °C. After 2 h, EtOH (15.0 mL) was added to the reaction mixture and evaporated under reduced pressure. The residue thus obtained was separated by preparative TLC (silica gel) using 6% MeOH in CH2Cl2 as eluant to give 15 (2.1 mg, 19%) and 16 (8.5 mg, 72%).

27-Acetylwithaferin A (15). White solid; mp 218–220 °C; [α]D +25.8° (c 0.8, CHCl3); 1H NMR (500 MHz, CDCl3) δ 6.90 (dd, J = 9.9, 5.8 Hz, 1H, H-3), 6.18 (d, J = 9.9 Hz, 1H, H-2), 4.88 (d, J = 11.8 Hz, 1H, H-7a), 4.84 (d, J = 11.8 Hz, 1H, H-27b), 4.36 (dt, J = 13.6, 3.3 Hz, 1H, H-27c), 3.74 (brd, J = 21.1 Hz, 1H, H-5), 2.22 (s, 3H, H4), 2.51 (dd, J = 13.2, 10.9 Hz, 2H), 2.12 (dd, J = 14.9, 6.3, 2.6, 1H, H-7a), 2.05 (s, 3H, H-28), 2.18 (brs, 2H, 1H, H-1), 1.53–1.43 (m, 2H), 1.39 (s, 3H, H-12), 1.25–1.18 (m, 1H), 1.02 (d, J = 6.6 Hz, 3H, H-21), 0.91–0.82 (m, 2H), 0.69 (s, 3H, H3-19); HRMS (ESI): [M + H] + calcd for C21H31O5Na+: 353.2387; found, 353.2380.

4,27-Diacetylwithaferin A (16). White solid; mp 223–234 °C; 1H NMR data were consistent with those reported; APCI-MS (+) m/z [M + 1]+: 555.
2.2, 10.3 Hz, 1H), 6.29 (dd, J = 4.8, 19.0 Hz, 1H, H-3), 6.18 (d, J = 10.3 Hz, 1H, H-2), 4.87 (d, J = 4.8, 12.1 Hz, 1H, H-27a), 4.37 (d, J = 11.6 Hz, 1H, H-27b), 4.36 (d, J = 16.6, 3.3 Hz, 1H, H-27a), 3.73 (d, J = 5.8 Hz, 1H, H-4), 3.21 (s, 1H, H-6), 2.44 (dd, J = 17.4, 13.5 Hz, 1H, H-23a), 2.13 (m, 1H, H-7a), 2.04 (3H, CH3), 1.97–1.91 (m, 2H), 1.80 (dq, J = 14.2, 3.6 Hz, 1H, 1.68–1.58 (m, 3H), 1.52–1.43 (m, 2H), 1.39 (s, 3H, H-9), 1.37–0.99 (m, 11H, 0.95 (d, J = 8.8 Hz, 3H, H-21), 0.87 (s, 18H, 3 x CH3), 0.68 (s, 3H, H-19), 0.07 (s, 3H, SiCH3); APCI-MS (+) m/z: [M + H]+ 585.

4-Acetylxylophan A 27-tert-Butyldimethylsilyl Ether (40). Acetylation of 39 (20.0 mg) by the usual procedure (Ac2O/pyridine) afforded 40 as a white solid (21.0 mg, 98%); APCI-MS (+) m/z: [M + H]+ 627.

4-Acetylxylophan A 14. Deprotection of 40 (19.0 mg) was carried out by treatment of a solution of it in THF (0.3 mL) and MeOH (0.05 mL) with 2 N HCl (0.05 mL) at 0 °C for 1 h. The reaction mixture was diluted with H2O, evaporated under reduced pressure, and extracted with EtOAc. The EtOAc layer was evaporated under reduced pressure, and the residue was purified by preparative TLC (silica gel) using 5% MeOH in CH2Cl2 as eluant to give 14 as a white solid (15 mg, 96%); mp 192–194 °C. 1H NMR data were consistent with those reported. 13C NMR data were consistent with those reported. 1H NMR (500 MHz, CDCl3) δ 6.80 (dd, J = 10.1, 1.5 Hz, 1H, H-3), 5.97 (dd, J = 10.1, 2.5 Hz, 1H, H-2), 4.64 (brs, 1H, H-4), 4.37 (dt, J = 13.5, 3.3 Hz, 1H, OAc), 2.01–1.92 (m, 4H), 1.47–1.13 (m, 18H), 0.93 (s, 3H, H-19), 0.07 (s, 3H, SiCH3); APCI-MS (+) m/z: [M + H]+ 531.2847; found, 513.2852.

