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Using the Heat-Shock Response To Discover Anticancer Compounds that Target Protein Homeostasis

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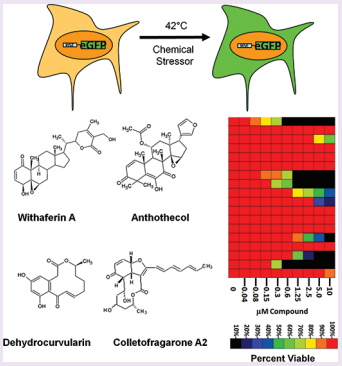
Supporting Information

ABSTRACT: Unlike normal tissues, cancers experience profound alterations in protein homeostasis. Powerful innate adaptive mechanisms, especially the transcriptional response regulated by Heat Shock Factor 1 (HSF1), are activated in cancers to enable survival under these stressful conditions. Natural products that further tax these stress responses can overwhelm the ability to cope and could provide leads for the development of new, broadly effective anticancer drugs. To identify compounds that drive the HSF1-dependent stress response, we evaluated over 80,000 natural and synthetic compounds as well as partially purified natural product extracts using a reporter cell line optimized for high-throughput screening. Surprisingly, many of the strongly active compounds identified were natural products representing five diverse chemical classes (limonoids, curvularins, withanolides, celastraloids, and colletofragarones). All of these compounds share the same chemical motif, an α,β-unsaturated carbonyl functionality, with strong potential for thiol-reactivity. Despite the lack of *a priori* mechanistic requirements in our primary phenotypic screen, this motif was found to be necessary albeit not sufficient, for both heat-shock activation and inhibition of glioma tumor cell growth. Within the withanolide class, a promising therapeutic index for the compound withaferin A was demonstrated *in vivo* using a stringent orthotopic human glioma xenograft model in mice. Our findings reveal that diverse organisms elaborate structurally complex thiol-reactive metabolites that act on the stress responses of heterologous organisms including humans. From a chemical biology perspective, they define a robust approach for discovering candidate compounds that target the malignant phenotype by disrupting protein homeostasis.



To prosper, cancer cells must accommodate a wide variety of stressors.[1] One source of stress is the hostile tumor microenvironment. Less widely appreciated are cell autonomous sources of stress such as the accumulation of mutated proteins and dysregulation of the protein translation machinery itself.[2] Innate adaptive responses are mobilized to counteract these challenges.[3] The heat-shock response (HSR) is a key component of this cytoprotective process.[4] Driven by Heat Shock Factor 1 (HSF1), high level expression of molecular chaperones and other potent pro-survival mediators helps cells to cope.[5,6] Additional powerful, interconnected transcriptional responses counteract malignancy-associated pressures placed on the protein degradation machinery, DNA replication and repair, energy metabolism, and the maintenance of redox balance.[1] Together, these systems collaborate to foster cell survival and proliferation at levels of stress that would otherwise be lethal.

The intense pressure faced by malignant cells presents an intriguing therapeutic opportunity, namely, the use of agents to stress tumor cells beyond their capacity to compensate.[4]

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Piperlongumine, a potent generator of reactive oxygen species, has recently been shown to selectively kill cancer cells.[7] Compounds such as geldanamycin and radicicol that inhibit HSP90 and lactacystin that inhibits the proteasome are examples of potent anticancer natural products that disrupt protein homeostasis. In doing so, they selectively impair the ability of cells to cope with imbalanced protein synthesis as a result of aneuploidy.[8] To further exploit this therapeutic strategy, we now report the results of a high-throughput phenotypic screening campaign designed to identify small molecule disruptors of protein homeostasis. We tested over 80,000 pure compounds and partially purified natural product extracts for their ability to activate the HSR. One such extract was subjected to bioactivity-guided fractionation to isolate the active compound. Five diverse classes of small-molecule natural products bearing thiol-reactive enone moieties were identified, and these were subsequently evaluated for their potential anticancer activity against human glioma cells in culture. Given its potential for good central nervous system (CNS) penetration and the unmet need for curative therapies for high-grade brain cancers, withaferin A (WA) was tested and found to be active in an orthotopic human glioma xenograft model in mice. Our findings demonstrate that both plants and fungi provide a rich bioresource of structurally complex thiol-reactive secondary metabolites capable of acting on the stress responses of animals. From a chemical biology perspective, our approach provides a robust strategy to identify structurally diverse compounds that target the malignant phenotype by disrupting protein homeostasis.

RESULTS AND DISCUSSION

Heat-Shock Response as a High-Throughput Biosensor. The heat-shock response plays a key role in enabling cells to accommodate the drastic alterations in physiology that accompany malignant transformation. Compared to normal diploid fibroblasts (IMR-90), multiple highly malignant human glioma cancer lines (LN428, LN837, and U87) demonstrate a marked increase in levels of HSF1 and its classical downstream transcriptional targets such as Heat-Shock Proteins HSP90 and HSP27 (Figure 1, panel A). Dependence on increased HSF1 function in these cancer cells was confirmed when levels of HSF1 were knocked down using RNAi technology. Infection with HSF1-targeted lentiviral constructs that effectively suppress HSF1 levels (Figure 1, panel B) led to a 60−80% reduction in viable cells while infection with 2 different control viruses had no such effect (Figure 1, panels C and D). These data confirm constitutive activation of the HSR in glioma cells. Further, they suggest that malignant transformation imposes increased demands on underlying mechanisms responsible for maintaining protein homeostasis in cells.

