The Quinone Methide Aurin Is a Heat Shock Response Inducer That Causes Proteotoxic Stress and Noxa-dependent Apoptosis in Malignant Melanoma Cells*

Received for publication, June 29, 2014, and in revised form, November 13, 2014. Published, JBC Papers in Press, December 4, 2014, DOI 10.1074/jbc.M114.592626

Angela L. Davis, Shuxi Qiao, Jessica L. Lesson, Montserrat Rojo de la Vega, Sophia L. Park, Carol M. Seanez, Vijay Gokhale, Christopher M. Cabello, and Georg T. Wondrak

From the Department of Pharmacology and Toxicology, College of Pharmacy and Arizona Cancer Center, University of Arizona, Tucson, Arizona 85724

Pharmacological induction of proteotoxic stress is rapidly emerging as a promising strategy for cancer cell-directed chemotherapeutic intervention. Here, we describe the identification of a novel drug-like heat shock response inducer for the therapeutic induction of proteotoxic stress targeting malignant human melanoma cells. Screening a focused library of compounds containing redox-directed electrophilic pharmacophores employing the Stress & Toxicity PathwayFinder™ PCR Array technology as a discovery tool, a drug-like triphenylmethane-derivative (aurin; 4-[bis(7-oxyl)-hydroxyphenyl)methylene]-2,5-cyclohexadien-1-one) was identified as an experimental cell stress modulator that causes (i) heat shock factor transcriptional activation, (ii) up-regulation of heat shock response gene expression (HSPA6, HSPA1A, DNAJB4, HMOX1), (iii) early unfolded protein response signaling (phospho-PERK, phospho-eIF2α, CHOP (CCAAT/enhancer-binding protein homologous protein)), (iv) proteasome impairment with increased protein-ubiquitination, and (v) oxidative stress with glutathione depletion. Fluorescence polarization-based experiments revealed that aurin displays activity as a geldanamycin-competitive Hsp90α-antagonist, a finding further substantiated by molecular docking and ATPase inhibition analysis. Aurin exposure caused caspase-dependent cell death in a panel of human malignant melanoma cells (A375, G361, LOX-IMVI) but not in nonmalignant human skin cells (HaCaT keratinocytes, primary melanocytes) undergoing the aurin-induced heat shock response without impairment of viability. Aurin-induced melanoma cell apoptosis depends on Noxa up-regulation as confirmed by siRNA rescue experiments demonstrating that siPMAIP1-based target down-regulation suppresses aurin-induced cell death. Taken together, our data suggest feasibility of apoptotic elimination of malignant melanoma cells using the quinone methide-derived heat shock response inducer aurin.

Proteotoxic stress (i.e. deviations from proteostasis) occurs in response to cytotoxic stimuli that cause accumulation of unfolded and/or misfolded proteins including heat shock, oxidative stress, calcium dysregulation, and inhibition of proteasomal or autophagic-lysosomal function (1–5). It is now widely accepted that tumor cells are exposed to high levels of endogenous proteotoxic stress originating from mutation-driven expression of misfolded proteins, aneuploidy, and adverse conditions associated with the tumor microenvironment including hypoxia, energy crisis, and redox dysregulation (6). In contrast, it is thought that normal cells display lower constitutive levels of endogenous proteotoxic stress together with a diminished dependence on proteostasis-ensuring mechanisms including proteasomal and heat shock protein chaperone functions. Therefore, constitutively elevated levels of proteotoxic stress may represent a specific molecular vulnerability of malignant cells amenable to chemotherapeutic intervention (3–13).

Melanoma, a malignant tumor derived from melanocytes, causes the majority of deaths attributed to skin cancer. Despite recent progress in the design of melanoma-targeted therapies such as the V600E mutation-directed BRAF-inhibitor vemurafenib (14–18), efficacy of chemotherapeutic intervention directed against the metastatic stage of the disease remains limited, creating an urgent need for the identification and development of improved antimelanoma agents (19, 20). Dysregulation of proteotoxic stress has been observed in human melanoma tissue contributing to the notorious chemoresistance of metastatic melanoma cells. Cumulative evidence suggests the involvement of autophagic dysregulation in melanogenesis and the emerging role of autophagy as a prognostic factor and therapeutic target in melanoma has been substantiated recently (21–26). Moreover, pathological altera-

* This work was supported, in whole or in part, by National Institutes of Health Grants R01CA122484, R03CA167580, R21CA166926, ES007091, ES006694, and Arizona Cancer Center Support Grant CA023074. Preliminary data from this research were part of an oral presentation at the 19th Annual Meeting of the Society for Free Radical Biology and Medicine, November 17, 2012, San Diego, CA.

1 To whom correspondence should be addressed: University of Arizona, College of Pharmacy and Arizona Cancer Center, 1515 North Campbell Ave., Tucson, AZ 85724. Tel.: 520-626-9009; E-mail: wondrak@pharmacy.arizona.edu.

VOLUME 290 • NUMBER 3
JANUARY 16, 2015
1623
© 2015 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
Aurin Causes Lethal Proteotoxic Stress in Melanoma Cells

Effects of aurin on the expression of heat shock proteins (including Hsp27, Hsp70, Hsp90, and GRP78) have been documented in human melanoma tissue (27–33). Specifically, Hsp90 serves as an essential factor stabilizing oncogenic BRAF in melanoma cells, and its inhibition has emerged as a promising strategy for antimelanoma intervention (34–37). Therefore, strategies that aim at increasing proteotoxic stress through pharmacological modulation of proteosomal, autophagic-lysosomal, or heat shock response functions are now pursued for experimental and investigational chemotherapeutic intervention targeting malignant melanoma (27–33, 37–40).

In an effort to identify novel drug-like molecules that might target malignant melanoma cells through the induction of proteotoxic and/or oxidative stress, we recently screened a focused library of compounds containing redox-directed electrophilic pharmacophores using the Stress & Toxicity PathwayFinder™ PCR Array technology (12, 39–44). Here, we report for the first time that in a series of drug-like phenolic triphenylmethane derivatives, the quinone methide aurin (CAS #143-74-8; 2-[bis(p-hydroxyphenyl)methylene]-2,5-cyclohexadien-1-one; rosalic acid; see Fig. 1A) was identified as a potent inducer of lethal proteotoxic stress in human A375 and other malignant melanoma cells. Further analysis revealed that aurin displays inhibitory activity targeting Hsp90α, causing early heat shock factor (HSF) transcriptional activation, up-regulation of heat shock response gene expression, and unfolded protein response (UPR) signaling followed by induction of Noxa-dependent apoptotic cell death.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals were purchased from Sigma.

**Cell Culture**—Malignant human melanoma (A375) and human metastatic melanoma (LOX-IMVI, G-361) cells from ATCC (Manassas, VA) were cultured in RPMI medium (10% FBS and 2 mM l-glutamine) or McCoy's 5a medium (10% FBS), respectively. Dermal neonatal foreskin Hs27 fibroblasts from ATCC and human immortalized HaCaT keratinocytes were cultured in DMEM containing 10% fetal bovine serum. Primary dermal foreskin Hs27 fibroblasts from ATCC (Manassas, VA) were cultured in RPMI medium (10% FBS) or McCoy's 5a medium (10% FBS), respectively. Dermal neonatal foreskin Hs27 fibroblasts from ATCC and human immortalized HaCaT keratinocytes were cultured in DMEM containing 10% fetal bovine serum. Primary human epidermal melanocytes (adult skin, lightly pigmented; HEMa-LP was from Invitrogen) were cultured using Medium 154 supplemented with HMGS-2 growth supplement. HEMa-LP cells were passaged using recombinant trypsin/EDTA and defined trypsin inhibitor. Cells were maintained at 37 °C in 5% CO₂, 95% air in a humidified incubator.

**Human Stress and Toxicity Pathfinder™ RT² Profiler PCR Expression Array**—After pharmacological exposure, total cellular RNA (3 × 10⁶ cells) was prepared according to a standard procedure using the RNeasy kit (Qiagen, Valencia, CA).

Reverse transcription was performed using the RT² First Strand kit (Superarray, Frederick, MD) and 5 μg of total RNA as described previously (40–42). The RT² Human Stress and Toxicity Pathfinder® PCR Expression Array (SuperArray) profiling the expression of 84 stress-related genes was run using the following PCR conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s alternating with 60 °C for 1 min (Applied Biosystems 7000 SDS). Gene-specific product was normalized to ACTB and quantified using the comparative (ΔΔCt) Ct method as described in the ABI Prism 7000 sequence detection system user guide as published earlier (42, 45). Expression values were averaged across three independent array experiments, and standard deviation was calculated for graphing.

**DDIT3, HSPA1A, HSPA6, HMOX1, and PMAIP1 Expression Analysis by Real Time RT-PCR**—For expression analysis by real time RT-PCR, total cellular RNA (3 × 10⁶ cells) was prepared using the RNeasy kit from Qiagen. Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Roche Molecular Biochemicals) and 200 ng of total RNA in a 50-μl reaction. Reverse transcription was primed with random hexamers and incubated at 25 °C for 10 min followed by 48 °C for 30 min, 95 °C for 5 min, and a chill at 4 °C. Each PCR reaction consisted of 3.75 μl of cDNA added to 12.5 μl of TaqMan Universal PCR Master Mix (Roche Molecular Biochemicals), 1.25 μl of gene-specific primer/probe mix (Assays-by-Design; Applied Biosystems, Foster City, CA), and 7.5 μl of PCR water. PCR conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, alternating with 60 °C for 1 min using an Applied Biosystems 7000 SDS and Applied Biosystems Assays On Demand primers specific to DDIT3 (assay ID Hs00358796_g1), HSPA6 (assay ID Hs00275682_s1), HSPA1A (assay ID Hs00359163_s1), HMOX1 (assay ID Hs00157965_m1), PMAIP1 (assay ID Hs00560402_m1), and ACTB (β-actin, assay ID Hs99999903_m1). Gene-specific product was normalized to ACTB and quantified using the comparative (ΔΔCt) Ct method as described before (42, 45).

