Hsp72 Is an Intracellular Target of the $\alpha,\beta$-Unsaturated Sesquiterpene Lactone, Parthenolide

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Supporting Information

ABSTRACT: The electrophilic natural product parthenolide has generated significant interest as a model for potential chemotherapeutics. Similar to other $\alpha,\beta$-unsaturated carbonyl electrophiles, parthenolide induces the heat shock response in leukemia cells, potentially through covalent adduction of heat shock proteins. Other thiol-reactive electrophiles have also been shown to induce the heat shock response as well as to covalently adduct members of the heat shock protein family, such as heat shock protein 72 (Hsp72). To identify sites of modification of Hsp72 by parthenolide, we used high-resolution tandem mass spectrometry to detect 10 lysine, histidine, and cysteine residues of recombinant Hsp72 as modified in vitro by 10 and 100 $\mu$M parthenolide. To further ascertain that modification of Hsp72 by parthenolide occurs inside cells and not simply as an in vitro artifact, an alkyne-labeled derivative of parthenolide was synthesized to enable enrichment and detection of protein targets of parthenolide using copper-catalyzed [3 + 2] azide–alkyne cycloaddition. The alkyne-labeled parthenolide derivative displays an half maximal inhibitory concentration (IC$_{50}$) in undifferentiated acute monocytic leukemia cells (THP-1) of 13.1 ± 1.1 $\mu$M, whereas parthenolide has an IC$_{50}$ of 4.7 ± 1.1 $\mu$M. Concentration dependence of protein modification by the alkyn–parthenolide derivative was demonstrated, as well as in vitro adduction of Hsp72. Following treatment of THP-1 cells in culture by the alkyn–parthenolide, adducted proteins were isolated with neutravidin resin and detected by immunoblotting in the enriched protein fraction. Hsp70 proteins were detected in the enriched proteins, indicating that Hsp70 proteins were adducted intracellularly by the alkyn–parthenolide derivative.

INTRODUCTION

Recent studies have identified parthenolide (1, Figure 1), a sesquiterpene lactone known as the active ingredient in feverfew (Tanacetum parthenium), as a potential model compound for new ways to treat leukemia and other cancers.1,2 Parthenolide contains an electrophilic $\alpha,\beta$-unsaturated carbonyl group, and its mechanism of action is attributed to its ability to undergo Michael addition with biological nucleophiles, resulting in covalent modification of proteins and peptides.3 Thiol-reactive electrophiles, such as parthenolide, are known to affect multiple cellular pathways, many of which are implicated in cancer and degenerative diseases.4 Recent clinical interest in parthenolide and other sesquiterpene lactones arose due to evidence for its selective eradication of leukemic stem cells after treatment, which can potentially reduce the risk of recurrence in treated patients.5,6 Leukemic stem cells, in particular primitive (CD34+) cells, are susceptible to parthenolide largely due to their low intracellular concentrations of glutathione and upregulation of their glutathione metabolic pathway.6 Although the glutathione pathway has proven to be important for understanding the leukemic stem cells’ susceptibility to parthenolide, there are many other cellular pathways affected by parthenolide, which should be considered in the evaluation of parthenolide and its derivatives as clinical treatments.

Cellular targets of parthenolide include many common targets of thiol-reactive electrophiles, such as nuclear factor kappa B (NFkB) and thioredoxin.7–11 Inhibitor of kappa B kinase was the first directly confirmed target and represents part of the inhibition mechanism of nuclear factor kappa B (NFkB) by parthenolide. Direct inhibition of NFkB by parthenolide is due to the covalent adduction of cysteine 38 in the p65 subunit,12 whereas adduction of caspase 1 by parthenolide results in loss of protease activity and inhibition of pyroptotic cell death.10 Analysis by Pei et al. using a biotinylated parthenolide derivative identified key components of the glutathione and oxidative stress pathways as targets of parthenolide in CD34+ primary leukemia cells, including glutamate–cysteine ligase, glutathione peroxidase, and thioredoxin.6