The ongoing clinical evaluation of several well-characterized disruptors of protein homeostasis, namely, inhibitors of HSP90 and the proteasome, suggests that cancer-related demands on protein homeostasis constitute an intrinsic vulnerability susceptible to therapeutic attack. A major component of the anticancer activity of these drugs appears to involve overwhelming the ability of cancer cells to cope with additional stress on their already severely taxed homeostatic mechanisms. Although none of these compounds directly target HSF1, their ability to activate the HSR is absolutely dependent on this transcription factor and its binding to regulatory heat-shock elements (HSEs) within the promoters of relevant genes. To identify compounds with which to target protein homeostasis

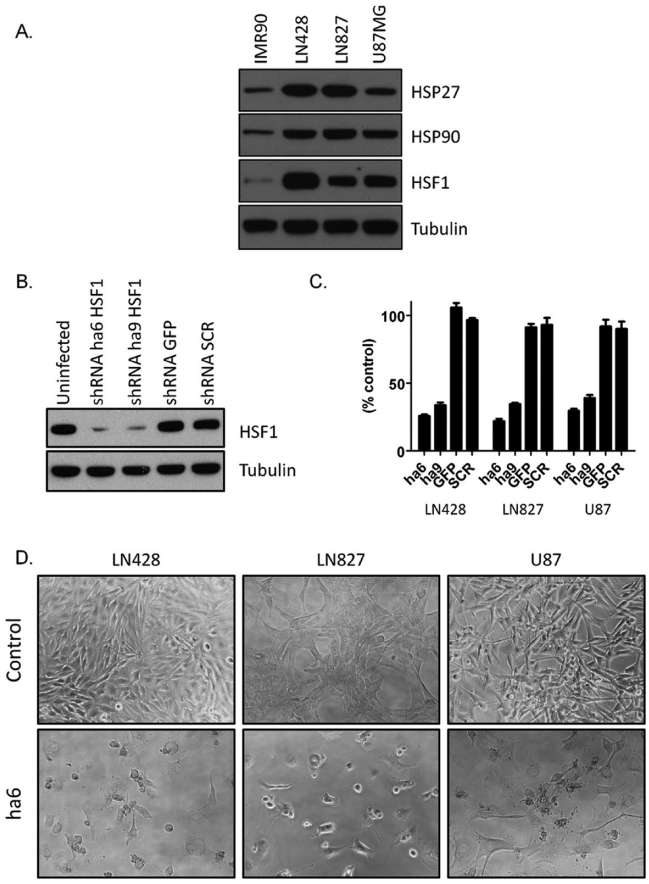

Figure 1. Glioma cells experience proteotoxic stress and are dependent on HSF1 for proliferation and survival. (A) Immunoblot analysis of normal diploid cell line IMR90 and established glioma cell lines LN428, LN827, and U87 with antibodies to HSP27, HSP90, and HSF1. α-Tubulin, loading control. (B) Immunoblot of LN428 cells 3.5 days following lentivirus-mediated knockdown of HSF1 with shRNA. shRNA-ha6 and ha9 target HSF1.[5] shRNA-GFP and shRNA-SCR (scrambled) are controls that do not target HSF1. (C) Viability of glioma cells 6 days following infection with indicated lentiviruses. Percent viability relative to uninfected control is plotted (six replicates per condition). (D) Photomicrographs of glioma cells without lentivirus infection (control, top row) and following lentivirus-mediated HSF1 knockdown with shRNA-ha6 (bottom row). Photomicrographs at 6 days following infection.

in new, as yet undiscovered ways, we hypothesized that the transcriptional HSR could be exploited as a sensitive, mechanistically unbiased biosensor. To pursue this hypothesis, a reporter cell line was constructed in which expression of enhanced green fluorescent protein (eGFP) is controlled by a minimal consensus HSE-containing promoter (Supplementary Figure 1). Upon thermal stress or exposure to inhibitors of HSP90 or the proteasome, these reporter cells robustly express eGFP.

After optimization through several rounds of fluorescence-activated cell sorting (FACS) and single cell cloning, the reporter cell line was deployed in a high-throughput screen of synthetic compounds, purified natural products derived from plant-associated fungi of the southwest U.S.[9,10] and partially purified fractions of fungal extracts from the National Institute for Biodiversity in Costa Rica (INBio). Approximately 80,000 samples were screened at a single concentration in duplicate to generate quantitative Z-scores as a measure of their relative

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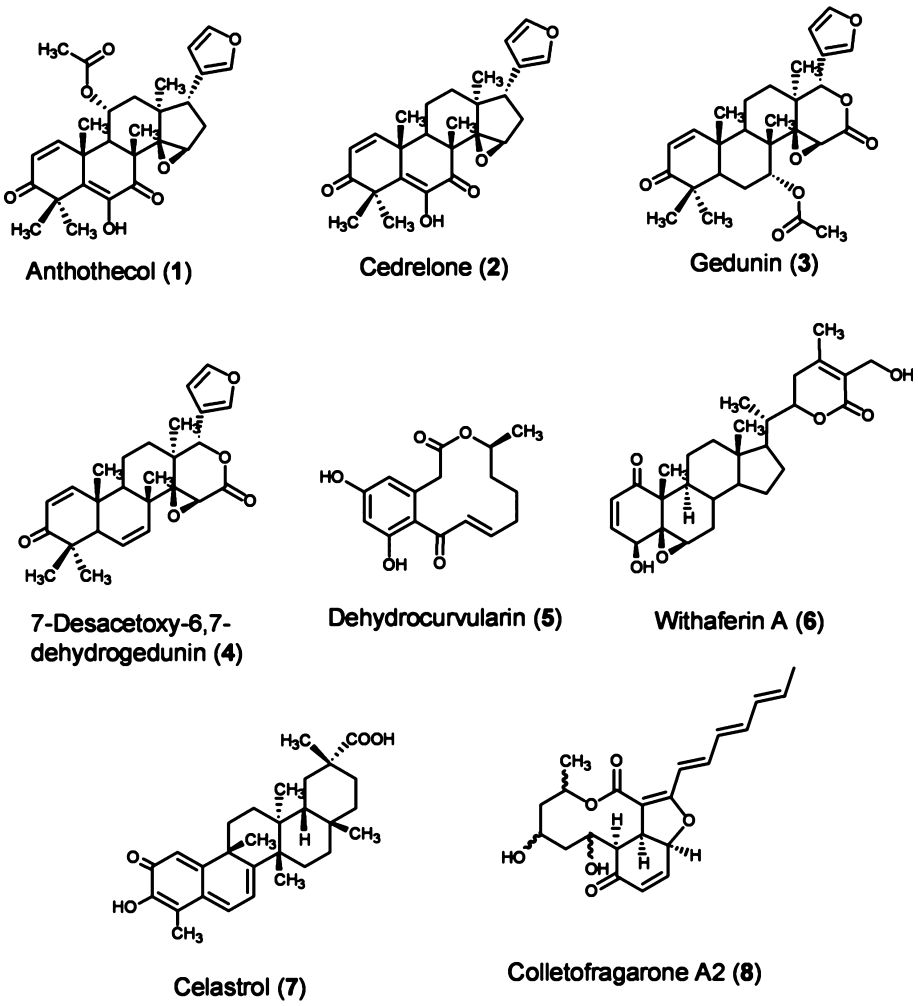