**siRNA Transfection Targeting PMAIP1 Expression**—A375 cells were transiently transfected with a 100-nmol pool of four small interfering RNA (siRNA) oligonucleotides (oligos) targeting PMAIP1 or a 100-nmol pool of four non-targeting siRNA oligos using the DharmaFECT 1 transfection reagent (Dharmacon RNA Technologies, Lafayette, CO) following a standard procedure as published recently (13, 44). The sequences of siGENOME PMAIP1 SMARTpool (PMAIP1 siRNA; GenBank™ NM021127) were AACUGAACCUCGGCCAGA, AUUCUGAUUCCACCU, CUGGAAGCAGUGUCU, and GCAAGAACCUCGACGAG. Cells were either harvested for confirmation of PMAIP1 knockdown by Noxa immunoblot analysis or exposed to aurin followed by viability assessment using flow cytometric analysis of annexin V (AV)-FITC/propidium iodide (PI)-stained cells.

**Immunoblot Analysis**—Sample preparation, SDS-PAGE, transfer to nitrocellulose, and development occurred as described earlier (40, 45). Gel percentage was 12%. Antibodies were purchased from the following manufacturers: Cell Signaling Technology (Danvers, MA); anti-HSF1 rabbit polyclonal (4356), anti-phospho-PERK (Thr980; 16F8) rabbit monoclonal, anti-PERK (C33E10) rabbit monoclonal, anti-phospho-eIF2α (Ser51; 9721) rabbit polyclonal, anti-eIF2α (total; 9722) rabbit poly-

---

2 The abbreviations used are: Hsp, heat shock protein; HSF1, heat shock factor protein 1; HSE, heat shock element; A, aurin; ATa, aurin-tricarboxylic acid; AV, annexin V; CHOP, CCAAT/enhancer-binding protein homologous protein; DDIT3, DNA-damage-inducible transcript 3; Glide, grid-based ligand docking with energetics; HSPA6, heat shock 70-kDa protein 1A; HSPA6, heat shock 70-kDa protein 6; HMOX1, heme oxygenase-1; PI, propidium iodide; UPR, unfolded protein response; AV, annexin V; zVADfmk, benzyl-oxycarbonyl-VAD-fluoromethyl ketone; AhR, aryl hydrocarbon receptor; ER, endoplasmic reticulum; PERK, protein kinase R-like endoplasmic reticulum kinase.
Aurin Causes Lethal Proteotoxic Stress in Melanoma Cells

clonal, anti-CHOP (L63F7) mouse monoclonal; Enzo Life Sciences (Farmingdale, NY): anti-Hsp70 mouse monoclonal (C92F3A-5, SPA-810; this antibody recognizes inducible Hsp70, also called Hsp72 or HspA1A, but does not react with constitutively expressed Hsc70), anti-Hsp60 mouse monoclonal (Mab11–13; SPA-829), anti-Hsp90 rabbit polyclonal (SPA-836), anti-Hsp27 rabbit polyclonal (SPA-803); Santa Cruz Biotechnology Inc. (Santa Cruz, CA): anti-ubiquitin (P4D1) mouse monoclonal; EMD Chemicals (Gibbstown, NJ): anti-Noxa (OP180) mouse monoclonal. The following secondary antibodies were used: HRP-conjugated goat anti-rabbit antibody or HRP-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Equal protein loading was examined by β-actin-detection using a mouse anti-actin monoclonal antibody (Sigma). For nuclear extraction (HSF1), the NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Waltham, MA) was used.

Heat Shock Element (HSE)-luciferase Reporter Assay—Heat shock factor-responsive TransLucent™ reporter vector, pHSE-Luc, and empty TransLucent control reporter vector were purchased from Panomics (Fremont, CA). pHSE-Luc contains multiple copies of the heat shock factor binding cis-acting promoter element (HSE; 5′-CGTGAATTTCTAGTGGAGATTTCTAGACTGGAAATTTTCTAGA-3′) upstream of the herpes simplex virus thymidine kinase TATA box promoter driving luciferase gene expression. A375 human melanoma cells were grown to 60% confluency in a 24-well plate. For transduction, 0.4 μg of HSF-responsive TransLucent reporter vector, 0.8 ng of promoter-less Renilla reporter vector (phRG-B; Promega, Madison, WI), 1.32 μl of FuGENE 6 (Roche Molecular Biochemicals), and 20.68 μl of Opti-MEM I (Invitrogen) were added per well. Twenty-four hours after transfection cells were exposed to test the compounds for 8 h. Cells were then lysed in 100 μl of passive lysis buffer using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Luciferase activity was measured with a Turner Designs TD20/20 luminometer. Data are reported as normalized averages of the firefly/Renilla luciferase ratio.

Cell Death Analysis—Viability and induction of cell death (early and late apoptosis/necrosis) were examined by AV-FITC/PI dual staining of cells followed by flow cytometric analysis as published previously (40). Cells (100,000) were seeded on 35-mm dishes and received drug treatment 24 h later. Cells were harvested at various time points after treatment, and cell staining was performed using an apoptosis detection kit according to the manufacturer’s specifications (APO-AF, Sigma).

Flow Cytometric Detection of Cleaved Procaspase-3—Treatment-induced proteolytic caspase-3 activation was examined using an antibody directed against cleaved/activated caspase-3 (Asp-175) (Alexa Fluor 488 conjugate; Cell Signaling) following by flow cytometric analysis as published (45, 46).

Proteasome Activity Assay—The Proteasome-Glo™ Cell-Based Assay kit (Promega) was used according to manufacturer’s specifications. Cells were seeded at 4000 cells/well of an opaque 96-well plate. After 24 h cells were treated with test compound. At the end of the treatment period, cells were washed with PBS and an equal volume of reagent containing the appropriate luminogenic substrate (Suc-LLVY-aminoluciferin for chymotrypsin-like activity) was added to each well. Plates were incubated at room temperature (10 min) before measuring luminescence signal using a Synergy 2 microplate reader. Data are expressed as % activity relative to untreated control cells (means ± S.D. (n = 3)). Likewise, inhibition of 20 S chymotrypsin-like proteasomal activity was examined using the isolated human erythrocyte 20 S proteasome obtained from a commercial kit (EMD Millipore).

Detection of Intracellular Oxidative Stress by Flow Cytometric Analysis—Induction of intracellular oxidative stress by test compound was analyzed by flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) as a sensitive non-fluorescent precursor dye according to a published standard procedure (46).

Determination of Reduced Cellular Glutathione Content—Intracellular reduced glutathione was measured using the GSH-Glo glutathione assay kit (Promega, San Luis Obispo, CA) as described recently (41). Cells were seeded at 100,000 cells/dish on 35-mm dishes. After 24 h, cells were treated with test compound. At selected time points after the addition of test compound, cells were harvested by trypsinization and then counted using a Coulter counter. Cells were washed in PBS, and 10,000 cells/well (50 μl) were transferred onto a 96-well plate. GSH-Glo reagent (50 μl) containing luciferin-NT and glutathione S-transferase was then added followed by 30 min of incubation. After the addition of luciferin detection reagent to each well (100 μl) and 15 min of incubation, luminescence reading was performed using a BioTek Synergy 2 Reader (BioTek, Winooski, VT). Data are normalized to GSH content in untreated cells and expressed as the means ± S.D. (n = 3).

Mitochondrial Transmembrane Potential—Mitochondrial transmembrane potential (ΔΨm) was assessed using the potentiometric dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; Sigma, T4069) following our published procedure (41, 44). In brief, cells were trypsinized, washed in PBS, resuspended in 300 μl of PBS containing 5 μg/ml JC-1 for 15 min at 37 °C and 5% CO2 in the dark, then washed twice in PBS and resuspended in 300 μl of PBS. Bivariate analysis was performed by flow cytometry with excitation at 488 nm, and mitochondrial function was assessed as JC-1 green (depolarized mitochondria, detector FL-1) or red (polarized mitochondria, detector FL-2) fluorescence.

Fluorescence Polarization Assay for Hsp90α-directed Geldanamycin Competitive Binding—Hsp90-directed inhibitory activity was assessed employing fluorescence polarization measuring competitive binding of fluorescently labeled geldanamycin (FITC-geldanamycin) to recombinant Hsp90α as a function of test compound concentration following the commercial BPS HSP90α assay kit procedure (BPS Bioscience, San Diego, CA) (47, 48). In brief, reactions were conducted at room temperature for 3 h in a 100-μl mixture containing assay buffer, HSP90α (human NM_005348, recombinant, full-length, C-terminal His tag; 48 ng), FITC-geldanamycin conjugate (5 nM), and test compound (aurin or positive control (geldanamycin); concentration range 1 nM–10 μM; 1% DMSO final). Activity assays were performed in duplicate at each concentration monitoring fluorescence intensity (excitation/emission wavelengths, 470/528 nm; Tecan Infinite M1000 microplate reader).
Fluorescence intensity was then converted to fluorescence polarization using the Tecan Magellan software, and the fluorescence polarization data were analyzed using the computer software Graphpad Prism. The fluorescence polarization (FP) in the absence of the compound in each data set was defined as 10% activity. In the absence of protein and test compound, the value of fluorescence polarization (FP) in each data set was defined as 0% activity. The % activity in the presence of the test compound was calculated according to the equation,

\[
\% \text{ activity} = \frac{(FP - FP_b)/(FP_t - FP_b)}{FP_t - FP_b} \times 100%
\]  

where FP = fluorescence polarization in the presence of the compound.