4-epi-Withaferin A (28). To a stirred solution of 27 (6.0 mg) in MeOH (1.0 mL) and THF (0.5 mL) was added CeCl3·7H2O (17 mg). The reaction mixture was cooled to 0 °C in an ice bath, and NaBH4 (2.0 mg) was added. After 30 min at 0 °C, the reaction mixture was evaporated, and the residue was separated on preparative TLC (silica gel) using 6% MeOH in CH2Cl2 as eluant to give 28 as a white solid (4.2 mg, 70%); mp 227–228 °C; [α]20D +29.9 (c 1.0, CHCl3). 1H NMR (500 MHz, CDCl3 + CD3OD) δ 6.80 (dd, J = 10.1, 1.5 Hz, 1H, H-3), 5.97 (dd, J = 10.1, 2.5 Hz, 1H, H-2), 4.64 (brs, 1H, H-4), 4.37 (dt, J = 13.5, 3.3 Hz, 1H, OAc), 2.01–1.92 (m, 4H), 1.47–1.13 (m, 18H), 0.93 (s, 3H, H-19), 0.07 (s, 3H, SiCH3); APCI-MS (+) m/z: [M + H]+ 531.2847; found, 513.2852.

4-epi-Withaferin A 27-tert-Butyldimethylsilyl Ether (41). To a solution of 27 (11.3 mg) in DMF (0.5 mL) were added t-BDMS-Cl (63 mg) and 4-PPP (78 mg), and the mixture was stirred at 60 °C for 3 h, after which the reaction mixture was diluted with EtOAc, washed with brine, dried over anhydrous Na2SO4, and evaporated under reduced pressure, and the residue was separated on preparative TLC (silica gel) using 3% MeOH in CH2Cl2 as eluant to give 41 as a white solid (21.0 mg, 90%); mp 249–250 °C; [α]20D +42.4 (c 1.0, CHCl3). 1H NMR (500 MHz, CDCl3) δ 6.63 (dd, J = 10.2, 1.4 Hz, 1H, H-3), 6.00 (dd, J = 10.2, 2.5 Hz, 1H, H-2), 4.88 (d, J = 11.9 Hz, 1H, H-27a), 4.85 (d, J = 11.9 Hz, 1H, H-27b), 4.71 (s, 1H, H-4), 4.38 (dt, J = 13.2, 3.3 Hz, 1H, H-22), 3.63 (s, 1H, H-6), 2.50 (dd, J = 17.6, 13.3 Hz, 1H, H-23a), 2.03 (s, 3H, OAc), 2.01 (s, 3H, CH3), 1.92 (m, 4H). 13C NMR (100 MHz, CDCl3) δ 201.4, 167.1, 153.0, 148.4, 120.8, 125.6, 65.8, 63.3, 58.0, 55.9, 55.8, 52.0, 48.1, 45.5, 42.5, 39.5, 38.8, 34.6, 31.8, 30.8, 30.1, 29.6, 27.3, 24.2, 22.6, 20.9, 20.8, 14.6, 14.1, 13.3, 11.7; HRMS (ESI): [M + H]+ calc for C30H41O7, 471.2747; found, 471.2764.

4-epi-Withaferin A 27-tert-Butyldimethylsilyl Ether (42). To a solution of 41 (9.5 mg) in THF (0.2 mL) and MeOH (0.2 mL) at 0 °C was added CeCl3·7H2O (125 mg) and NaNH2 (small portion), and the mixture was stirred at 0 °C. After 10 min, a small ice cube was added to the reaction mixture, the solvent and water were evaporated
under reduced pressure, which was partitioned between H₂O and EtOAc. The EtOAc layer was dried over anhydrous Na₂SO₄ and evaporated under reduced pressure, and the residue was separated on preparative TLC (silica gel) using 2% MeOH in CH₂Cl₂ as eluant to give 42 (7.5 mg, 70%) as a white solid; APPI-MS (+) m/z: [M + H]+ 585.