Figure 2. Small-molecule natural products with an α,β-unsaturated carbonyl moiety identified in a screen for heat-shock response activators.

heat-shock inducing activity. Multiple natural products belonging to five diverse classes but sharing a conserved electrophilic α,β-unsaturated carbonyl structural motif were identified (Figure 2). These included the limonoids, anthothecol (**1**), cedrelone (**2**), gedunin (**3**), and 7-desacetoxy-6,7-dehydrogedunin (**4**), the macrocyclic lactone dehydrocurvularin (DHC) (**5**), the steroidal lactone withaferin A (WA) (**6**), and the triterpenoid quinone-methide celastrol (**7**). A fifth class of natural products, represented by colletofragarone A2 (**8**), was identified by HSR-guided fractionation of an ethanolic extract of the fungus *Colletotrichum* sp. which was active in the primary screen.

α,β-Unsaturated Carbonyl Motif Is Associated with Heat-Shock Activity. Limited structure–activity relationships (SARs) for these molecules were investigated using the HSR assay. The steroidal lactone WA (**6**) induced a peak transcriptional response at 1.25 μM (Figure 3, panel A). Its analogue, 2,3-dihydrowithaferin A (**9**) (Supplementary Figure 2), lacking the α,β-unsaturated carbonyl moiety, demonstrated no detectable heat shock induction. Withanolide analogues pubesenolide (**10**) and viscosalactone B (**11**), which also lack the reactive enone moiety present in WA, were inactive for heat shock induction (Supplementary Figure 2). The induction of endogenous heat-shock proteins by WA (**6**) was confirmed by immunoblot in LN428 cells (Figure 3, panel B). For the limonoids, anthothecol (**1**), cedrelone (**2**), and 7-desacetoxy-6,7-dehydrogedunin (7-desacetoxy-6,7-DHG) (**4**) (Supplementary Figure 3) were the most potent inducers of the HSR with compound **4** demonstrating activity across a broader concentration range (Figure 3, panel A). Compounds **14**–**16**, all of which lack the reactive enone moiety, were each inactive for heat shock induction. DHC (**5**) (Figure 2) demonstrated peak heat shock inducing activity at 5 μM, and curvularin (**17**), which lacks only the α,β-unsaturated carbonyl moiety, was inactive for heat shock induction (Supplementary Figure 4). In addition, the known thiol-reactive molecule celastrol (**7**) (Figure 2) was identified in this screen, demonstrating peak activity at 600 nM, and colletofragarone A2 (**8**) induced a peak HSR at 2.5 μM. Conventional DNA-damaging chemotherapeutic agents such as cisplatin and doxorubicin are inactive in stimulating the HSR.[11] It is noteworthy that the library of 80,000 samples we screened contained many cytotoxic chemotherapeutics, but these were not identified as heat-shock active hits. From this, we infer that heat shock inducing compounds can be cytotoxic, but cytotoxicity *per se* is not sufficient to activate the HSR. In addition, the presence of an α,β-unsaturated carbonyl moiety in compounds that do not induce a HSR, *e.g.*, withanolides A (**12**) and B (**13**) (Supplementary Figure 2), suggests that this structural motif is necessary but not sufficient to induce a heat-shock response.

Thiol-Reactivity of Compounds with α,β-Unsaturated Carbonyl Motif and Its Consequences. We previously