Induction of the heat shock response (HSR) by thiol-reactive electrophiles, such as parthenolide, in cultured cells and animal
The similarities in chemistry and genetic response between 4-HNE and parthenolide are substantial and suggest that parthenolide may interact with the heat shock response system in a similar manner to 4-HNE. It was our hypothesis that parthenolide can adduct Hsp72, and to identify such covalent modification sites, we used high-resolution liquid chromatography–tandem mass spectrometry (LC–MS/MS) to detect modification of recombinant Hsp72 in vitro at increasing concentrations of parthenolide. Addition of the protein with parthenolide was detected at lysine, histidine, and cysteine residues, representing the first identifications of noncysteine adducts of parthenolide. We have also synthesized a versatile bio-orthogonal derivative of parthenolide (2), which contains an alkyne moiety enabling affinity capture and labeling within a biological context, to evaluate its covalent modification of Hsp72 both in vitro and in treated cells. The toxicity of the derivative to undifferentiated human acute monocytic leukemia cells (THP-1) was compared to parthenolide using a water-soluble cellular proliferation reagent to ascertain that our modification did not affect parthenolide’s cellular effects. Hsp72 was identified as a protein target of parthenolide both in vitro and in treatment of cultured THP-1 cells using derivative 2. Parthenolide derivative 2 was tested for modification of purified proteins in cell lysates and was used to isolate modified proteins in cultured and undifferentiated THP-1 cells.

The involvement of parthenolide with the heat shock response system has implications for the effect of similar or derivative therapeutic molecules on cellular responses to therapy. The heat shock system is a cytoprotective mechanism, the inducement of which can be counterproductive in chemotherapeutic regimens. Conversely, some cancers depend largely on an upregulated heat shock system for increased cell growth and stamina, and dysregulation of that function can lead to increased apoptotic events. Naturally occurring sesquiterpene lactones, such as parthenolide, and similarly reactive synthetic molecules are currently under evaluation for their effects. As more potential therapeutics are developed that contain the α,β-unsaturated lactone functionality, its reactivity must be comprehensively characterized.

## RESULTS AND DISCUSSION

### Hsp72 Is Modified In Vitro by Parthenolide at Cysteine, Histidine, and Lysine Residues.

To identify potential sites of modification, recombinant purified Hsp72 was incubated with 10 and 100 μM parthenolide overnight at 37 °C, followed by proteolytic in-gel digestion and analysis by liquid chromatography–mass spectrometry (LC–MS). The raw data were searched with a Mascot database and then parsed and filtered using Scaffold. Each reported modification was within 5 ppm error of its predicted precursor mass-to-charge ratio, and the respective MS² spectrum included coverage of b or y ions across the modified amino acid. The total sequence coverage of Hsp72 peptides in our experiment ranged from 83 to 91% for each set of experimental samples (Figure S1, Supporting Information). For recombinant human Hsp72, 10 modifications were validated (representative MS² spectra are found in Figures S2–S12, Supporting Information). Parthenolide’s reactive moiety is the α,β-unsaturated lactone, not the epoxide. A reduced compound retaining the epoxide was not found to be reactive with human serum albumin, whereas parthenolide did react with a cysteine available on the protein.

In this work, two cysteine residues were identified to be modified by parthenolide at a concentration of 100 μM, and...
several lysine and histidine residues also exhibited modification (Table 1). Both cysteines identified as targets of parthenolide were not available: 2e88 for the N-terminal nucleotide-binding domain and 4po2 for the C-terminal substrate-binding domain. The calculated pK_a’s of the adducted lysines (Table 1, Supporting Information) range from 10.27 to 11.41, and surface exposure ranges from 0 to 25% buried. We expected to find that the adducted lysines had both lower pK_a values and more surface exposure than the unadducted lysines; however, there was no consistent consensus for pK_a value. Most of the lysines in Hsp72 were, as expected, close to the surface. For the five cysteines in Hsp72, two were involved in a disulfide bond (C574 and C603) and C17 was very deeply buried within the protein. The two adducted cysteines were calculated to be deeply buried at 85 and 79% for C267 and C306, respectively; however, their calculated pK_a values were lower than those of the other three cysteine residues. Previous results from Szapacs et al. have shown that lower pK_a can correlate with faster kinetic reaction of histidine with 4-HNE.31