For IC\text{50} measurements, the values of % activity versus a series of compound concentrations were then plotted using non-linear regression analysis of a sigmoidal dose-response curve generated with the equation,

\[
Y = B + \frac{(T - B)}{1 + 10^{(\log IC_{50} - X) \times \text{Hill Slope}}}
\]

where \(Y\) = % activity, \(B\) = minimum % activity, \(T\) = maximum % activity, \(X\) = logarithm of compound, and Hill Slope = slope factor or Hill coefficient. The IC\text{50} value was defined as the compound concentration causing half-maximal % activity.

**Hsp90 ATPase Activity Assay**—Inhibition of Hsp90 ATPase enzymatic activity by test compounds was assessed in 96-well plate format as described previously (58). This assay determines the amount of free phosphate generated due to enzymatic ATP hydrolysis and is based on the reaction of a phosphomolybdate complex with malachite green. Per reaction, human Hsp90 richia coli (ab48801; Abcam, Cambridge, MA) was incubated with test compound in a buffer containing 50 mM Hepes (pH 7.5), 6 mM MgCl\text{2}, 20 mM KCl, 1 mM ATP (50 \(\mu\)M total volume; 6 h, 25 °C). Using the malachite green phosphate assay kit from Cayman Chemical (Ann Arbor, MI), the reaction was terminated by the addition of 5 \(\mu\)l of malachite green acidic solution and 15 \(\mu\)l of MB blue solution to each well. After 25 min at room temperature, the absorbance at 620 nm was determined. Data are expressed as % ATPase activity relative to Hsp90α incubated with solvent control (means ± S.D. (n = 3)).

**Molecular Modeling**—Docking analysis was performed using Glide software (“grid-based ligand docking with energetics”; available from Schrödinger Inc.). X-ray crystal structure coordinates of geldanamycin with Hsp90α (Protein Data Bank code 1YET) were used for docking (49). Glide-generated ligand poses pass through a series of hierarchical filters that evaluate the ligand interaction with the target. Initial filters test the spatial fit between ligand and target examining complementarity of ligand-target interactions using a grid-based method patterned after the empirical ChemScore function. Final scoring is carried out on energy-minimized poses; Glide scores (G scores) approximating the ligand binding free energy were calculated for aurin and geldanamycin with the Hsp90α target protein (50).

**Statistical Analysis**—Unless indicated differently, the results are presented as the mean ± S.D. of at least three independent experiments, and data were analyzed employing one-way analysis of variance with Tukey’s post hoc test using the Prism 4.0 software. Differences were considered significant at \(p < 0.05\). Means without a common letter differ (\(p < 0.05\)).

**RESULTS**

Array Analysis Reveals Global Up-regulation of Heat Shock Response Gene Expression in Human A375 Malignant Melanoma Cells Exposed to Aurin—in an attempt to identify novel small molecules for the therapeutic modulation of oxidative and proteotoxic stress targeting human malignant melanoma cells, we recently employed a simple phenotypic screen examining modulation of HSPA1A gene expression in A375 malignant melanoma cells exposed to members of a collection of drug-like electrophiles (39–44). Inspired by structural features displayed by established small molecule heat shock response inducers including the benzoquinone ansamycin antibiotic geldanamycin (Gel) and the quinone-methide celastrol (Cel; Fig. 1A) (54–57, 58–63), we also examined the activity of synthetic triphenylmethane derivatives with phenolic and/or quinone-methide functional groups including aurin-tricarboxylic acid (ATA; 5,5’-(3-carboxy-4-oxocyclohexa-2,5-dien-1-yldiene)bis(2-hydroxybenzoic acid), CAS #4431-00-9; Fig. 1A), an experimental therapeutic tested previously for suppression of thrombocyte aggregation and cardioprotection (51–53). In addition, we examined heat shock modulatory activity of ATA-related triphenylmethane derivatives including aurin (A; 4-[bis(4-hydroxyphenyl)methylene]-2,5-cyclohexadienone, CAS #143-74-8), phenol red (phenolsulfonphthalein (PR), 143-74-8), and pararosaniline chloride (PRC, CAS #569-61-9). Chemical structures are depicted in Fig. 1A. We observed that aurin, a triphenylmethane dye with an electrophilic quinone methide group, displayed micromolar activity as an inducer of HSPA1A gene expression (10 \(\mu\)M, 24 h) in A375 cells, detectable both at the mRNA (~25-fold up-regulation) and protein levels (Fig. 1, B and C). In contrast, none of the other triphenylmethane-derived test compounds (ATA, phenol red (PR), pararosaniline chloride (PRC) caused HSPA1A up-regulation (Fig. 1, B and C), whereas pronounced up-regulation of HSPA1A was induced in response to either celastrol or geldanamycin, an observation consistent with the published literature (54–57, 59–63).

Next, in an attempt to further characterize the cellular stress response elicited by aurin treatment, we performed gene expression array analysis. To this end, aurin-induced modulation of stress and toxicity response gene expression was examined in A375 melanoma cells using the RT\(^2\) Profiler\textsuperscript{TM} PCR Expression Array technology (SuperArray, Frederick, MD; aurin, 10 \(\mu\)M, 6 h; scatter blot; Fig. 2A) as summarized in Fig. 2B (39–41, 43). Of 85 stress-related genes contained on the array, aurin-induced expression changes in A375 cells affected 10 genes by at least 3-fold over untreated control cells, 8 of which are known to be inducible in response to stimuli causing proteotoxic stress: HSP\text{A9a} (encoding the stress-responsive Hsp70 family member Hsp70\text{B}, −179-fold), HMOX1 (encoding heme oxygenase-1, −67-fold), HSPA1A (encoding the inducible heat shock 70-kDa protein 1A (Hsp70, also called Hsp72), −17-fold), DD\text{IT}3 (encoding the ER-stress response transcription factor DNA damage-inducible transcript 3/CHOP (GADD153), −7-fold), HSPA\text{IL} (encoding the Hsp70 family member heat shock
Aurin Causes Lethal Proteotoxic Stress in Melanoma Cells

To further elucidate the molecular mechanisms underlying aurin-induced heat shock response up-regulation, aurin-modulation of HSF transcriptional activity was assessed in A375 cells transfected with an HSE-luciferase reporter construct employing a dual luciferase reporter assay (Fig. 3D). Indeed, aurin exposure caused a pronounced (~9-fold) activation of firefly luciferase expression indicative of HSF1 transcriptional activation. The established heat shock response inducer celastrol was employed as a positive control (54–57). Immunoblot analysis (comparing cytosolic and nuclear fractions) indicated that aurin-induced HSF1 nuclear translocation occurs within 10 min exposure time (Fig. 3E).

Importantly, viability analysis using flow cytometric analysis of annexin V-PI-stained cells indicated that prolonged aurin-exposure (10 μM, 24 h) causes caspase-dependent cell death that was antagonized if aurin-exposure occurred in the presence of the pancaspase inhibitor zVADfmk (40 μM; Fig. 3H).

Aurin-induced Heat Shock Response Elicited in Non-Malignant Human Skin Cells (Hs27 Fibroblasts, HaCaT Keratinocytes, Primary Melanocytes) Occurs without Impairment of Viability—Next, the effects of aurin exposure on viability and HSPA1A expression were examined in a panel of cultured cells including malignant melanoma (A375, G361, LOX-IMVI) and nonmalignant skin cells (Hs27 fibroblasts, HaCaT keratinocytes, primary melanocytes). Employing flow cytometric analysis of annexin V-PI-stained cells, we observed that in malignant melanoma cells, exposure to aurin (24 h) caused pronounced induction of apoptotic cell death (LD_{50} ± S.D.); A375, 5.4 ± 3.4 (Figs. 3H and 7A); G361, 8.1 ± 1.8 (Fig. 4A); LOX-IMVI, 2.6 ± 1.4 (data not shown)). As observed in A375 melanoma cells (Fig. 4H), aurin-induced loss of cell viability was antagonized when G361 metastatic melanoma cells were cotreated with the pancaspase inhibitor zVADfmk (Fig. 5A). Moreover, as observed with A375 melanoma cells, aurin treatment (1–10 μM; 6 h) caused pronounced up-regulation of HSPA1A and HMOX1.
Aurin Causes Lethal Proteotoxic Stress in Melanoma Cells

![Graph](image)