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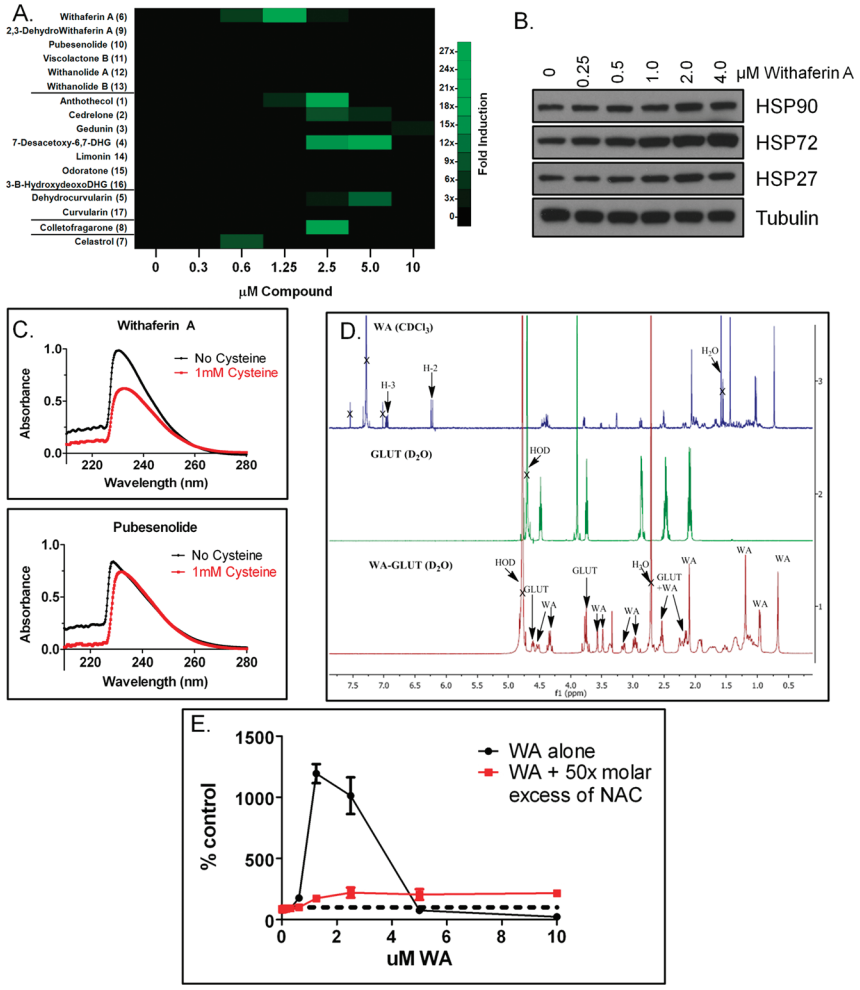

Figure 3. Thiol-reactive natural products induce a heat-shock response. (A) Heat map of HSE-eGFP induction compared to vehicle only. Determinations were in triplicate. (B) Immunoblot analysis of LN428 following treatment with indicated amounts of WA (**6**) for 16 h. (C) UV absorption spectra of WA (**6**) and pubesenolide (**10**) with or without L-cysteine. (D) NMR spectra of WA (**6**) with or without glutathione. (E) Heat-shock reporter activation by WA alone and in the presence of a 50× molar excess of *n*-acetylcysteine (NAC). Dashed line at 100% indicates baseline level of reporter signal. Decrease below 100% reflects cytotoxicity.

demonstrated covalent adduct formation between WA and cysteine residue 133 of annexin II,[12] one of the several reported protein targets of this small molecule natural product.[13,14] To further investigate the presumed thiol reactivity of WA, several independent approaches were pursued. UV spectrophotometry revealed a marked hypochromic shift when WA was incubated with L-cysteine, indicative of adduct formation (Figure 3, panel C),[15] but addition of L-cysteine had a minimal effect on the UV spectrum of pubesenolide (**10**), which lacks the reactive α,β-unsaturated carbonyl moiety (Figure 3, panel C). Using ^{1}H NMR (Figure 3, panel D), adduct formation was readily detected between WA and the thiol group of glutathione, a major component of the intracellular redox defense network. Interestingly, simultaneous addition of *N*-acetyl cysteine (NAC) and WA to the heat-shock reporter cell line led to a near complete suppression of heat shock activation (Figure 3, panel E). Moreover, as seen in another system,[16] the cytotoxicity of WA was counteracted by excess NAC (Figure 3, panel E). Taken together, these data support the importance of the reactive α,β-unsaturated carbonyl moiety of WA in thiol adduct formation and cytotoxicity.

Thiol-reactive compounds play important roles as chemical messengers in nature.[17] Such compounds are often produced by organisms experiencing unfavorable conditions. In the laboratory setting, for instance, increased production of WA can be triggered in plant cells by exposure to copper sulfate.[18] In addition to local effects within the elaborating organism itself, thiol-reactive molecules are also encountered by neighboring plants or fungi or are even ingested by animals. In these heterologous organisms, the compounds can act as chemical cues of impending adversity. By inducing low-level proteotoxic stress in an organism exposed to such compounds, potent protective responses can be unleashed in anticipation of looming environmental challenges, thus priming these organisms to endure otherwise lethal conditions. This process, which has been termed xenohormesis,[19] can be considered akin to the more well established phenomenon of thermotolerance.[20] Unlike thermotolerance, however, xenohormesis is not a cell-autonomous process. Instead, acquisition of protection depends upon communication between organisms. A noteworthy example of such biology is provided by monocillin I, a metabolite produced by the rhizosphere fungus *Parapheosphaeria quadriseptata*. This natural product is not thiol-reactive (unpublished data), but we have shown that it selectively targets HSP90 and at low concentrations can dramatically enhance plant thermotolerance.[21]

