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Celastrols as Inducers of the Heat Shock Response and Cytoprotection*[S]

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Alterations in protein folding and the regulation of conformational states have become increasingly important to the functionality of key molecules in signaling, cell growth, and cell death. Molecular chaperones, because of their properties in protein quality control, afford conformational flexibility to proteins and serve to integrate stress-signaling events that influence aging and a range of diseases including cancer, cystic fibrosis, amyloidoses, and neurodegenerative diseases. We describe here characteristics of celastrol, a quinone methide triterpene and an active component from Chinese herbal medicine identified in a screen of bioactive small molecules that activates the human heat shock response. From a structure/function examination, the celastrol structure is remarkably specific and activates heat shock transcription factor 1 (HSF1) with kinetics similar to those of heat stress, as determined by the induction of HSF1 DNA binding, hyperphosphorylation of HSF1, and expression of chaperone genes. Celastrol can activate heat shock gene transcription synergistically with other stresses and exhibits cytoprotection against subsequent exposures to other forms of lethal cell stress. These results suggest that celastrols exhibit promise as a new class of pharmacologically active regulators of the heat shock response.

Modulation of the heat shock response has gained attention as a potential therapeutic modality in human disease for cancer, ischemia-reperfusion, trauma, transplantation surgery, and diabetes and has been implicated in longevity and aging (1–6). Consequently, the identification of pharmacologically active small molecules that influence the levels of molecular chaperones has gained some attention. Examples include sodium salicylate and other nonsteroidal anti-inflammatory drugs, which directly activate heat shock transcription factor 1 (HSF1)[1]; the benzoquinones ansamycin, geldanamycin, and radicicol, which bind to and inhibit Hsp90 and activate a compensatory heat shock response; and inhibitors of the proteasome, which indirectly activate chaperone expression in response to an increased flux of proteins targeted for degradation (1, 3, 7–11). Chaperones are central to many vital cellular functions, including protein folding, signal transduction, immunity, and apoptosis, and they have critical roles in protecting the cell against a wide range of physiological stressors. The regulation of the heat shock response, therefore, may be directly beneficial for a variety of diseases including those associated with aberrant cell growth, such as cancer, and those associated with damaged and misfolded proteins, such as the neurodegenerative diseases of aging.

In response to a flux of misfolded proteins, mammalian cells induce the heat shock response as mediated by the stress-induced transcription factor HSF1. Under "normal" conditions of cell growth, the majority of HSF1 exists in a repressed state associated transiently with the molecular chaperones Hsp90, Hsp70, and Hsp40 and distributed in the cytoplasm and nucleus of human cells. Upon activation, HSF1 undergoes a multi-step process involving relocalization within the nucleus, oligomerization to a trimeric DNA-binding competent state, binding to heat shock promoter elements, and hyperphosphorylation at serine residues resulting in the coordinated elevated transcription of a large family of heat shock genes (1, 12–17). The high level of heat shock gene transcription, induced by heat stress, occurs rapidly and transiently and is autoregulated by molecular chaperones that feedback through HSF1 to attenuate transcription.

To identify new lead compounds for the pharmacological treatment of neurodegenerative diseases, we participated together with a consortium of 26 laboratories to screen a library of Federal Drug Association-approved or biologically active drugs to identify small molecules that suppress properties associated with the expression of mutant Huntingtin and superoxide dismutase, respectively (18, 19). The 283 positive small molecules identified in the primary screen were subsequently screened in human cells with an hsp70.1 promoter-luciferase reporter to establish whether any of these compounds increased expression of the inducible hsp70 promoter. Of the several positive compounds in our assay, one compound, celas-

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[1] The abbreviations used are: HSF1, heat shock transcription factor 1; Hsp, heat shock protein; NF-κB, nuclear factor κB.

trol, a natural product derived from the *Celastraceae* family of plants (20, 21), was of particular interest because it was also identified independently in five laboratories using six different cell-based screens for Huntingtin aggregation and neurotoxicity. Extracts from the *Celastraceae* family of plants have been used in traditional Chinese medicine for the treatment of fever, chills, joint pain, inflammation, edema, rheumatoid arthritis, and bacterial infection (22, 23).