**B**

| gene (accession number) | gene description | fold change | p-value |
|-------------------------|------------------|-------------|---------|
| aurin (10 µM; 6h)       |                  |             |         |
| HSPA6 (NM_002155)       | Heat shock 70Da protein 6 (HSP70B) | 178.49      | 0.0000  |
| HMOX1 (NM_002133)       | Heme oxygenase (decycling) 1 | 66.58       | 0.0000  |
| HSPA1A (NM_005345)      | Heat shock 70Da protein 1A | 17.27       | 0.0000  |
| GDF15 (NM_004864)       | Growth differentiation factor 15 | 8.48        | 0.0000  |
| PMAIP1 (NM_006039)      | Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) | 6.67 | 0.0011 |
| DDIT3 (NM_004063)       | DNA-damage-inducible transcript 3 | 6.55 | 0.0000 |
| HSPA1L (NM_005527)      | Heat shock 70Da protein 1-like | 6.13 | 0.0001 |
| DNAJB4 (NM_007034)      | DnaJ (Hsp40) homolog, subfamily B | 5.50 | 0.0000 |
| CRYAB (NM_011865)       | Crystallin, alpha B | 4.63 | 0.0000 |
| HSPB1 (NM_001540)       | Heat shock 27Da protein 1 | 3.03 | 0.0000 |
| HSPH1 (NM_006444)       | Heat shock 105Da/13Da protein 1 | 2.96 | 0.0000 |
| GADD45A (NM_001904)     | Growth arrest and DNA-damage-inducible, alpha | 2.81 | 0.0001 |
| ERP17 (NM_019644)       | Early growth response 1 | 2.63 | 0.0007 |
| SERPIN1ET (NM_000602)   | Serpin peptidase inhibitor, clade E | 2.42 | 0.0000 |
| CDK9 (NM_003386)        | Cyclin-dependent kinase inhibitor 1A (p21, Cip1) | 2.16 | 0.0000 |
| DNAJ1 (NM_001539)       | DnaJ (Hsp40) homolog, subfamily A, member 1 | 2.10 | 0.0000 |
| HSP90AA1 (NM_005348)    | Heat shock 90Da protein 1, alpha | 1.95 | 0.0026 |
| HSPA8 (NM_007034)       | Heat shock 70Da protein 8 | 1.60 | 0.0001 |
| HSP56AB1 (NM_007355)    | Heat shock 90Da protein 1, beta | 1.42 | 0.0000 |
| HSPF1 (NM_002157)       | Heat shock 10Da protein 1 (chaperonin 10) | 1.33 | 0.0002 |
| HSPA4 (NM_002154)       | Heat shock 70Da protein 4 | 1.32 | 0.0010 |
| HSPD1 (NM_002156)       | Heat shock 60Da protein 1 (chaperonin) | 1.15 | 0.0057 |
| HSPA5 (NM_005347)       | Heat shock 70Da protein 5 (BiP/Gp78) | -1.33 | 0.0028 |
| HSPA2 (NM_021979)       | Heat shock 70Da protein 2 | -1.52 | 0.0005 |
| CXXC10 (NM_001565)      | Chemokine (C-X-C motif) ligand 10 | -2.06 | 0.0104 |
| IL1B (NM_000576)        | Interleukin 1, beta | -2.20 | 0.0001 |
| IL6 (NM_000800)         | Interleukin 6 (interferon, beta 2) | -2.98 | 0.0092 |
| CYP1A1 (NM_000499)      | Cytochrome P450, family 1, subfamily A, polypeptide 1 | -5.64 | 0.0000 |

**C**

| cytokin (10 µM; 6h) | gene description | fold change | p-value |
|---------------------|------------------|-------------|---------|
| PTGS2 (NM_000962)   | Prostaglandin-endoperoxide synthase 1 | 170.86 | 0.0001 |
| CCL3 (NM_002683)    | Chemokine (C-C motif) ligand 3 | 68.75 | 0.0041 |
| TNFSF10 (NM_003816) | Tumor necrosis factor (ligand) superfamily, member 10 | 64.45 | 0.0430 |
| HSPA1A (NM_005345)  | Heat shock 70Da protein 1A | 14.66 | 0.0001 |
| HSPA6 (NM_002155)   | Heat shock 70Da protein 6 (HSP70B) | 10.29 | 0.0002 |
| CXXC10 (NM_001565)  | Chemokine (C-X-C motif) ligand 10 | 7.19 | 0.0006 |
| CRAB (NM_001865)    | Crystallin, alpha B | 5.29 | 0.0000 |
| TNF (NM_000594)     | Tumor necrosis factor (TNF superfamily, member 2) | 4.98 | 0.0000 |
| IL6 (NM_000800)     | Interleukin 6 (interferon, beta 2) | 4.25 | 0.0001 |
| IL1B (NM_000576)    | Interleukin 1, beta | 4.24 | 0.0001 |
| CSF2 (NM_000788)    | Colony stimulating factor 2 (granulocyte-macrophage) | 3.39 | 0.0001 |
expression in G361 melanoma cells, observable both at the mRNA and protein levels (Fig. 4, B and C).

Next, we examined aurin effects on heat shock response gene expression and viability in non-malignant cells (HaCaT keratinocytes (Fig. 4, D–F), Hs27 dermal fibroblasts (Fig. 4, G–I), primary melanocytes (Fig. 4, J–L)). Remarkably, even though aurin exposure caused pronounced up-regulation of HSPA1A expression at the mRNA and protein levels (1–10 μM; 3–24 h) in HaCaT keratinocytes (Fig. 4, E and F), Hs27 fibroblasts (Fig. 4, H and I), and melanocytes (Fig. 4, K and L), cellular viability of these non-malignant cells was not impaired by aurin treatment (10 μM, 24 h; Figs. 4, D, G, and J, respectively).

Aurin Induces UPR Signaling, Proteasomal Impairment, and Oxidative Stress in Human A375 Malignant Melanoma Cells—Led by HSF1 nuclear translocation observed within minutes of aurin exposure followed by early up-regulation of proteotoxic stress and heat shock response gene expression (HSPA6, HSPA1A, HSPA1L, DNAJB4, CRYAB, HSPB1, HSPH1, DNAJA1, and DDIT3) as observed by array analysis (Fig. 2), we further examined the occurrence of UPR stress signaling in aurin-treated A375 melanoma cells (Fig. 5, A and B). Time course analysis by quantitative RT-PCR revealed early up-regulation of DDIT3 expression encoding CHOP (CCAAT/enhancer-binding protein homologous protein), the ER stress-inducible transcription factor (Fig. 5A). DDIT3 mRNA levels exceeded control by almost 7-fold within 6 h of exposure. Immunoblot detection indicated that up-regulation of CHOP protein levels occurred within 1–3 h of continuous exposure, reaching a maximum within 12 h (Fig. 5B). As a positive control, CHOP protein up-regulation was also monitored in response to thapsigargin (T), an established inducer of ER stress and UPR through SERCA inhibition (sarco/endoplasmic reticulum)-

**Figure 2.** Gene expression array analysis performed in human A375 malignant melanoma cells exposed to aurin or celastrol. A, the scatter blot depicts differential gene expression as detected by the RT2 Human Stress and Toxicity Profiler™ PCR Expression Array technology (aurin, 10 μM, 6 h exposure). Upper and lower lines, cut-off indicating 4-fold up- or down-regulated expression, respectively. Arrays were performed in three independent repeats and analyzed using the two-sided Student’s t test. B, numerical expression changes (p < 0.05) elicited by aurin. C, for comparison, an analogous experiment was performed using the established heat shock response inducer celastrol (10 μM, 6 h exposure), and celastrol-induced numerical expression changes are listed (p < 0.05).
Ca\(^{2+}\) ATPase (Fig. 5B) (39). Inhibitory phosphorylation of eIF2\(\alpha\) (eukaryotic translation initiation factor) through activation of PERK (double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase) is an established hallmark of cytotoxic ER stress known to occur upstream of DDIT3/CHOP up-regulation. We, therefore, examined aurin-induced changes affecting phosphorylation status of eIF2\(\alpha\) and PERK (Fig. 5B). Consistent with aurin induction of cytotoxic ER stress, pronounced PERK activation (autophosphorylation of its cytoplasmic kinase domain (Thr-980)) together with inhibitory phosphorylation of eIF2\(\alpha\) (Ser-51) was detectable within 6 h of exposure and was sustained over the 24-h observation period. Likewise, inhibitory phosphorylation of eIF2\(\alpha\) was detectable over a 24-h time course (Fig. 5B).

After confirming aurin induction of ER stress signaling, we examined aurin-induced changes in protein ubiquitination status and inhibition of proteasomal enzymatic activity (Fig. 5, C and D). Consistent with aurin induction of a pronounced proteotoxic insult in A375 cells, elevated levels of ubiquitinated proteins were detectable within 1–3 h of exposure, further increasing over a 12-h observation time (Fig. 5C). Moreover, using a luminescent protease assay that measures proteasome-dependent release of luciferin from specific precursor substrates, we observed that aurin displays activity as an inhibitor of chymotrypsin-like proteasomal function. Specifically, chymotrypsin-like activity in cytosolic extracts prepared from aurin-treated A375 cells was impaired by 

![FIGURE 4. Aurin-induced heat shock response elicited in non-malignant human skin cells (Hs27 fibroblasts, HaCaT keratinocytes, primary melanocytes) occurs without impairment of viability. Viability of aurin-exposed (10 \(\mu\)M, 24 h) cells was monitored using flow cytometric analysis (annexin V-PI staining); human metastatic G361 melanoma cells (A), HaCaT keratinocytes (D), Hs27 dermal fibroblasts (G), primary melanocytes (HEMa-LP; J). Numbers in the quadrants indicate viable (AV-negative, PI-negative) in percent of total gated cells (mean \(\pm\) S.D., \(n = 3\)). In G361 melanoma cells, aurin exposure also occurred in the presence of the pancaspase inhibitor zVADfmk (40 \(\mu\)M; panel A). B, E, H, and K, bar graphs depict analysis of HSPA1A/HMOX1 expression changes induced by aurin treatment (\(\leq 10\ \mu\)M, 24 h) at the mRNA level (quantitative RT-PCR; \(n = 3\)). C, F, I, and L, Hs70/Hsp27/HMOX1 expression changes induced by aurin-treatment (1–10 \(\mu\)M, \(\geq 24\) h) at the protein level; immunoblot analysis representative of three independent repeats is depicted.](https://www.jbc.org/content/290/3/1630)
Aurin Causes Lethal Proteotoxic Stress in Melanoma Cells

Next, we examined the occurrence of oxidative stress in aurin-exposed A375 malignant melanoma cells using flow cytometric determination of 2',7'-dichlorodihydrofluorescein (DCF) fluorescence and luminometric detection of reduced glutathione levels (Fig. 5, E and F). Aurin treatment was associated with the induction of pronounced oxidative stress as obvious from significant 2',7'-dichlorodihydrofluorescein fluorescence increase (almost 10-fold more than control levels) and depletion of reduced glutathione (by ~35%) detectable within 12–18 h of exposure time. The fact that aurin-induced redox dysregulation was detectable only after HSF1 nuclear translocation (Fig. 3E), transcriptional activation (Fig. 3D), and up-regulation of early heat shock response gene expression (Fig. 2, A and B) have already occurred suggests that oxidative stress does not represent the upstream event that triggers aurin-induced stress responses. However, consistent with a mechanistic involvement of redox dysregulation in the apoptotic mode and cytotoxic potency of aurin, it was observed that cellular glutathione depletion employing L-buthionine-S,S-sulfoximine (BSO; 1 mM, 24 h of pretreatment) strongly sensitized A375 cells to the apoptogenic activity of aurin (Fig. 5G).