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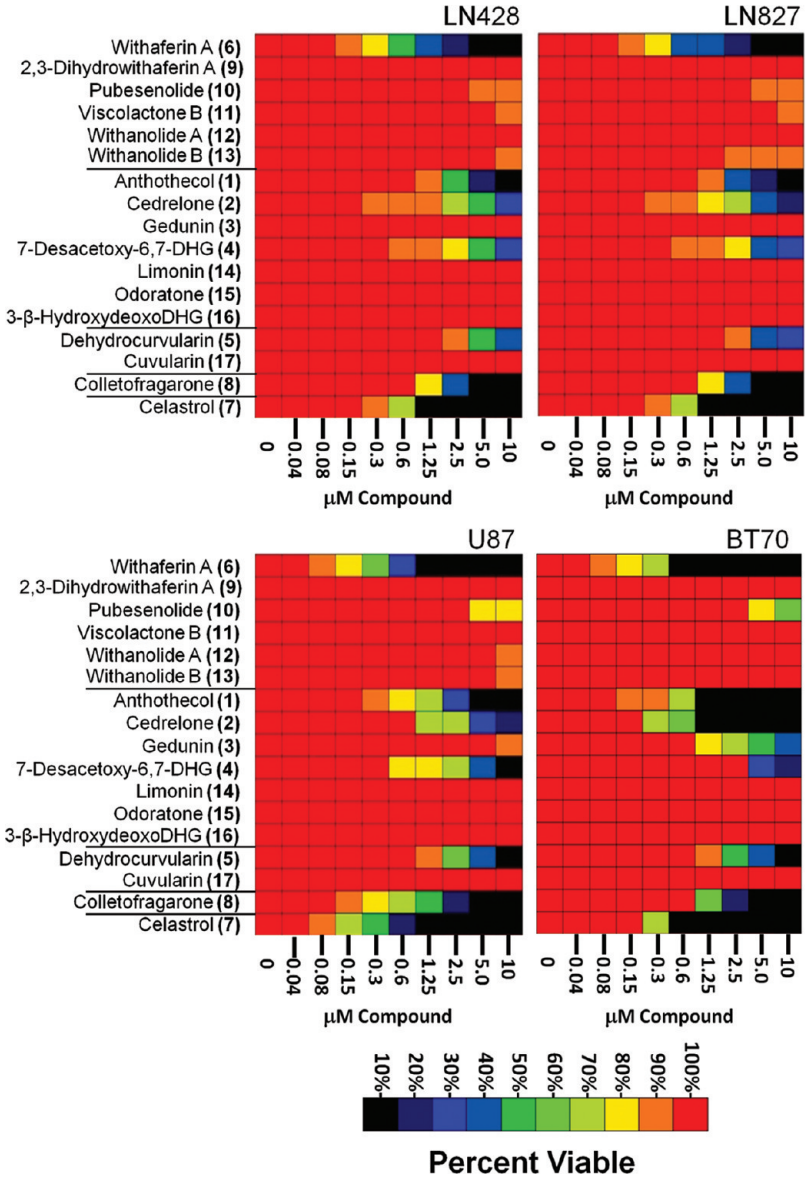

Figure 4. Thiol-reactive natural products are cytotoxic to glioma cell lines. Heat maps of glioma cell survival in the presence of thiol-reactive natural products and analogues. LN428 cells have wild-type *PTEN* and a p53 V173M/R282W mutation. LN827 cells have an Exon 3 splice acceptor mutation of *PTEN* and a *p53* mutation. U87 cells have an Exon 3 splice acceptor mutation of *PTEN* and a wild-type *p53*. BT70 cells have a G44 V mutation in *PTEN* and R273C mutation in *p53*. Cells were treated with the indicated concentrations of the compounds, and viability was measured using resazurin 48 h later (in triplicate).

In nature, rapid dilution ensures that the concentration of any bioactive secondary metabolites released into the environment will be exceedingly low. Adduct formation as enabled by thiol-reactivity may provide a solution to this problem. The frequent occurrence of a reactive α,β-unsaturated carbonyl moiety in hits from our screen suggest the strategy has been widely adopted to allow these molecules to exert effects in the environment at low concentration in a rapid and predominantly irreversible manner. These adducts amount to stable post-translational modifications that may serve important functional roles including modulation of cytoskeletal dynamics,[12] enzyme function,[17] chaperone activity, epigenetic regulation of gene expression, and the triggering of adaptive responses. But is it not paradoxical that compounds that launch cytoprotective responses could have the ability to actually kill cancer cells? While the effects of dehydrocurvularin (**5**)[22] and colletofragarone A2 (**8**)[23] are not yet understood, celastrol (**7**),[24,25] the synthetic triterpenoid CDDO,[15] HSP90 inhibitors, limonoids,[26−28] and WA[29] have all demonstrated both protective and cytotoxic activities. Cellular context (normal *versus* malignant) and exposure conditions (low *versus* high concentration) likely dictate the ultimate biological outcome resulting from exposure to these compounds.

Heat Shock Induction and Anticancer Activity. To further examine the effects of heat-shock active compounds from our primary screen, we evaluated their potential antiglioma activity using standard dose−response cytotoxicity assays (Figure 4). The glioma cell lines used for these studies bear diverse molecular genetic defects including clinically relevant mutations that impair *PTEN* and *p53* tumor suppressor function. Heat shock active screen hits demonstrated concentration-dependent antiproliferative activity against all three established human glioma cell lines (LN428, LN827, and U87) irrespective of their specific underlying