Here, we show that an important consequence of celastrol exposure is the induction of heat shock protein gene expression by activation of HSF1. Our analysis of synthetic celastrol analogs and other chemically related multi-ring compounds suggest that the activity of celastrol is highly dependent on its molecular structure. Celastrol treatment is cytoprotective against subsequent exposures to lethal stress in HeLa cells and SH-SY5Y neuronal cells. We propose that a common mechanism for celastrol could be its effect on the expression of heat shock proteins including Hsp70, Hsp40, and Hsp27, which may be responsible for its cytoprotective properties.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions—Human HeLa, SH-SY5Y, and 293T cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. MCF7, BT474, and H157 cells were grown in RPMI medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The cells were maintained at 5% CO_2. The compounds were dissolved in Me_2SO and added at the indicated concentrations and for the indicated times to the cells. Control cells were treated with an equivalent concentration of Me_2SO. Heat shock was induced by submersion of cells in a prewarmed circulating water bath at 42 °C.

Preparation of Chemical Reagents—Celastrol was obtained from the GAIA Chemical Corporation (Gaylordsville, CT), and derivatives and other multi-ring compounds were prepared as described in the supplemental text.

Plasmid Constructs—The hsp70.1pr-luc construct was made by PCR amplifying the hsp70.1 promoter (−188 to +150, GenBank™ accession number M11717) from LSN-WT (24) using the 5′ primer (5′-CGGGATCCGAAGAGTCTGGAGAGTTCTG-3′) and the 3′ primer (5′-CGGGATCCCCAGCTGAACGGTCTGGTTA-3′). Both primers contain BamHI sites. Purified PCR products were digested with BamHI and subcloned into BglII site of the pEGFP-N2 (CMVpr), for which the cytomegalovirus promoter was deleted using VspI and NheI sites. The luciferase gene was then subcloned from pRSLL/V (25) to hsp70.1pr-luc using HindIII and SalI sites. The GAL4-HSF1 fusion construct contains GAL4 residues 1–147 and HSF1 residues 124–503 (26).

Transfection and Generation of Stable Cell Lines—Transient transfections were performed with Polyfect (Qiagen) according to the manufacturer's protocol. The hsp70.1pr-luc cell line was generated by transfecting HeLa cells with the hsp70.1pr-luciferase plasmid using Lipofectamine (Invitrogen). Colonies were selected for G418 resistance (600 μg/ml). The resistant colonies were then tested for the induction of luciferase expression 5 h after heat shock (15 min at 44 °C) or cadmium (20 μM) treatment, and the colony with the highest luciferase induction was selected for our studies.

HSF1 Antibody—We have generated a new polyclonal antibody (HSF1 r2) against a murine glutathione *S*-transferase-HSF1 fusion protein that recognizes the various phosphorylated states of murine and human HSF1 with characteristics comparable with our previously published HSF1A antibody (27). For additional antibody information see Supplemental Fig. 1.

Luciferase Assays—The cells were plated at 7.5×10^3 cells/well in 96-well plates 24 h before compound treatment. The indicated compounds, dissolved in Me_2SO at a concentration of 10 mM, were diluted in medium and added at the indicated concentrations to the cells. Twenty-four hours after compound addition, the cells were harvested for luciferase activity using the Bright-Glo reagent (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was quantified using a 96-well plate luminometer (Molecular Devices, Sunnyvale, CA).

Gel Mobility Shift Analysis—Electrophoretic mobility shift analysis was performed as previously described (28) using a ^{32}P-labeled probe containing the proximal heat shock element from the human hsp70.1 gene promoter. The intensities of the shifted bands were quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Supershifts were performed by incubating 1 μl of polyclonal antibodies specific for HSF1 (27) with the whole cell extracts for 20 min at room temperature prior to the HSF-heat shock element binding reaction.