Fluorescence Polarization and Molecular Modeling Identify Aurin as a Geldanamycin-competitive Inhibitor of Hsp90 Causing Down-regulation of Hsp90 Client Proteins in A375 Malignant Melanoma Cells—It has been shown earlier that heat shock response gene expression and UPR may be up-regulated in response to pharmacological inhibition of Hsp90, an established pathway leading to heat shock response gene expression as observed with the Hsp90 inhibitor geldanamycin (Fig. 1, B and C) (59–63). Specifically, up-regulation of HSPA1A and other heat shock response genes downstream of Hsp90 antagonism may represent a counter-regulatory mechanism involving the known repression of heat shock transcription factor (HSF1) activation by Hsp90 that forms a stress-sensitive complex with HSF1 (59–62). We, therefore, tested the hypothesis that aurin induction of heat shock gene expression and UPR may occur in part as a result of Hsp90-directed inhibitory activity.

First, we examined the possibility that aurin-induced impairment of Hsp90 function may cause destabilization and subsequent down-regulation of established Hsp90 client proteins in melanoma cells. It has recently been shown that V600E-B-Raf (as expressed in A375 malignant melanoma cells) requires Hsp90 chaperone function for stability and that V600E B-Raf is degraded in response to Hsp90 inhibitor treatment (34, 37).
Likewise, Hsp90 inhibitors suppress aryl hydrocarbon receptor (AhR)-mediated activation of the AhR target gene CYP1A1 (63). Indeed, immunoblot analysis revealed a pronounced down-regulation of these Hsp90 client proteins in A375 melanoma cells exposed to aurin (10 μM; 24 h; Fig. 6A). Consistent with pronounced reduction of cellular AhR protein levels, we also observed that aurin treatment suppressed expression of CYP1A1 at the mRNA level observable within 3 h of exposure time (Fig. 6B). This finding is in agreement with our initial expression array-based identification of CYP1A1 as the gene displaying the most pronounced down-regulation in response to aurin treatment in A375 melanoma cells (Fig. 2, A and B).

Next, using a validated commercial fluorescence polarization assay platform, we directly examined the possibility that aurin displays activity as a competitive Hsp90α N-terminal inhibitor. Based on the competition between a fluorescently labeled high affinity probe derived from the standard Hsp90 inhibitor geldanamycin (FITC-geldanamycin) and the test compound for binding to human recombinant Hsp90α, reduction in FITC-geldanamycin fluorescence polarization was assessed to quantify competitive target binding (47, 48). Measuring displacement of the geldanamycin-FITC/Hsp90α interaction over a broad concentration range of aurin versus geldanamycin (positive control) (Fig. 6, C and D), aurin displayed a significant geldanamycin-competitive Hsp90α target affinity (geldanamycin, 22.0 ± 2.3 nM; aurin, 574.0 ± 15.2 nM; IC50 values (inhibitory activity on Hsp90α; data are the mean ± S.E.; n = 2)). However, Hsp90α-directed ligand activity of aurin was ~25-fold smaller than that displayed by geldanamycin.

Further experimental evidence in support of an Hsp90-directed antagonistic activity of aurin was obtained by assessment of aurin-induced inhibition of ATPase enzymatic activity of
Aurin Causes Lethal Proteotoxic Stress in Melanoma Cells

recombinant human Hsp90α (Fig. 6E) (58). Using the malachite green assay for the photometric detection of phosphate generated from enzymatic ATP hydrolysis, it was observed that low micromolar concentrations of aurin (10–40 μM) were sufficient to cause an almost 40% reduction in Hsp90α ATPase activity. Under our assay conditions, aurin surpassed the inhibitory activity of geldanamycin (~20% inhibition of ATPase activity at 40 μM), an observation that is consistent with the known dependence of geldanamycin and its derivatives on NQO1/NADH-mediated bioreductive activation for full inhibitory activity (58).

A computational strategy was then used to explore the potential molecular basis of aurin-induced target modulation by modeling ligand binding of aurin to Hsp90α. It is well established that Hsp90 chaperone function depends on the conformational changes driven by its ATPase activity. Binding of the ansamycin antibiotic geldanamycin to the N-terminal ATP pocket restrains Hsp90 in its ADP-bound conformation, preventing the subsequent “clamping” of Hsp90 around a client protein (49, 64). The N-terminal geldanamycin binding domain of Hsp90 (amino acid residues 9–232) reveals a pronounced structural similarity toward Hsp90 around a client protein (49, 64). The N-terminal geldanamycin binding domain of Hsp90 (amino acid residues 9–232) reveals a pronounced structural similarity to geldanamycin-competitive structure-based target inhibition, an empirical scoring function that approximates the probability of a molecule being a specific target in the active site (formed by ATP/ADP switch domain regulating Hsp90 conformation, and it has been demonstrated that geldanamycin adopts a compact structure inside this pocket, thereby competing with substrate binding and blocking the conformational maturation and refolding reaction of Hsp90 client proteins (49). To assess geldanamycin-competitive structure-based target inhibition, molecular modeling was performed based on the x-ray crystal structure coordinates of geldanamycin with Hsp90α (Protein Data Bank code 1YET) employing the Glide docking methodology, an empirical scoring function that approximates the ligand binding free energy (Fig. 6, F–H) (49, 50). Glide score (G score) analysis indicated that aurin displays significant binding affinity toward Hsp90α, a finding consistent with the fluorescence polarization-based observation that aurin may operate as a structure-based Hsp90α inhibitor. A common hallmark of known Hsp90 N-terminal domain inhibitors is their ability to engage in molecular interaction with Asp-93, conserved throughout all known Hsp90 homologs from at least 35 species, positioned at the bottom of a mostly hydrophobic pocket. Indeed, comparative Glide docking revealed that aurin may interact favorably with the Hsp90α active site (formed by antiparallel β-sheets S3, S4, and S7 at the bottom surrounded by α-helices H2, H4, and H7) through hydrogen bonding interactions involving Asp-93, Asn-106, and Lys-58, residues also engaged in geldanamycin binding (G score = −3.8 versus G score = −5.3, respectively; Fig. 6, F–H).

Aurin-induced Melanoma Cell Apoptosis Depends on Up-regulated PMAIP1 Gene Expression—Next, we aimed at elucidating details of the molecular pathway underlying aurin-induced A375 melanoma cell death. To this end, viability was monitored using flow cytometric analysis of annexin V–PI-stained cells defining the dose-response relationship and time course of aurin-induced cell death (Fig. 7, A and B). Remarkably, viability of A375 cells exposed to aurin (10 μM, 24 h) was maintained upon cotreatment with zVADfmk but not with the caspase 8-specific inhibitor IETD-CHO pointing toward a causative involvement of the intrinsic (mitochondrial) pathway of apoptosis (Figs. 3H and 7C). We also observed that pretreatment using the thiol antioxidant and glutathione precursor N-acetyl-l-cysteine (10 mM, 24 h) rescued cells from aurin-induced apoptosis, an observation consistent with the prior observation that glutathione-depletion caused sensitization to aurin-induced cell death (Fig. 5, F and G). Aurin-induced proteolytic procaspase-3 activation was observable within 12 h of continuous exposure (Fig. 7D), a time point at which a significant disruption of the mitochondrial transmembrane potential (Δψm) became apparent (Fig. 7E). In the context of a possible mitochondrial involvement in aurin-induced apoptosis, we then focused on the up-regulated expression of the proapoptotic gene PMAIP1 encoding the BH3-only Bcl2 family member Noxa as detected earlier at the mRNA level by expression array profiling (Fig. 2, A and B), confirmed subsequently by a more detailed time course analysis (Fig. 7F). Due to the established causative involvement of PMAIP1 up-regulation in cancer cell mitochondrial apoptosis that occurs in response to various genotoxic, proteotoxic, and oxidative stress stimuli (39, 41, 44, 65, 66, 68), a genetic target modulation approach employing siRNA-based antagonism of PMAIP1 expression was used to further substantiate the causative involvement of Noxa in aurin-induced melanoma cell apoptosis. First, immunoblot analysis confirmed that aurin-induced up-regulation of Noxa protein levels (detectable within 12 h of exposure time) was fully suppressed in siPMAIP1–cotreated A375 melanoma cells (Fig. 7G). Strikingly, siPMAIP1-based suppression of Noxa expression protected A375 melanoma cells against aurin-induced (10 μM; 24 h) apoptosis, a specific rescue effect that was not observed in siControl–treated cells exposed to aurin (Fig. 7H).