Alkyne-Labeled Parthenolide Derivative 2 Retains Toxicity against THP-1 Cells. Alkylate-modified parthenolide derivative 2 was synthesized from (+)-parthenolide in a two-step process (Figure S3, Supporting Information). Parthenolide was first oxidized to melampomagnolide B with selenium dioxide and tert-butyl hydroperoxide, as previously reported.37,38 Esterification of melampomagnolide B with hexynoic acid using Mitsunobu coupling was unsuccessful; however, the Steglich esterification using dimethylaminopyridine and dicyclohexylcarbodiimide was successful, with an overall yield of 63%. The resulting compound was verified by 1H NMR spectroscopy and liquid chromatography—mass spectrometry.

To ascertain that the parthenolide derivative retained the cellular effects of the parent molecule, the toxicities of parthenolide and alkyne-parthenolide were determined in undifferentiated THP-1 cells. In this experiment, THP-1 monocytic cells were treated in RPMI medium supplemented with 10% fetal bovine serum (FBS) for 48 h prior to analysis using WST-1 to determine cell viability. The IC_50 of 2 was calculated to be 13.1 ± 1.1 μM in comparison to parthenolide’s IC_50 of 4.7 ± 1.1 μM (Figure 1). The IC_50 of the derivative increased less than 3-fold, and the 95% confidence interval between the two means ranged from 5.06 to 11.74 μM, which suggest that the addition of alkyne did not practically affect the potency of the parthenolide derivative. The small statistically significant change in toxicity may be due to lower membrane permeability of the alkyne-parthenolide with the addition of hexynoic acid to the molecule; however, the derivative retains the low micromolar toxicity of parthenolide.

Alkyne-Labeled Parthenolide Derivative 2 Adducts Multiple Proteins after Incubation with Both Cell Lysates and Cultured THP-1 Cells. To test the capacity of 2 not only to adduct proteins but also to allow specific detection through bio-orthogonal chemistry, cell lysates were treated in vitro and then subjected to copper-catalyzed azide−alkyne cycloaddition (CuAAC) with an azide−biotin tag.39,40 The appearance of multiple streptavidin-reactive bands on an immunoblot of treated and untreated lysates was evident through bio-orthogonal chemistry, cell lysates were treated in vitro and then subjected to copper-catalyzed azide−alkyne cycloaddition (CuAAC) with an azide−biotin tag.39,40

Table 1. Sites of in Vitro Parthenolide Modification on Recombinant Hsp72

| adduction site on Hsp72 | peptide sequence* | precursor resolutions (ppm) |
|------------------------|-------------------|----------------------------|
| K77 KFGDPVQSDM(...)K   | 0.55, 1.1         |
| K100 HWPQYINDKR(...)PK| 1.5, 1.0          |
| K112 GET(...)FTPEIEMSS(...) | 1.3, 3.1 |
| VLTK                   |                   |
| H227 ATAGDTH(...)LGEDFDNR| 0−0.38        |
| K251 K(...)DISQNK      | 0.34             |
| C267S                  | −0.33            |
| C306S                  | 1.3, 2.1         |
| K319 STLEPVE(...)ALR   | 2.1              |
| K451 AMTK(...)DNLLGR   | 2.2              |
| K500 ANK(...)TITNDK    | 1.7, 1.6, 1.2    |