4-Acetyl-4-epi-witaferin A 27-tert-Butylidimethylsilyl Ether (43). A solution of 42 (7.5 mg) in pyridine (0.3 mL) and ACF (0.2 mL) was stirred at 25 °C for 4 h. The reaction mixture was evaporated under reduced pressure to give 43 (8.0 mg) as a white solid; APPI-MS (+) m/z: [M + H]+ 627.

4-Acetyl-4-epi-witaferin A (34). To a solution of 43 (8.0 mg) in THF (0.5 mL) and MeOH (0.3 mL) at 0 °C was added 2 N HCl (2.5 mL), which was combined with EtOAc (200 mL), dried over anhydrous Na₂SO₄, and evaporated to give 35 (0.5 mg) in pyridine (0.3 mL) and Ac₂O (0.2 mL), and the mixture was stirred at 25 °C for 24 h. The reaction mixture was diluted with H₂O, MeOH and THF were evaporated under reduced pressure and extracted with EtOAc (3 × 15 mL), the combined EtOAc layer was washed with H₂O, dried over anhydrous Na₂SO₄ and evaporated under reduced pressure, and the residue was separated on preparative TLC (silica gel) using 5% MeOH in CH₂Cl₂ as eluant to give 36 (1.0 mg) in pyridine (0.3 mL) and Ac₂O (0.2 mL), and the mixture was stirred at 25 °C for 4 h. The reaction mixture was evaporated under reduced pressure and by adding EtOH, and the residue was purified on preparative TLC (silica gel) using 4% MeOH in DCM as eluant to give 37 (1.2 mg, 95%) as a white amorphous solid. 13C NMR data were consistent with those reported. 38 13C NMR (100 MHz, pyridine-d₅) δ 209.8 (C-9), 174.3 (COS-CO₂H), 166.7 (C-26), 154.2 (C-3), 128.9 (C-2), 121.9 (C-25), 88.1 (C-17), 80.9 (C-4), 79.5 (C-22), 78.9 (C-20), 65.3 (C-18), 64.3 (C-24), 127.8 (C-25), 78.7 (C-22), 77.4 (C-2), 65.4 (C-5), 59.5 (C-14), 56.1 (C-27), 56.3 (C-14), 54.0 (C-SH), 52.3 (C-17), 51.4 (C-10), 44.8 (C-3), 43.9 (C-8), 43.0 (C-14), 41.1 (C-2), 39.6 (C-15), 39.5 (C-20), 34.9 (Cys-CH₂), 31.9 (C-25), 30.4 (C-8), 30.5 (C-7), 27.6 (C-12), 24.8 (C-15), 23.4 (Cys-CH₂), 22.1 (C-11) 20.5 (C-28), 16.0 (C-19), 13.9 (C-21), 11.9 (C-18); LR-APClMS (+) m/z [M + Na]+ 566, [M + H]+ 534, [M + H-NAC]+ 471.