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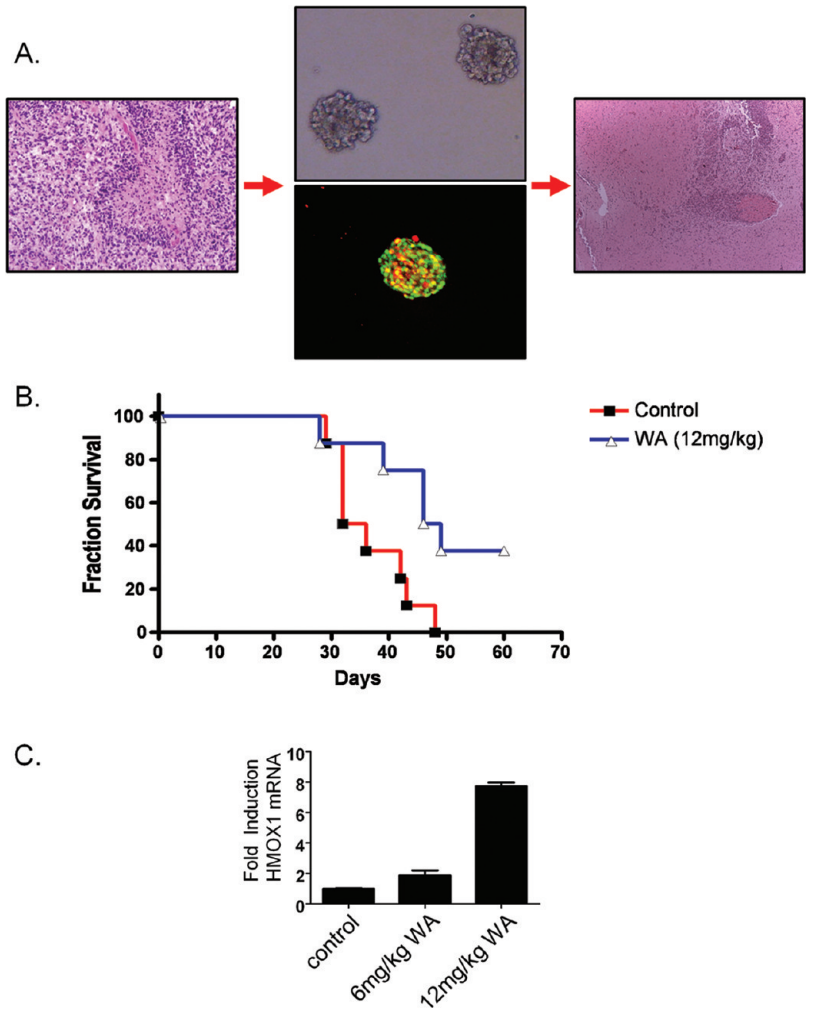

Figure 5. WA has anticancer activity in a glioma stem cell-based orthotopic xenograft model in mice. (A) Schematic depiction of the previously characterized BT70 glioma orthotopic model.[30] The cells were derived from a human glioblastoma (example H&E shown). Tumor spheres are immunoreactive for MIB1 (red) and OLIG2 (green) and form infiltrative tumors (H&E). (B) Kaplan–Meier analysis of mice bearing orthotopic BT70 xenografts treated with WA (12 mg/kg) or vehicle control. (C) *HMOX1* (*HSP32*) mRNA expression in orthotopic BT70 xenografts is modulated by WA treatment. Measurements were in duplicate using the nCounter system (error bars SD, $p = 0.09$ for 6 mg/kg WA, $p < 0.001$ for 12 mg/kg WA).

genetic defects. Potent antiproliferative activities were seen with compounds **1**, **2**, and **4**–**8** (Figure 4). Importantly, all molecules capable of inducing a transcriptional HSR were able to inhibit the survival/proliferation of glioma cells *in vitro*, but compounds **9**–**17** (Supplementary Figures 2–4) lacking an α,β-unsaturated carbonyl moiety were all inactive against the glioma cells or showed markedly reduced activity. The activity of compounds was also tested against the highly malignant glioma progenitor/stem cell line BT70,[30] which was used for subsequent xenograft studies in mice. This line bears a G44V mutation in PTEN and R273C mutation in p53. Growth of this cell line was also sensitive to heat-shock active compounds (Figure 4). Against each of the cell lines, WA was the most active of all natural products tested with an IC_{50} of 300 nM against the BT70 glioma progenitor/stem cells (Figure 4). In comparison, 100 μM temozolomide achieves only a partial response against established glioma lines and glioma tumor spheres in cell culture.[31]

WA was selected for evaluation in mice because it demonstrated the most potent antiglioma activity *in vitro* and its lipophilic, steroidal scaffold suggested the potential for good central nervous system penetration. To provide maximal clinical relevance, we adopted a very stringent orthotopic xenograft model involving intracranial implantation of BT70 glioma progenitor cells. These cells, unlike many established glioma cell lines, maintain expression of important transcription factors of glioma lineage such as Olig2 (Figure 5, panel A). In addition, these cells form infiltrative tumors that mimic many of the features of high-grade gliomas (Figure 5, panel A). Ten days following orthotopic implantation, mice were administered WA (12 mg/kg) or vehicle three times weekly. The WA treatment group had a 40% increase in median survival (47.5 days *versus* 34 days; log rank test $p = 0.015$) (Figure 5, panel B). This is a more dramatic response than achieved by temozolomide, the current standard of care, on established glioma cell lines and glioma tumor neurospheres.[31] To monitor the impact of WA treatment on stress response pathways within the intracranial tumor mass, we measured levels of *HMOX1* (*HSP 32*) mRNA following two doses of WA (6 mg/kg or 12 mg/kg). *HMOX1* was chosen because genome-wide expression profiling had previously found it to be one of the most highly activated genes following WA exposure.[32] Encouragingly, WA treatment at

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systemically well-tolerated levels resulted in a dose-dependent stress response within the orthotopic tumor xenograft as evidenced by an average 7.7-fold increase in *HMOX* mRNA levels ($p < 0.0001$) (Figure 5, panel C). The cell culture and animal model results with the promising hit, WA, provide clear proof of principle that systemically well-tolerated compound exposures can exert marked antibrain cancer activity, at least in part by imposing additional stress on tumors growing within the central nervous system. WA has been shown to trigger the production of reactive oxygen species,[16,33] so it may in part share a common mechanism of anticancer activity with piperlongumine.[7]

Ashwaghanda (derived from roots of *Withania somnifera* and a rich source of WA) and numerous other plant extracts containing thiol-reactive molecules have been used for millennia in traditional medicines. Nonetheless, there are major challenges facing the introduction of purified thiol-reactive molecules into conventional therapeutic practice. As we show using multiple modalities, the α,β-unsaturated group of WA reacts with cysteine residues. Because thiol-reactive compounds have the potential to react with many proteins bearing accessible cysteine residues, should this preclude their use as anticancer therapeutics? Evidence for a useable therapeutic index is provided by our orthotopic glioma study. Indeed, considerable precedent exists for very useful anticancer agents that are chemically reactive, *e.g.*, DNA alkylating agents. Many of these agents show a preference for areas of open chromatin structure such as the promoter regions of actively transcribed genes. It is very likely that thiol-reactive molecules behave in a similar manner. Rather than reacting with proteins indiscriminately and broadly disrupting protein conformation, it is likely that compounds with an electrophilic α,β-unsaturated moiety selectively react with particularly nucleophilic residues. Such amino acids may be poised to react by virtue of local electronic and structural constraints as well as the particular pH maintained in specific intracellular compartments.