* Indicates modified residue. (+) Indicates oxidized methionine present in some spectra. 3Cysteine residues identified as modified by other electrophilic compounds.14,30

are known targets of other thiol-reactive compounds. Previously, 4-HNE was found to modify C267 of human Hsp72 in vitro at concentrations of 10 and 100 μM after incubation overnight at 37 °C and that 4-HNE treatment reduced Hsp72 chaperone refolding activity. Given the extent of inhibition of chaperone refolding resulting from 4-HNE treatment (up to 75%), modification of C267 is very likely the site of the causal adduction.14 Oxidation of C306 in human Hsp72 by methylene blue has been reported, and oxidation of C267 is suspected. Mutation of both C306 and C267 to serine renders Hsp72 insensitive to methylene blue oxidation and reduction in adenosinetriphosphatase activity, suggesting that these residues are targets for other cysteine-reactive compounds, such as 4-HNE and parthenolide.30 Similarly, the yeast Hsp72 homolog, Ssa1, requires C267 and C306 for electrophilic activation of the heat shock response through covalent modification.13 From these previous results, we expected to observe at least modification at C267 and C306, both of which were detected after treatment with 100 μM parthenolide (Table 1), but neither with the 10 μM treatment.

We also report here eight previously unidentified modifications of Hsp72 at one histidine and seven lysines at both 10 and 100 μM treatments (Table 1). These modifications were verified as described above. Modification of lysine and histidine residues by electrophilic compounds containing α,β-unsaturated carbonyls have been reported in the literature.4,31,32 Although parthenolide modification of such residues on proteins under aqueous conditions has not been reported previously, the reaction of primary and secondary amines with parthenolide in methanol is a synthetic route for the development of new chemotherapeutic drugs.2,33 Furthermore, electrophilic modification of histidine by electrophiles 4-HNE and biliatresone (an α,β-unsaturated ketone) suggests that modification is possible with a secondary amine as nucleophile, such as an imidazole group.52,34,35 Hsp72 has 50 lysine residues, 7 of which we found to be modified. To try to understand why those seven were targeted as well as the other cysteines and histidine, we used the PDB2PQR server to run a PROPKA3 calculation to find pK_a and surface exposure for all of the ionizable amino acids in Hsp72.36,37 We used two PDB files to calculate the pK_a’s for Hsp72 because a single crystal structure...
potentially representing previously identified protein targets of parthenolide adduction as well as potential new targets. Adducted and biotinylated proteins from treated cell lysates were isolated using neutravidin affinity purification at various treatment concentrations (Figure 2B). The efficacy of this method at isolating different amounts of modified proteins is shown in Figure 2B, with a concentration-dependent increase in the quantity of biotinylated proteins from cells treated with 50, 100, and 200 μM alkyne–parthenolide. Proteins within THP-1 cells were adducted by 2 despite the presence of 1.0 mM reduced glutathione in the RPMI medium.

Recombinant Hsp72 Is Adducted in Vitro by 2. To test that our derivative 2 would covalently modify Hsp72 in a similar manner to parthenolide, purified recombinant protein was treated in vitro with 100 μM 2 for 1 h and subjected to CuAAC with an azide tag to biotinylate the modification sites. Modification and biotinylation of Hsp72 are observed in Figure 3A through recognition of the biotinylation with an antibiotic–horseradish peroxidase (HRP) antibody, whereas lanes containing incomplete CuAAC reactions or treatment with unmodified parthenolide present no signal. The presence of Hsp72 protein in each lane is represented by the anti-His6 lower blot, with signal shown in each lane. The upper band (>70 kDa) observed in the full reaction lane is likely due to the dimerization of modified Hsp72. This upper band suggested that parthenolide may cross-link two Hsp72 monomers together, however, we saw no evidence of cross-linking by parthenolide in model thiol reactions with 3-nitrobenzyl mercaptan detected with LC–MS (R. Connor, unpublished observations).