DISCUSSION

Pharmacological induction of proteotoxic stress is rapidly emerging as a promising strategy for cancer cell-directed chemotherapy intervention (3, 7–9, 13). Cumulative experimental evidence suggests that therapeutic impairment of proteostasis may be harnessed for the apoptotic elimination of cancer cells that display constitutively elevated levels of proteotoxic stress originating from endogenous factors such as aneuploidy, mutation-driven expression of misfolded proteins, and adverse conditions associated with the tumor microenvironment (3–11).

Here, we report the identification of aurin, a phenolic triphenylmethane–derived quinone–methide, as an apoptogenic inducer of proteotoxic and oxidative stress in human cultured malignant melanoma cells. Our data indicate that in A375 malignant melanoma cells, aurin treatment causes (i) early nuclear translocation and transcriptional activation of HSF1 (Fig. 3, D and E), (ii) up-regulation of heat shock response gene expression (Figs. 1, B and C, and 2, A and B), (iii) UPR induction (phosphorylation of PERK and eIF2α, up-regulation of DDIT3/CHOP, Fig. 5, A and B), (iv) proteasome inhibition with accumulation of ubiquitinylated proteins (Fig. 5, C and D), (v) down-regulation of Hsp90 client proteins including BRAF and AhR (Fig. 6A), (vi) oxidative stress with glutathione depletion (Fig. 5, E–G), and (vii) induction of caspase-dependent melanoma cell...
death downstream of PMAIP1 (Noxa) up-regulation and impairment of mitochondrial transmembrane potential. Furthermore, fluorescence polarization-based experimentation revealed that aurin displays activity as a geldanamycin-competitive Hsp90/H9251 antagonist (Fig. 6, C and D), a finding further substantiated by aurin inhibition of ATPase enzymatic activity of recombinant human Hsp90/H9251 (Fig. 6, E). One experiment (histogram) representative of three similar repeats is displayed. E, mitochondrial transmembrane potential was determined using flow cytometric analysis of JC-1 stained cells (time course; 10 μM, 24 h). Numbers indicate cells with polarized mitochondria (circle) in percent of total cells (mean ± S.D., n = 3)). F, time course of PMAIP1 expression changes induced by aurin (10 μM) and molecular docking analysis of Noxa protein levels in aurin (10 μM, 24 h) exposed cells with siControl or siPMAIP1 cotreatment. G, viability of aurin-exposed (10 μM; 24 h) cells was monitored as a function of siControl or siPMAIP1 cotreatment using flow cytometric analysis (annexin V-PI staining; mean ± S.D., n = 3)).
Hsp90 inhibition (Fig. 1, C and D), earlier experiments have demonstrated that Hsp90-directed pharmacological inhibitors up-regulate mRNA and protein levels of heat shock response genes by inhibiting the association of Hsp90 with HSF1 causing nuclear translocation and increased transcriptional activity of the latter (59–62).

Earlier research has demonstrated that Hsp90 antagonism, proteasome inhibition, and UPR induction can all be associated with redox dysregulation and oxidative stress occurring downstream of the initial proteotoxic event (8, 66). Indeed, our data indicate that redox dysregulation represents an additional factor involved in aurin-induced apoptosis as evident from antioxidant rescue (using N-acetyl-l-cysteine (NAC), Fig. 7C) and cell sensitization caused by pharmacological glutathione depletion (using 1-buthionine-sulfoximine, Fig. 5G). Importantly, a detailed time course analysis indicated that induction of proteotoxic stress (as evident from HSF1 nuclear translocation observable within 10 min of aurin exposure (Fig. 3E), heat shock response gene expression (Figs. 1, B and C, and 2, A and B), DDIT3/CHOP up-regulation (Fig. 5, A and B), and protein ubiquitination (Fig. 3C)) precedes loss of proteasomal enzymatic activity (Fig. 3D), redox dysregulation (Fig. 5, E and F), and mitochondrial impairment (Fig. 7E) detectable only at later time points. This suggests that it is the Hsp90-directed inhibitory activity of aurin that is the upstream factor causing the induction of a cytotoxic heat shock response in malignant melanoma cells. It should also be mentioned that prior research has documented the specific mitochondriotoxic activity of cationic aniline-triphenylmethane dyes including crystal (gentian) violet that target the mitochondrion based on cationic lipophilic properties (71, 72). However, at physiological pH aurin (rosolic acid) exists as an anionic phenolate devoid of mitochondrial tropism (70), and our data indicate that early proteotoxic effects of aurin precede loss of mitochondrial transmembrane potential.

However, more detailed follow up experimentation must aim at elucidating the mechanistic interplay between Hsp90-, proteasome-, and redox-directed effects elicited by aurin in A375 malignant melanoma cells. Importantly, our data identify aurin-induced PMAIP1 up-regulation (Figs. 2, A and B and 7, F–H) as a key factor underlying Noxa-dependent apoptosis in A375 melanoma cells. A tentative mechanistic scheme presenting the suggested molecular mechanism underlying induction of lethal proteotoxic stress in aurin-exposed malignant melanoma cells is presented in Fig. 8.

Remarkably, in non-malignant human skin cells (Hs27 fibroblasts, HaCaT keratinocytes, primary melanocytes) an aurin-induced heat shock response (as indicated by HSPA1A up-regulation) occurred without impairment of viability (Fig. 4, D–L), an observation consistent with the emerging concept that normal cells display lower constitutive levels of endogenous proteotoxic stress together with a diminished dependence on proteostasis-ensuring mechanisms including proteasomal and heat shock protein chaperone functions pointing toward a molecular vulnerability of malignant cells amenable to therapeutically intervention as proposed before (4–6, 39, 40). It is interesting to note that one prior publication has reported the cytoprotective activity of aurin observed in cultured human endothelial cells where aurin caused up-regulation of heme oxygenase 1 (HO-1) without loss of cell viability (70), consistent with our observation that in non-malignant human cultured cells aurin treatment up-regulates the heat shock response without inducing cell death (Fig. 4, D–L).

Due to its essential role in the stabilization of oncogenic client proteins and its emerging role in the suppression of mitochondrial apoptosis, the molecular chaperone Hsp90 represents a valid molecular target for chemotherapeutic intervention (64). Small molecule inhibitors of Hsp90 are, therefore, an important class of experimental and investigational chemotherapeutics that have reached the stage of clinical testing. Hsp90-directed inhibitory activity of aurin seems to be of particular relevance in the context of melanoma chemotherapy, as Hsp90 inhibition has emerged as a particularly promising therapeutic strategy based on the finding that \( V600E \)-B-Raf is an obligatory Hsp90 client protein essential to the clonal evolution of melanoma tumors and that Hsp90 inhibitors cause the degradation of \( V600E \)-B-Raf (34, 37, 63). Moreover, it has now been observed that \( V600E \)-BRAF melanoma cells that have acquired resistance to vemurafenib due to a variety of molecular changes retain sensitivity to Hsp90 inhibition suggesting feasibility of
Aurin Causes Lethal Proteotoxic Stress in Melanoma Cells

aurin-based Hsp90-directed interventions targeting melanoma (35, 36).

Based on the results of our prototype fluorescence polarization and ATPase enzymatic activity assays combined with Glide molecular docking analysis, aurin was identified as a novel structure-based geldanamycin-competitive Hsp90 antagonist (Fig. 6). Future research efforts must explore the structure activity relationship underlying aurin-induced Hsp90-directed proteotoxic stress. Interestingly, aurin contains a quinone methide functional group reminiscent of the tricarboxylated celastrol, a reference heat shock inducer and Hsp90 inhibitory agent containing an essential quinone methide pharmacophore. However, our comparative transcriptional profiling analysis revealed that apart from the common induction of Hsp70-directed gene expression changes aurin (Fig. 2, A and B)- and celastrol-induced (Fig. 2C) responses differed significantly in A375 melanoma cells. Interestingly, in contrast to aurin targeting the N-terminal ATP pocket, it has been demonstrated that celastrol displays Hsp90 C-terminal domain-directed antagonistic activity inhibiting Hsp90 interaction with its cochaperone, cdc37 (67). Remarkably, in addition to Hsp90-directed antagonistic activity, proteasome-directed inhibitory activity and depletion of cellular thiols are thought to be involved in celastrol-induced cancer cell apoptosis, suggesting a mechanistic overlap between celastrol-induced proteotoxic and oxidative stress pathways reminiscent of aurin-induced changes observed by us in malignant melanoma cells (54–57). Interestingly, Noxa-induced apoptosis downstream of ER stress causing PERK/phospho-eIF2α/ATF4-dependent PMAIP1 up-regulation has been substantiated earlier in celastrol-exposed hepatocellular carcinoma cells (65). In this context, it will be interesting to examine how Hsp90-directed antagonistic activity synergizes with proteasomal impairment, UPR signaling (resulting in up-regulated expression of the pro-apoptotic transcription factor CHOP), and oxidative stress, all of which are potentially critical factors contributing to aurin-induced Noxa-dependent melanoma cell apoptosis (11, 39, 65, 66, 68).

In the context of our preclinical study examining feasibility of experimental intervention targeting malignant melanoma cells using the small molecule drug-like quinone-methide aurin, it should be mentioned that prior preclinical and clinical developmental studies have focused on the pharmacological activity of the tricarboxylated aurin derivative ATA (Fig. 1A), a promiscuous experimental therapeutic acting as an inhibitor of protein-protein and DNA-protein interactions (51–53, 69). However, ATA was not active in our HSE-luciferase reporter (Fig. 3D), mRNA- (HSPA1A, HSPA6, HMOX1; Figs. 1B and 3F), and protein-directed (Figs. 1C and 3G) assays examining heat shock response induction. Based on the prototype evidence presented above, our ongoing preclinical experimentation aims at testing efficacy of aurin-induced proteotoxic stress and apoptosis for therapeutic intervention in relevant murine xenograft models of human malignant melanoma.