But what are the "real" targets of these compounds? Answering this question is certainly a challenge. While targets for many thiol-reactive molecules have been proposed, it remains unclear if these are the most biologically meaningful interactions.[12,13,27] More comprehensive proteomic techniques will be required to globally capture the spectrum of protein targets of these compounds.[34] Because of the similar reactive groups and the similar transcriptional response that they generate, it is possible that they may even share some targets. Recently, efforts to define the array of potential targets and the relevant functional cysteine residues in these proteins have been undertaken.[35−38] One approach has been quantitative reactivity profiling with isotopically labeled small molecule electrophiles.[38] Here, proteins with highly reactive, functional cysteines were identified at low electrophile concentrations, and progressive recruitment of additional targets was seen with increasing concentration of the electrophile. Some of the hyper-reactive proteins identified by this approach are perhaps the fundamental electrophile-sensors in cells that serve as first-line defenses for launching adaptive transcriptional and post-transcriptional responses.[19,39]

The natural products encountered in our work seem best understood as containing a structurally complex targeting moiety that could easily confer considerable selectivity and a more generic effector motif that confers thiol-reactivity (Michael addition reaction). Specificity may well be provided by the targeting moiety in a concentration-dependent manner, but it is likely that these regions are dispensable for adduct formation with the target. At high concentrations of compound, however, selective targets are probably overwhelmed and more promiscuous adduct formation occurs leading to effects that are independent of stress pathway modulation. The complex biology and chemical reactivity of the heat-shock inducing natural products identified in our screen will make elucidating their mechanisms of action challenging. Efforts to move beyond simple chemical reductionist approaches that attempt to pair one small molecule with one target protein will be essential,[40] but the effort will be worthwhile. Rather than poison a molecular target of presumed importance based on our still limited understanding of oncogenesis, these compounds have been honed by nature over eons to alter phenotypes by engaging ancient transcriptional responses such as the HSR that act system-wide. In this regard, they could prove less susceptible to the rapid emergence of target-related resistance and complement current efforts to disrupt the signal transduction and other molecular derangements that underlie specific cancers.

METHODS

Materials. Anthothecol (**1**), cedrelone (**2**), gedunin (**3**), and 7-desacetoxy-6,7-dehydrogedunin (**4**) were from Gaia Chemical. Celastrol (**7**) and withanolide B (**13**) were purchased from Chromadex. Dehydrocurvularin (**5**) was isolated from the rhizosphere fungus, *Aspergillus* sp.[22] WA (**6**), pubesenolide (**10**), viscosalactone B (**11**), and withanolide A (**12**) were obtained from aeroponically grown *Withania somnifera* plant.[41,42] 2,3-Dihydrowithaferin A (**9**)[41] and curvularin (**17**)[43] were prepared by the catalytic reduction of WA (**6**) and DHC (**5**), respectively, and their identities were confirmed by comparison of spectroscopic data (NMR and MS) with those reported.

Cell Culture. LN428 (kind gift of Rosalind Segal, DFCI), LN827, and U87MG cells (ATCC) were maintained under 5% CO_2 in DMEM (ATCC) supplemented with 10% FBS. Reporter cell line 3T3-Y9 was maintained under 5% CO_2 in Opti-MEM medium with 2.5% FBS.[44] BT70 glioma stem cells were obtained from the UCSF (David James). BT70 culture medium consisted of serum-free neural stem cell medium, human recombinant EGF (20 ng/mL; Sigma) and bFGF (20 ng/mL; Upstate), and Neuronal Survival Factor (NSF,1x; Clonetics).

High-Throughput Screening. Screening was performed with the Broad Institute Chemical Biology Platform. Test substances (final concentration ∼10 μg/mL) were added to 3T3-Y9 reporter cells (20,000/well in 384-well clear-bottom tissue culture plates). Relative fluorescence intensity per well was measured 16−18 h post compound addition using a Tecan Safire plate reader (excitation 483 nm, emission 507 nm). Assays were in duplicate. Deviation from the median value of DMSO-treated wells was calculated (Z-score). Primary screening data are available at http://chembank.broadinstitute.org/; HeatShockModulation screen 1119).

Countering screening for autofluorescence was performed by pinning hits in PBS.

UV Experiments. Solutions of WA (**6**) or pubesenolide (**10**) (final concentration 100 μM) were prepared in 20 mM Tris (pH 8.0), 0.2% DMSO and 0.075% Triton-X 100. UV spectra (210−340 nm) were acquired in quartz cuvettes using a double beam spectrophotometer (Hitachi U-3900). Subsequently, L-cysteine was added (Sigma Aldrich Cat, no. C-7352, final concentration 1 mM) and incubated for 15 min at RT before repeat UV spectra were acquired.