Adducted Hsp70 Proteins Are Isolated from Cells Treated with 2. Given that 2 modifies Hsp72 in vitro, we expected to find Hsp72 isolated from 2-treated cells. As shown in Figure 3B, Hsp70 proteins are present in the enriched fraction of adducted proteins from THP-1 cells treated with 100 μM 2 for 1 h. Enriched proteins from cells treated with parthenolide or DMSO (Figure 3B, lanes 1, 2, and 6) do not contain Hsp70 as indicated by detection with an Hsp70 antibody. Conversely, the positive control (Figure 3B, lane 3) and the two enriched samples from 2-treated cells do display Hsp70 signals. The enriched proteins were first eluted from the resin competitively with 3 mM D-biotin and then finally by heating the resin in loading buffer. Both methods show enrichment of Hsp70 proteins in the eluted samples, indicating that adduction occurs intracellularly by αβ-unsaturated carbonyl compounds, such as parthenolide and 2.

Biotinylated parthenolide derivatives have previously been used to identify protein targets; however, the addition of a biotin moiety is a substantial modification to the molecule. Since the first report on parthenolide–biotin in 2001, the CuAAC reaction has emerged as a very facile and efficient reaction for bio-orthogonal chemistry under aqueous conditions. The relatively small addition of functional groups, such as an alkyne or azide, to a target molecule represents a smaller change to a molecule versus the larger biotin functionality label and affinities of Hsp70 target proteins. The broad distribution of adduction sites across the Hsp70 protein illustrates the reactivity of parthenolide, an electrophilic natural product and a potential chemotherapeutic compound.

Figure 2. Proteins in both cell lysates (A) and cultured cells (B) are modified by parthenolide derivative 2. (A) Modification of proteins in whole cell lysates from THP-1 cells. Each lane contains 15 μg of protein as follows: (1) THP-1 cell lysate treated with 2 and labeled with CuAAC. (2) THP-1 cell lysate treated with 2, but lacking the azide–biotin in the CuAAC reaction. (3) THP-1 lysate treated with DMSO and labeled with CuAAC. (4) Biotinylated superoxide dismutase as positive control. (B) Recovery of modified proteins from cells treated with increasing concentrations of 2. THP-1 cells were treated at confluence in RPMI medium with parthenolide derivative 2 for 1 h, lysed, protein concentration normalized to 3 mg/mL, and subjected to CuAAC. After the CuAAC treatment, modified proteins were isolated with neutravidin resin.

Figure 3. Hsp70 is modified by parthenolide and related electrophiles both in vitro and in THP-1 cells. (A) Recombinant Hsp72 is modified in vitro after treatment with 100 μM 2, as shown by its biotinylation signal in lane 1. All lanes contain 7.5 μg of protein. Lanes 2 and 3 are Hsp72 protein incubated with 2, but (2) without the azide tag in the labeling reaction and (3) without the ligand and catalyst. Lane 4 is the full CuAAC reaction with parthenolide. Each of the four lanes contains Hsp70 protein, as shown with anti-His-tag antibody detection. (B) Modification of Hsp70 proteins in THP-1 cells is exhibited by neutravidin capture from cells treated with 100 μM 1, 2, and DMSO for 1 h. Each cell lysate was normalized to 4 mg/mL total protein prior to CuAAC. Lanes 1 and 4 show elution from the neutravidin beads with 3 mM D-biotin; thus, lane 4 contains Hsp70 proteins from adduction by 2. Lanes 2, 5, and 6 were eluted by boiling in Laemmli sample buffer and only lane 5 contains Hsp70 proteins from adduction by 2. Lane 3 contains 15 μg of protein from whole cell lysate of treated THP-1 cells as positive control for detection of Hsp72.