Reverse Transcription-PCR—The cells were harvested, and RNA was generated using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. After the reverse transcription reaction, PCR was performed using PCR primers specific for hsp70.1 and 18 S rRNA. The hsp70.1 primers were: 5′-AGAGCCGAGCCGACAGAG-3′ (forward) and 5′-CACCTTGCCGTGTTGGAA-3′ (reverse). The 18 S rRNA PCR primers were 5′-CGTCTGCCCTATCAACTTTCG-3′ (forward) and 5′-TGCCTTCCTTGGATGTGGTAG-3′ (reverse). PCRs were carried out for 25 cycles.

Western Blot Analysis—Ten μg of whole cell extracts were run on 7.5% SDS-PAGE gels and transferred to nitrocellulose. Western analysis was performed with the Odyssey system (Li-COR, Lincoln, NE). For HSF1, the HSF1 r2 antibody was used at a 1:5,000 dilution. For Hsp70, a mouse monoclonal antibody to Hsp70 (4g4; Affinity Bioreagents, Inc., Golden, CO) was used at a dilution of 1:5,000. The anti-β-actin antibody (3103, Oncogene Science, Cambridge, MA) was used to verify equal protein loading.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation reactions were performed essentially as described (29). The samples generated from HeLa-S3 cells (3×10^7) were immunoprecipitated with 10 μl of anti-HSF1 r2 (see Supplemental Fig. 1) at 4 °C overnight. Primers used for the hsp70.1 promoter were (forward) 5′-GGCGAAACCCCTGGAATATTCCCGA-3′ and (reverse) 5′-AGCCTTGGGACAACGGGAG-3′. Primers used for the dihydrofolate reductase promoter were: 5′-GGCCTCGCCTGCACAAATAGGG-3′ (forward) and 5′-GGGCAGAAATCAGCAACGGGC-3′ (reverse).

Cytoprotection Analysis—The cells were pretreated with 3 μM celastrol for 1 h, washed three times and given fresh medium, and then recovered for 5 h prior to a 45 °C heat treatment for the indicated times. As a control, the cells were pretreated with a 42 °C heat shock for 1 h. The cells were then assayed for the percentage of cell death by trypan blue uptake 24 h later. Apoptotic cell death was detected by measuring the increase in cytoplasmic nucleosomes using the Cell Death Elisa Plus assay system (Roche Applied Science) according to the manufacturer's instructions.

RESULTS

Celastrol Activation of a Heat Shock Promoter Is Chemically Selective—Celastrol, a quinone methide triterpene, was shown by our laboratory to induce expression of an hsp70.1 promoter-luciferase reporter gene stably integrated into HeLa cells as part of a screen performed by multiple laboratories to identify compounds with potential for treating neurodegenerative disease (18). To establish the mechanism by which celastrol activates the heat shock response, we first characterized the EC_{50} of celastrol in HeLa cells stably expressing the hsp70.1 promoter-luciferase reporter (hsp70.1pr-luc) and determined an optimal concentration of 3 μM (Fig. 1*A*). Celastrol activates the hsp70 promoter reporter in diverse cell types to levels comparable with or greater than that obtained by heat shock (42 °C; data not shown) or treatment with other chemical stressors ($CdCl_2$ treatment; data not shown). For example, induction of the hsp70.1pr-luc reporter of 10-fold or greater was observed in the human breast cancer cell lines MCF7 and BT474, the human nonsmall cell lung carcinoma cell line H157, and the human neuroblastoma cell line SH-SY5Y (Fig. 1*B*). These results demonstrate that celastrol can activate the hsp70 reporter independent of cell type.