NMR Experiments. NMR spectra were recorded in $CDCl_3$ or D_2O using a Bruker DRX-600 instrument at 600 MHz for ^{1}H NMR and a Bruker DRX-500 instrument at 125 MHz for ^{13}C NMR, respectively, using residual solvents as internal standards. Chemical shift values are given in parts per million (ppm), and coupling constants are in Hz. Low- and high-resolution MS were recorded, respectively, on Shimadzu LCMS-8000 QPα and JEOL HX110A

spectrometers. For colletofragarone A2 (**8**), NMR was carried out on a Varian INOVA 600 MHz spectrometer.

Cell Proliferation Assay. Cells (2,500/well) were dispersed into 96-well tissue culture plates, allowed to adhere overnight, infected with lentiviruses encoding the indicated shRNA, and cultured for 6 days. Photomicrographs were captured, and cell viability was measured using resazurin (Invitrogen, 1:200 final dilution). Fluorescence intensity as a measure of relative viable cell number was measured (Tecan Safire reader, excitation 540 nm, emission 590 nm). Lentiviral shRNA plasmids targeting HSF1 and procedures for generating infectious virus and transducing cells have been described.[5] Plasmids are from the Broad Institute/RNAi platform (deposited in Open Biosystems). For cytotoxicity testing, cells were treated for 48 h and relative viability was assessed with resazurin.

Identification of the Fungal Strain from Which Colletofragarone A2 (8) was Isolated. Fungal strain CR222K was isolated at the National Biodiversity Institute (INBio, Costa Rica). For identification by internal transcribed spacer (ITS) sequencing, CR222K was cultured in potato dextrose broth (PDB) for 5 days. The mycelium was retrieved by filtration and ground in liquid N_2. Genomic DNA was extracted (Wizard Genomic DNA Purification Kit, Promega), large subunit rDNA was amplified with PCR primers LR5 (5′-TCCTGAGGGAAACTTCG-3′) and LROR (5′-ACCCGCTGAACTTAAGC-3′), cloned with TOPO-TA Cloning Kit (Invitrogen), sequenced, and found to be 99% identical to *Colletotrichum* sp.

Bioassay-Guided Isolation and Characterization of Colletofragarone A2 (8). The fungus was grown at 25 °C on yeast malt plates supplemented with 30 μg/mL streptomycin and 12 μg/mL chlortetracycline. Agar plugs were used to inoculate 10 mL rich media seed cultures (5 g tryptone peptone, 10 g dextrose, 3 g yeast extract and 10 g malt extract per liter, pH 6.2), which were incubated at 25 °C with shaking (150 rpm, 6 days). Contents were poured into 300 mL of 0.66% malt extract broth (pH 6.2), and cultures were incubated at 25 °C with shaking (150 rpm, 14 days). Seven days before the end of fermentation, 10 g of sterile HP-20 resin (Diaion) was added to each 300-mL culture. Following fermentation, fungal cultures were filtered. HP-20 resin and mycelia were collected, and filtrates were discarded. Resin and mycelia were sonicated three times in 95% ethanol (200 mL ethanol per 10 g HP-20). Ethanolic extracts were concentrated to dryness. Crude extracts were fractionated on HP-20 resin and sequentially eluted with 100% water, 7:3 water/ethanol, 1:1 water/ethanol, 3:7 water/ethanol, 100% ethanol, 100% ethyl acetate. The first fraction was active for heat-shock induction and was fractionated further by reversed-phase HPLC (C18 column, eluted with 25:75 water/acetonitrile) to yield pure colletofragaraone A2 (**8**). Chromatographic purification was performed (Agilent 1100 series HPLC) using a semipreparative Discovery HS-C18 column (Supelco, 25 cm × 10 mm, 10 μm particle size).

Immunoblotting. Cell lysates were prepared in TNEK buffer (50 mM Tris, pH 7.4; NP-40 1%; EDTA 2 mM; KCl 200 mM and protease inhibitor cocktail (Roche Diagnostics, Cat, no. 11836153001). Samples (15 μg total protein/lane) were analyzed by SDS-PAGE and immunoblotting using the following antibodies: anti-HSP27 (clone G3.1, 1:1000, Enzo Life Sciences), anti-HSP90 (clone 16F1, Enzo Life Sciences, 1:1000), anti-HSP72 (clone C92F3A-5, Stressmarq Biosciences, 1:3000), anti-HSF1 antibody cocktail (Ab4, Thermo Scientific, 1:500), and anti-α-tubulin (clone DM1A, Sigma, 1:3,000).

RNA Extraction and NanoString nCounter Analysis. Fragments of tumor (30−50 mg) were lysed in RLT buffer plus 1% β-mercaptoethanol; 100 ng of total RNA purified using a Qiagen RNeasy Mini Kit (Qiagen Cat. no. 74104) was processed according to manufacturer's recommendations (NanoString Technologies). Data was collected with an nCounter Digital Analyzer and was processed according to manufacturer's guidelines.[45]

Orthotopic Glioma Model. All experimentation involving mice was performed in accordance with a protocol approved by the BWH and DFCI IUCAC and in accordance with all national and local guidelines and regulations. BT70 human glioma stem cells were suspended in sterile PBS at a density of 3×10^6 cells/mL, and 30,000 cells were implanted stereotactically in the right cerebral cortices of male athymic nude (nu/nu) mice (Charles River Laboratories). Ten days following orthotopic implantation, 10 mice per treatment group mice were randomly assigned to receive either control vehicle (Cremophor-EL 7.5%; DMSO 5%; PBS 87.5%) or WA (12 mg/kg) formulated in the same vehicle. Treatment was administered intraperitoneally on a 3×/wk schedule. Mice displaying behavioral changes consistent with tumor progression were sacrificed. The brain was removed for histologic analysis. For pharmacodynamic experiments, mice bearing intracranial xenografts (4 per group) were treated with two doses of WA separated by 12 h or vehicle control. Tumors were harvested 6 h following the last treatment.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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