Cytotoxicity Assays. Ewing’s sarcoma cell line CHP-100 and myeloma cell line H929 were cultured at 37 °C under 5% CO₂ in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), and 293T cells were grown in DMEM supplemented with 10% FBS. All cell lines were tested and found to be negative for Mycoplasma contamination. Cultures were passaged twice weekly, and cells exponential growth were used for experiments. Stock solutions of compounds were formulated in DMSO and maintained at −20 °C protected from light. To measure acute toxicity, CHP-100 cells were seeded in flat-bottom 96-well plates (7500 cells/well) and allowed to adhere overnight. Serial dilutions of compounds or DMSO vehicle control (not exceeding 0.2%) were added, and the relative viable cell number was determined by reduction of the dye resazurin (Alamar Blue) as previously described. 40 Heat-Shock Reporter Assays. Reporter cells were generated by infecting 293T cells (American Type Culture Collection) with a previously reported lentiviral vector encoding a fusion protein consisting of enhanced GFP fused to firefly luciferase under control of HSP70B+ promoter elements. 54 The plasmid encoding the fusion protein was generously provided by Khalid Shah (Massachusetts General Hospital, Boston, MA, USA). To isolate a homogeneous population of high responding cells, a transduced culture was heat-shocked at 42 °C for 1 h and then processed 8 h later by fluorescence-activated cell sorting (FACS). Prior to use, cells were reverse-selected by FACS to eliminate a minority population of cells constitutively expressing the reporter in the absence of induction. To evaluate compounds, cells were seeded in white 384-well plates (20 000 cells/well) and allowed to adhere overnight. Measurement of relative luciferase activity was achieved using an Envision plate luminometer (PerkinElmer) and Steady-Glo reagent (Promega) per the manufacturer’s recommendations. As a confirmatory assay for some compounds, 3T3-Y9-B12 reporter cells were seeded in black flat-bottom 96-well plates (20 000/well) and allowed to adhere overnight as previously reported. 33 Cells were then incubated for 24 h in the presence of WA or analogues (1, 2, or 4 µM). After washing with PBS, fluorescence was quantified using an Analyst AD (LJL Biosystems) plate reader with excitation and detection at 230 nm [ WAA and DMSO control (not exceeding 0.2%) were added, and the relative viable cell number was determined by reduction of the dye resazurin (Alamar Blue) as previously described. 40 Heat-Shock Reporter Assays. Reporter cells were generated by infecting 293T cells (American Type Culture Collection) with a previously reported lentiviral vector encoding a fusion protein consisting of enhanced GFP fused to firefly luciferase under control of HSP70B+ promoter elements. 54 The plasmid encoding the fusion protein was generously provided by Khalid Shah (Massachusetts General Hospital, Boston, MA, USA). To isolate a homogeneous population of high responding cells, a transduced culture was heat-shocked at 42 °C for 1 h and then processed 8 h later by fluorescence-activated cell sorting (FACS). Prior to use, cells were reverse-selected by FACS to eliminate a minority population of cells constitutively expressing the reporter in the absence of induction. To evaluate compounds, cells were seeded in white 384-well plates (20 000 cells/well) and allowed to adhere overnight. Measurement of relative luciferase activity was achieved using an Envision plate luminometer (PerkinElmer) and Steady-Glo reagent (Promega) per the manufacturer’s recommendations. As a confirmatory assay for some compounds, 3T3-Y9-B12 reporter cells were seeded in black flat-bottom 96-well plates (20 000/well) and allowed to adhere overnight as previously reported. 33 Cells were then incubated for 24 h in the presence of WA or analogues (1, 2, or 4 µM). After washing with PBS, fluorescence was quantified using an Analyst AD (LJL Biosystems) plate reader with excitation and emission filters set at 485 and 530 nm, respectively.
tissue harvesting approximately 18 h postdosing. Organs were snap-frozen in liquid nitrogen and pulverized, and lysates were prepared in nonionic detergent buffer. Samples (15 μg of total protein/lane) were fractionated by SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies to HSP72 (clone C92F3A-S, Stressmarq Biosciences, 1:3000) and β-actin (mAbG6, Thermofisher, 1:1000). Chemiluminescent detection was performed using a ChemiDoc MP imaging system (Bio-Rad), and integrated band intensity was determined with Image Lab software (version 4.1, Bio-Rad). Plotting of the data and statistical analysis were performed using GraphPad Prism 6 software. The statistical significance cutoff for all comparisons was p < 0.05.

**ASSOCIATED CONTENT**

Supporting Information

Chemical characterization of new withanolide analogues 15, 17–19, 21–26, 28, 31, 33–35, and 38; screen for acute cytotoxic activity of 1 and analogues 2–37 using CHP-100 cells; HPLC evidence for time-dependent conversion of 31 to 1 in cell culture medium; and relative reactivity of 1 and 35 with N-acetylcycteine. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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**ABBREVIATIONS USED**

CHP-100, human Ewing’s sarcoma cell line; DMEM, Dulbecco’s modified Eagle’s medium; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; H929, plasmacytoma myeloma; HMBC, heteronuclear multibond correlation; HSA, heat-shock-inducing activity; HSE, heat-shock element; HSFI, heat-shock factor 1; HSP, heat-shock protein; HSR, heat-shock response; NOE, nuclear Overhauser effect; PBS, phosphate-buffered saline; SAR, structure—activity relationship; t-BDMS-Cl, tert-butylmethylsilyl chloride; TLC, thin-layer chromatography; WA, withaferin A; 293T, transformed human embryonic kidney cells

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(17) Development Therapeutics Program Mean Graph. http://dtp.ncti.nih.gov/dtp standard/ servers/ M e a n G r a p h S u m m a r y ? t e s t s h o r t n a m e = N C I + C a n c e r + S c r e e n + 1 0 % 2 F 2 0 0 9 + D a t a & s e a r c h t y p e = NCISearchlist-101088.


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