■ CONCLUSIONS

Using high-resolution mass spectrometry and bio-orthogonal chemistry, we have shown that Hsp72 is modified both in vitro and in cultured cells by parthenolide and derivative 2. We identified 10 sites of in vitro modification of Hsp72 by parthenolide: 2 previously reported targets (C267 and C306) and 8 new adduction sites on lysine and histidine residues. For verification of modification of Hsp72 in cells, we synthesized a versatile alkyne-bearing derivative of parthenolide that exhibits similar effects on THP-1 leukemia cells as parthenolide. Derivative 2 modifies Hsp72, both in vitro and in cell culture, as shown using CuAAC addition of a biotin affinity label and affinity purification of modified cellular proteins. The broad distribution of adduction sites across the Hsp72 protein illustrates the reactivity of parthenolide, an electrophilic natural product and a potential chemotherapeutic compound.
Reagents. All chemicals were purchased from Sigma-Aldrich and used as received, unless otherwise stated. (+)-Parthenolide was purchased from Selleckchem (Houston, TX). Mouse antibody–HRP antibody was purchased from Invitrogen (La Jolla, CA) and antirabbit secondary antibody was purchased from Rockland Immunochemicals (Limerick, PA). Both the Hsp70 (H-300) primary antibody and His-probe (H-3) HRP conjugate were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Isopropyl β-D-1-thiogalacto-pyranoside (IPTG) was purchased from Gold Biotechnology (St. Louis, MO). Tris-benzyltriazolylamine (TBT-A) and azide–biotin were synthesized as described previously.

Production of Recombinant Human Hsp72. Human gene Hsp70A1A was obtained from Dr. Richard Morimoto within plasmid pET-wt-Hsp70.35 This gene was amplified using the following DNA primers: pQE80Hsp70F, TACTCTACGT CATGCATGGCCAAAGCCGCG and pQE-Hsp70R, GGTTCCGAAGCTTCTAATCTACCTC. After amplification, the Hsp70 gene was ligated into the vector pQE80 to create pQEHsp70-His6. pQEHsp70-His6 was transformed into BL21 cells for expression in lysogeny broth medium. After cultivation, the Hsp70 gene was ligated into the vector pQE80 to create pQEHsp70R, GGTTCCGAAGCTTCTAATCTACCTC. After amplification, the Hsp70 gene was ligated into the vector pQE80 to create pQEHsp70-His6. pQEHsp70-His6 was transformed into BL21 cells for expression in lysogeny broth medium. After expression following IPTG induction for 4 h at 37 °C, the Hsp72 protein was purified by native nickel affinity chromatography (His-Pur Ni-NTA resin; Thermo Fisher) in filters (MilliporeSigma, Darmstadt, Germany).

Identification of Hsp72 Modification Sites by Parthenolide. Purified recombinant Hsp72 produced and purified as described previously was treated with DMSO (vehicle control), 10 and 100 μM parthenolide individually in 50 mM phosphate buffer at pH 7.4 overnight at 37 °C. This treatment was repeated twice so that each condition was analyzed in triplicate. After the overnight incubation, 6X sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer containing 50 mM dithiothreitol (DTT) was added to the samples prior to heating at 95 °C for 5 min. The adducted proteins were separated on a 10% Tris-glycine polyacrylamide gel, and the resulting bands were excised for analysis. The gel slices were sent to MS Bioworks for digestion and analysis using the following methods: The in-gel digestion was performed by a ProGest robot (Digilab) and consisted of an initial wash with 50 mM amionic bicarbonate followed by an acetonitrile wash. Subsequently, the proteins were reduced with 10 mM DTT at 60 °C and then alkylated with 50 mM iodoacetamide at room temperature. Tryptsin digestion was performed for 4 h at 37 °C and then quenched with formic acid for direct analysis by high-resolution LC−MS/MS on either a Fusion Lumos or a Q Exactive HF spectrometer equipped with a Waters nanoACQUITY high-performance liquid chromatography system (Thermo Fisher Scientific, Bremen, Germany). One set of three samples was analyzed with a Fusion Lumos, and the two other sets were analyzed with a Q Exactive for a total of nine samples. The peptides were identified using the MASCOT database, and parthenolide modifications were found by searching for dynamic modification with parthenolide (full adduct: +248.14 Da or adduct with loss of hydroxyl: +231.137 Da) at cysteine, lysine, and histidine residues. Other dynamic modifications in the search were methionine oxidation, N-terminal acetylation, deamidation, Pyro-Glu, and cysteine alkylation. The peptide mass tolerance was set at 10 ppm with a fragment tolerance of 0.01 Da. The resulting DAT files were parsed and filtered with the Scaffold software with 1% protein and peptide false discovery rate such that redundant proteins were removed. Scaffold PTM was used to assign site localization probabilities and generate an A-score for identified modifications. Parthenolide modifications were not detected in any of the three DMSO-treated control samples.