REFERENCES
1. Kim, I., Xu, W., and Reed, J. C. (2008) Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat. Rev. Drug Discov. 7, 1013–1030
2. Chen, D., Frezza, M., Schmitt, S., Kanwar, J., and Dou, Q. P. (2011) Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. Curr. Cancer Drug Targets 11, 239–253
3. Xu, W., Trepel, I., and Neckers, L. (2011) Ras, ROS, and proteotoxic stress: a delicate balance. Cancer Cell 20, 281–282
4. Dai, C., Dai, S., and Cao, J. (2012) Proteotoxic stress of cancer: implication of the heat-shock response in oncogenesis. J. Cell Physiol. 227, 2982–2987
5. Dobbelstein, M., and Moll, U. (2014) Targeting tumour-supportive cellular machineries in anticancer drug development. Nat. Rev. Drug Discov. 13, 179–196
6. Luo, J., Solimini, N. L., and Elledge, S. J. (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. Cell 136, 823–837
7. Obeng, E. A., Carlson, L. M., Gutman, D. M., Harrington, W. J., Jr., Lee, K. P., and Boise, L. H. (2006) Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. Blood 107, 4907–4916
8. Healy, S. J., Gorman, A. M., Moussavi-Shafaei, P., Gupta, S., and Samali, A. (2009) Targeting the endoplasmic reticulum-stress response as an anticancer strategy. Eur. J. Pharmacol. 625, 234–246
9. De Raadt, T., Walton, Z., Yecies, J. L., Li, D., Chen, Y., Malone, C. F., Maertens, O., Jeong, S. M., Bronson, R. T., Lebleu, V., Kalluri, R., Normant, E., Haigis, M. C., Manning, B. D., Wong, K. K., Macleod, K. F., and Cichowski, K. (2011) Exploiting cancer cell vulnerabilities to develop a combination therapy for ras-driven tumors. Cancer Cell 20, 400–413
10. Whitesell, L., and Lindquist, S. (2009) Inhibiting the transcription factor HSF1 as an anticancer strategy. Expert. Opin. Ther. Targets 13, 469–478
11. Benbrook, D. M., and Long, A. (2012) Integration of autophagy, proteasomal degradation, unfolded protein response, and apoptosis. Exp. Oncol. 34, 286–297
12. Wondrak, G. T. (2009) Redox-directed cancer therapeutics: molecular mechanisms and opportunities. Antioxid. Redox. Signal. 11, 3013–3069
13. Pan, J. A., Ullman, E., Dou, Z., and Zong, W. X. (2011) Inhibition of protein degradation induces apoptosis through a microtubule-associated protein 1 light chain 3-mediated activation of caspase-8 at intracellular membranes. Mol. Cell. Biol. 31, 3158–3170
14. Garbe, C., Eigentler, T. K., Keilholz, U., Hauschild, A., and Kirkwood, J. M. (2011) Systematic review of medical treatment in melanoma: current status and future prospects. Oncologist 16, 5–24
15. Chapman, P. B., Hauschild, A., Robert, C., Haenen, J. B., Ascierto, P., Larkin, J., Dummer, R., Garbe, C., Testori, A., Maio, M., Hogg, D., Lorigan, P., Lebev, C., Jouary, T., Schadendorf, D., Ribas, A., O’Day, S. J., Sosman, J. A., Kirkwood, J. M., Eggemont, A. M., Dreno, B., Nolop, K., Li, N., Nelson, B., Hou, J., Lee, R. J., Flaherty, K. T., McArthur, G. A., and BRIM-3 Study Group (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N. Engl. J. Med. 364, 2507–2516
16. Yang, H., Higgins, B., Kolinsky, K., Packman, K., Go, Z., Iyer, R., Kolis, S., Zhao, S., Lee, R., Grippio, J. F., Schostach, K., Simonco, M. E., Heimbrook, D., Bollag, G., and Su, F. (2010) RG7204 (PLX4032), a selective BRAFV600E inhibitor, displays potent antitumor activity in preclinical melanoma models. Cancer Res. 70, 5518–5527
17. Hoeflich, K. P., Gray, D. C., Eby, M. T., Tien, J. Y., Wong, L., Bower, J., Gogineni, A., Zha, J., Cole, M. J., Stern, H. M., Murray, L. J., Davis, D. P., Zhao, S., Lee, R., Grippio, J. F., Schostach, K., Simonco, M. E., Heimbrook, D., Bollag, G., and Su, F. (2010) RG7204 (PLX4032), a selective BRAFV600E inhibitor, displays potent antitumor activity in preclinical melanoma models. Cancer Res. 70, 5518–5527
18. Kudchadkar, R., Paraiso, K. H., and Smalley, K. S. (2012) Targeting mutant melanoma. Nat. Rev. Clin. Oncol. 9, 227–238
19. Kudchadkar, R., Paraiso, K. H., and Smalley, K. S. (2012) Targeting mutant melanoma. Nat. Rev. Clin. Oncol. 9, 227–238
20. Kwong, L. N., and Davies, M. A. (2014) Targeting therapy for melanoma: rational combinatorial approaches. Oncogene 33, 1–9
21. Sheen, J. H., Zoncu, R., Kim, D., and Sabatini, D. M. (2011) Defective regulation of autophagy upon leucine deprivation reveals a targetable liability of human melanoma cells in vitro and in vivo. Cancer Cell 19, 613–628
22. Armstrong, J. L., Corazzari, M., Martin, S., Pagliarini, V., Falasca, L., Hill, D. S., Ellis, N., Al Sabah, S., Redfern, C. P., Finia, G. M., Piacentini, M., and Lovat, P. E. (2011) Oncogenic B-RAF signaling in melanoma impairs the
therapeutic advantage of autophagy inhibition. *Clin. Cancer Res.* 17, 2216–2226.

23. Marino, M. L., Pellegrini, P., Di Lernia, G., Djavaheri-Mergny, M., Brnjic, S., Zang, X., Hägg, M., Linder, S., Fäis, S., Codognò, P., and De Miltio, A. (2012) Autophagy is a protective mechanism for human melanoma cells under acidic stress. *J. Biol. Chem.* 287, 30664–30676.

24. Ma, X. H., Piao, S., Wang, D., McAfee, Q. W., Nathanson, K. L., Lum, J. I., Li, L. Z., and Amaravadi, R. K. (2011) Measurements of tumor cell autophagy predict invasiveness, resistance to chemotherapy, and survival in melanoma. *Clin. Cancer Res.* 17, 3478–3489.

25. Xie, X., White, E. P., and Mehnert, J. M. (2013) Coordinate autophagy and mTOR pathway inhibition enhances cell death in melanoma. *PLoS ONE* 8, e55096.

26. Fleming, A. (2011) Cancer: autophagy presents Achilles heel in melanoma. *Nat. Rev. Drug Discov.* 10, 491.

27. Coss, R. A., Storck, C. W., Daskalakis, C., Berd, D., and Wahl, M. L. (2003) Intracellular acidification abrogates the heat shock response and compromises survival of human melanoma cells. *Mol. Cancer Ther.* 2, 383–388.

28. Deichmann, M., Polychronidis, M., Benner, A., Kleist, C., Thome, M., Kahlé, B., and Helmkne, B. M. (2004) Expression of the heat shock cognate protein HSP73 correlates with tumour thickness of primary melanomas and is enhanced in melanoma metastases. *Int. J. Oncol.* 25, 259–268.

29. Carta, F., Demuro, P. P., Zanini, C., Santona, A., Castiglia, D., D’Atri, S., Ascieri, P. A., Napolitano, M., Cossu, A., Tadolini, B., Turrini, F., Manca, A., Sini, M. C., Palmieri, G., and Rozzo, A. C. (2005) Analysis of candidate genes through a proteomics-based approach in primary cell lines from malignant melanomas and their metastases. *Melanoma Res.* 15, 235–244.

30. Ciocca, D. R., and Calderwood, S. K. (2005) Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell Stress Chaperones* 10, 86–103.

31. Kalogeraki, A., Garbagnati, F., Darvianaki, K., Delides, G. S., Santinami, M., Stathopoulos, E. N., and Zoras, O. (2006) HSP-70, C-myc, and HLA-DR expression in patients with cutaneous malignant melanoma. *Clin. Cancer Res.* 12, 239–250.

32. Marri, G. N. (2012) Suppression of heat shock protein 27 induces long-term dormancy in human breast cancer. *Int. J. Mol. Sci.* 13, 2052–2061.

33. Coss, R. A., Storck, C. W., Daskalakis, C., Berd, D., and Wahl, M. L. (2003) Intracellular acidification abrogates the heat shock response and compromises survival of human melanoma cells. *Mol. Cancer Ther.* 2, 383–388.

34. Grbovic, O. M., Basso, A. D., Sawai, A., Ye, Q., Fais, S., Codogno, P., and DeMilito, A. (2012) Autophagy is a protective mechanism for human melanoma cells but not primary melanocytes. *Biochem. Pharmacol.* 83, 1229–1240.

35. Qiao, S., Tao, S., Rojo de la Vega, M., Park, S. L., Vonderfecht, A. A., Jacobs, S. L., Zhang, D. D., and Wondrak, G. T. (2013) The antimalarial amodi-

aquine causes autophagic-lysosomal and proliferative blockade sensitizing human melanoma cells to starvation- and chemotherapy-induced cell death. *Autophagy* 9, 2087–2102.

36. Cabello, C. M., Lamore, S. D., Bair, W. B., 3rd, Qiao, S., Azimian, S., Lesson, J. L., and Wondrak, G. T. (2012) The redox antimalarial dihydroartemisinin targets human metastatic melanoma cells but not primary melanocytes with induction of NOXA-dependent apoptosis. *Invest. New Drugs* 30, 1289–1301.

37. Cabello, C. M., Lair, B. L., Baur, A. E., and Wondrak, G. T. (2009) Antimelanoma activity of the redox dye DCPIP (2,6-dichlorophenoindol- 

pheno) is antagonized by NQO1. *Biochem. Pharmacol.* 78, 344–354.

38. Davis, A. L., Cabello, C. M., Qiao, S., Azimian, S., and Wondrak, G. T. (2013) Phenotypic identification of the redox dye methylene blue as an antagonist of heat shock response gene expression in metastatic melanoma cells. *Int. J. Mol. Sci.* 14, 4185–4202.

39. Qiao, S., Cabello, C. M., Lamore, S. D., Lesson, J. L., and Wondrak, G. T. (2012) D-Penicillamine targets metastatic melanoma cells with induction of the unfolded protein response (UPR) and Noxa (PMAIP1)-dependent mitochondrial apoptosis. *Aptosis* 17, 1079–1094.

40. Cabello, C. M., Bair, W. B., 3rd, Ley, S., Lamore, S. D., Azimian, S., and Wondrak, G. T. (2009) The experimental chemotherapeutic A6-furfuryl-

ladenosine (kinetin-riboside) induces rapid ATP depletion, genotoxic stress, and CDKN1A (p21) up-regulation in human cancer cell lines. *Biochem. Pharmacol.* 77, 1125–1138.

41. Wondrak, G. T. (2007) NQO1-activated phenothiazinium redox cyclers for the targeted bioreductive induction of cancer cell apoptosis. *Free Radic. Biol. Med.* 43, 178–190.

42. Llauger-Bufi, L., Felts, S. J., Huezo, H., Rosen, N., and Chiosis, G. (2003) Synthesis of novel fluorescent probes for the molecular chaperone Hsp90. *Bioorg. Med. Chem. Lett.* 13, 3975–3978.

43. Howes, R., Barril, X., Dymock, B. W., Grant, K., Northfield, C. J., Roberts, A. G., Surgeon, A., Wayne, J., Wright, L., James, K., Matthews, T., Cheung, K. M., McDonald, E., Workman, P., and Drysdale, M. J. (2006) A fluorescence polarization assay for inhibitors of Hsp90. *Anal. Biochem.* 350, 202–213.

44. Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. (1997) Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell* 89, 239–250.

45. Friesner, R. A., Banks, J. L., Murphy, R. B., Halgren, T. A., Klicic, J. J., Mainz, D. T., Repasky, M. P., Knoll, E. H., Shelley, M., Perry, J. K., Shaw, D. E., Francis, P., and Shenkin, P. S. (2004) Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J. Med. Chem.* 47, 1739–1749.

46. Phillips, M. D., Moake, J. L., Nolasco, L., and Turner, N. (1988) Aurin tricarboxylic acid: a novel inhibitor of the association of von Willebrand factor and platelets. *Blood* 72, 1898–1903.

47. Strony, J., Phillips, M., Brands, D., Moake, J., and Adelman, B. (1990) Aurin triacarboxylic acid in a canine model of coronary artery thrombosis. *Circulation* 81, 1106–1114.

48. Lu, H., Wei, G., Wang, D., Yung, P., and Ying, W. (2008) Posttreatment with the Ca2+-Mg2+-dependent endonuclease inhibitor aurin triacarboxylic acid abolishes genotoxic agent-induced nuclear condensation and DNA fragmentation and decreases death of astrocytes. *J. Neurosci.* 86, 2925–2931.

49. Trotter, A., West, J. D., Kläi, L., Westerbeide, S. D., Silverman, R. B., Morimoto, R. I., and Morano, K. A. (2008) Activation of heat shock and antioxidant responses by the natural product celastrol: transcriptional signatures of a thiol-targeted molecule. *Mol. Biol. Cell* 19, 1104–1112.

50. Kläi, L., Trippier, P. C., Mishra, R. K., Morimoto, R. I., and Silverman, R. B. (2011) Remarkable stereospecific conjugate additions to the Hsp90 inhibitor celastrol. *J. Am. Chem. Soc.* 133, 19634–19637.

51. Peng, B., Xu, L., Cao, F., Wei, T., Yang, C., and Zheng, D. (2010) HSPO9 inhibitor, celastrol, arrests human monocytic leukemia cell U937 at Go/G1 in thiol-containing agents reversible way. *Mol. Cancer* 9, 79.

52. Wang, W. B., Feng, L. X., Yue, Q. X., Wu, W. Y., Guan, S. H., Jiang, B. H., Yang, M., Liu, X., and Guo, D. A. (2012) Parapostasia accompanied by autophagy and apoptosis was induced by celastrol, a natural compound with
influence on proteasome, ER stress, and Hsp90. J. Cell Physiol. 227, 2196–2206.

58. Guo, W., Reigan, P., Siegel, D., Zirrolli, J., Gustafson, D., and Ross, D. (2005) Formation of 17-allylamino-demethoxygeldanamycin (17-AAG) hydroquinone by NAD(P)H quinone oxidoreductase 1: role of 17-AAG hydroquinone in heat shock protein 90 inhibition. Cancer Res. 65, 10006–10015.

59. Guo, F., Rocha, K., Bal, P., Pranpat, M., Fiskus, W., Boyapalle, S., Kumarswamy, S., Balasis, M., Greedy, B., Armitage, E. S., Lawrence, N., and Bhalla, K. (2005) Abrogation of heat shock protein 70 induction as a strategy to increase antileukemia activity of heat shock protein 90 inhibitor 17-allylamino-demethoxy geldanamycin. Cancer Res. 65, 10536–10544.

60. McCollum, A. K., Lukasiewicz, K. B., Teneyck, C. J., Lingle, W. L., Toft, D. O., and Erlichman, C. (2008) Cisplatin abrogates the geldanamycin-induced heat shock response. Mol. Cancer Ther. 7, 3256–3264.

61. Goloudina, A. R., Demidov, O. N., and Garrido, C. (2012) Inhibition of HSP70: a challenging anti-cancer strategy. Cancer Lett. 325, 117–124.

62. Zou, J., Guo, Y., Guettouche, T., Smith, D. F., and Voellmy, R. (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell 94, 471–480.

63. Hughes, D., Guttenplan, J. B., Marcus, C. B., Subbaramaiah, K., and Dannenberg, A. J. (2008) Heat shock protein 90 inhibitors suppress aryl hydrocarbon receptor-mediated activation of CYP1A1 and CYP1B1 transcription and DNA adduct formation. Cancer Prev. Res. (Phila) 1, 485–493.

64. Neckers, L., and Workman, P. (2012) Hsp90 molecular chaperone inhibitors: are we there yet? Clin. Cancer Res. 18, 64–76.

65. Zhu, H., Yang, W., He, L. J., Ding, W. J., Zheng, L., Liao, S. D., Huang, P., Lu, W., He, Q. J., and Yang, B. (2012) Up-regulating Noxa by ER stress, celastrol exerts synergistic anti-cancer activity in combination with ABT-737 in human hepatocellular carcinoma cells. PLoS ONE 7, e52333.

66. Selimovic, D., Porzig, B. B., El-Khattouti, A., Badura, H. E., Ahmad, M., Ghanjati, F., Santourlidis, S., Haikcl, Y., and Hassan, M. (2013) Bortezomib/proteasome inhibitor triggers both apoptosis and autophagy-dependent pathways in melanoma cells. Cell. Signal. 25, 308–318.

67. Zhang, T., Hamza, A., Cao, X., Wang, B., Yu, S., Zhan, C. G., and Sun, D. (2008) A novel Hsp90 inhibitor to disrupt Hsp90/Cdc37 complex against pancreatic cancer cells. Mol Cancer Ther 7, 162–170.

68. Hassan, M., Alaoui, A., Feyer, O., Mirmohammadsebagh, A., Essmann, F., Tannapfel, A., Gulbins, E., Schulze-Osthoff, K., and Hengge, U. R. (2008) The BH3-only member Noxa causes apoptosis in melanoma cells by multiple pathways. Oncogene 27, 4557–4568.

69. Lukenberg, L. J., Kazi, A. A., and Lang, C. H. (2014) Salutary effect of aurantricarboxylic acid (ATA) on endothotoxin- and sepsis-induced changes in muscle protein synthesis and inflammation. Shock 41, 420–428.

70. Foresti, R., Hoque, M., Monti, D., Green, C. J., and Motterlini, R. (2005) Differential activation of heme oxygenase-1 by chalcones and rosinic acid in endothelial cells. J. Pharmacol. Exp. Ther. 312, 686–693.

71. Kandela, R. K., Bartlett, J. A., and Indig, G. L. (2002) Effect of molecular structure on the selective photocotoxicity of triarylmethane dyes towards tumor cells. Photochem. Photobiol. Sci. 1, 309–314.

72. Zhang, X., Zheng, Y., Fried, L. E., Du, Y., Montano, S. J., Sohn, A., Leftkove, B., Holmgren, L., Arbiser, J. L., Holmgren, A., and Lu, J. (2011) Disruption of the mitochondrial thioredoxin system as a cell death mechanism of cationic triphenylmethanes. Free Radic. Biol. Med. 50, 811–820.