Synthesis of Parthenolide Derivative 2. (+)-Partheno-lide was oxidized according to the previously reported methods to obtain melampomagnolide B.7,38 Melampomagnolide B was esterified by the Steglich esterification with slight modification.39 Specifically, melampomagnolide B (43.9 mg, 0.171 mmol) was dissolved in 5 mL of dehydrated CH2Cl2. To this solution, 4-dimethylaminopropionic acid (34.82 mg, 0.285 mmol) and 5-hexynoic acid (31.4 μL, 0.285 mmol) were added. After the addition, the reaction was cooled in an ice bath and stirred for 5 min. N,N′-Dicyclohexylcarbodimide (64.37 mg, 0.312 mmol) was added and stirred for 10 min, after which the reaction vessel was purged with nitrogen. The reaction was removed from the ice bath and allowed to slowly warm to room temperature with stirring for at least 3 h and up to 24 h until the reaction was judged complete by thin-layer chromatography. Upon completion, the reaction was filtered to remove insoluble material, and the filtrate was washed with saturated sodium bicarbonate to remove excess hexynoic acid. The organic layer was washed twice with deionized water and purified using flash chromatography (50% ethyl acetate/hexane) and isolated as a white solid in 63% yield: 1H NMR (300 MHz, CDCl3) δ: 6.3 (d, H-13a), 5.72 (t, H-1), 5.6 (d, H-13β), 4.6 (q, H-14), 3.9 (t, H-6), 2.9 (d, H-5), 2.85–2.99 (m, H-7), 2.5 (t, H-17), 2.37–2.56 (H-9β, H-2β), 2.3 (td, H-18), 2.14–2.28 (H-2α, H-3β, H-8α, H-9α), 2.03 (t, H-21), 1.89 (p, H-19), 1.65–1.74 (m, H-8β), 1.58 (s, H-15), 1.13 (t, H-3α).

Cell Culture. THP-1, human monocytes leukemia cells, were obtained from the American Tissue Culture Consortium and cultured in RPMI medium buffered with 10 mM N-(2-hydroxyethyl)piperazine-N’-ethanesulfonic acid (pH 7.4), containing minimum essential medium vitamins and 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) at 37 °C in a 5% CO2 atmosphere. Treatment of THP-1 cells was performed at confluence (1 x 106 cells/mL) in RPMI medium with 10% fetal bovine serum, as noted for each experiment.

Viability Assays. Water-soluble tetrazolium salt 1 (WST-1) was obtained from Roche and used as received. THP-1 cells were plated as monocytes at a density of 30,000 cells per well.47 Drug treatments were applied as 1000X dilutions in RPMI medium, resulting in a total DMSO content of 0.1% for each well. The treated cells were incubated for 24 h at 37 °C under 5% CO2 atmosphere. To each well, 10 μL of WST-1 reagent was added, and after incubation for approximately 45 min, absorbance at 440 nm with a reference wavelength of 600 nm was measured using a SpectraMax M3 plate reader (Molecular Devices, Sunnyvale, CA). The percent viability for each drug concentration was calculated as the treated WST response per DMSO vehicle control response. The IC50 was then determined by fitting percent viability with a nonlinear regression for a normalized response using GraphPad Prism (La Jolla, CA).

Immunoblotting. All gels were run using the Bio-Rad MINI-Protean system with Tris–glycine buffer. Proteins were transferred to nitrocellulose membranes using the Trans-Blot.
Turbo (Bio-Rad) semidyry system. Membranes were blocked with 3% milk in 1X Tris-buffered saline (TBS, pH 7.4), with 0.1% Tween-20 prior to incubation with antibodies. All antibodies, including secondary antibodies, were used at dilutions of 1:1000 in 3% milk in TBS-T. The SuperSignal West Pico luminescent reagent from Thermo Pierce (Waltham, MA) was used for detection of horseradish peroxidase signal on the membranes. Blots were imaged using a ProteinSimple FluorChem digital imager (San Jose, CA).

In Vitro Modification and CuAAC Biotinylation of Recombinant Hsp72 with 2. Recombinant Hsp72 protein was incubated in 50 mM phosphate buffer (50 μL) in the presence of compound 2 (see Results and Discussion for concentrations of 2 used). After incubation for 1 h, the reagents for CuAAC were added to final concentrations of 10 mM CuSO4, 1 mM tris(2-carboxyethyl)phosphine (TCEP), and 1 mM TBTA. The volume of the reaction was increased to 100 μL with phosphate-buffered saline (PBS, pH 7.4) and allowed to react overnight at 4 °C prior to separation and analysis by SDS-PAGE and immunoblotting.

Treatment of Cell Lysates with Alkyne–Parthenolide. Confluent THP-1 cells were washed twice with 1X PBS (pH 7.4) and then lysed in 1X PBS (pH 7.4) with 0.1% NP-40 and mammalian protease inhibitors (MilliporeSigma, St. Louis, MO), using a sonicator set to 30% amplitude with six 10 s pulses. After sonication, the lysates were clarified by centrifugation at 10 000 g for 15 min. Protein concentration was determined using the Bio-Rad Protein Assay. The cell lysates were diluted to 2 mg/mL, and 28 μL was determined using the Bio-Rad Protein Assay. The cell lysates were diluted to 2 mg/mL, and 28 μL was added to a concentration of 100 μM or 0.1% (DMSO). After incubation for 1 h, the reagents for CuAAC were added to final concentrations of 10 mM CuSO4, 1 mM tris(2-carboxyethyl)phosphine (TCEP), and 1 mM TBTA. The reaction was incubated at 4 °C for at least 1 h. Following incubation, the proteins were precipitated with 4 volumes of methanol and 1 volume of chloroform. This precipitate was pelleted by centrifugation at 13 000 g for 15 min. The supernatant was retained, and the pellet was treated with 100 μL of 10% SDS for resuspension of any remaining precipitated proteins. The SDS was diluted to 1% with 1X PBS (pH 7.4), and the solution was centrifuged again at 10 000 g for 10 min. The two supernatants from each step were combined and diluted to 10 mL with 1X PBS (pH 7.4). High-capacity neutravidin resin (Thermo Fisher) was added to each sample and incubated rotating overnight at 4 °C. After incubation, the resin was washed once with 1X PBS, twice with 6 M urea, twice with 1% SDS, and twice with 1X PBS (pH 7.4). Proteins bound to the resin were eluted either by incubation in SDS loading buffer at 95 °C for 5 min or competitively with 3 mM biotin in 1X PBS.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00954.

Representative MS2 spectra for all of the detected adducts and their corresponding precursor ion MS1 spectrum; peptide coverage maps for Hsp72 from all three sets of samples; table of calculated pKα values for cysteines, histidines, and lysines on Hsp72 (PDF)

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M.S., R.E.C., G.J.D., and A.M. designed and performed experiments; T.C. and T.C. performed experiments; M.S., A.M., and R.E.C. analyzed data; and R.E.C. wrote the manuscript.

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ABBREVIATIONS
Hsp72, heat shock protein 72; THP-1, acute monocytic leukemia cells; 4-HNE, 4-hydroxynonenal; CuAAC, copper-catalyzed azide–alkyne cycloaddition; TBTa, tris-benzyltriazolylamine; TCEP, tris(2-carboxyethyl)phosphine; PBS, phosphate-buffered saline; WST-1, 4(3-(4-iodophenyl)-2(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate
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