To test the chemical specificity of celastrol, we analyzed other celastrol derivatives (Fig. 2*A*, *structures 1–9*) and multi-ring compounds (Fig. 2*B*, *structures 10–19*). Our results show that in addition to celastrol (*structure 1*, 30-fold), celastrol methyl ester (*structure 2*, 9-fold) and dihydrocelastrol diacetate (*structure 7*, 28-fold) are also active at 3 μM, whereas celastrol butyl ester (*structure 4*) and dihydrocelastrol (*structure 5*) require a higher concentration for activity. By comparison, closely related chemical structures including celastrol benzyl ester (*structure 3*), pristimerol (*structure 6*), pristimerol diacetate (*structure 8*), and the triacetate of celastrol (*structure 9*)

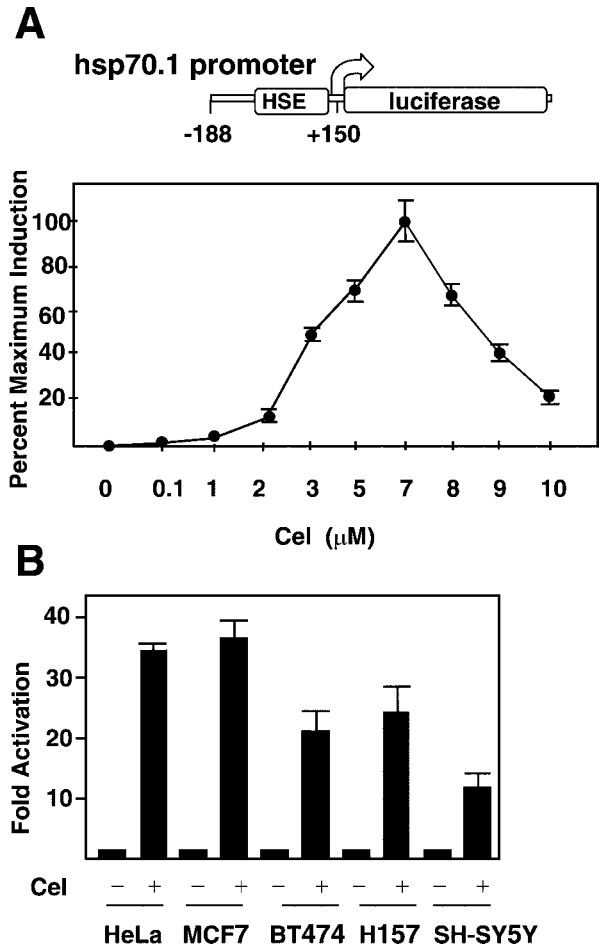

FIG. 1. **Celastrol activates a heat shock promoter reporter, and this activity is not cell-type specific.** *A*, celastrol activates the hsp70.1pr-luc with an EC_{50} of 3 μM. Stable HeLa hsp70.1pr-luc cells were treated with the indicated concentrations of celastrol, and luciferase activity was determined 24 h later. Each experiment was performed in triplicate. The hsp70.1pr-luc construct is diagrammed. *B*, celastrol activates the hsp70.1pr-luc reporter in a variety of cell types. HeLa, MCF7, BT474, H157, and SH-SY5Y cells were transiently transfected with the hsp70.1pr-luc reporter. 24 h later, the cells were treated with or without 3 μM celastrol for 24 h in triplicate and then harvested and assayed for luciferase activity.

were inactive at all concentrations tested for induction of the hsp70.1 promoter reporter. To further examine the selectivity exhibited by celastrol, we tested other multi-ring compounds in a concentration range of 1–10 μM, including various quinone-methides, anthrones, and anthraquinones, for activation of the heat shock response and found none of them to be active (Fig. 2*B*). Therefore, our results reveal that the activity of celastrol is highly selective, because only celastrol and a small number of celastrol derivatives can activate the hsp70.1pr-luc reporter. Because celastrol is the most potent of the small molecules tested, further analysis was performed with this compound.

Celastrol Induces HSF1 Activity—Stress-induced activation of the heat shock response is regulated by HSF1, which binds to the conserved heat shock element corresponding to reiterated inverted and adjacent arrays of the pentamer 5′-nGAAn-3′. To assess whether celastrol-dependent activation of the human hsp70 promoter was due to induction of HSF1 DNA binding-activity, we performed electrophoretic gel mobility shift assays using radiolabeled heat shock element-containing oligonucleotides. The results indicate that HSF1 DNA binding activity is induced within 10 min of exposure to celastrol and persists through to the last time point assayed of 240 min (Fig. 3*A*, *upper panel*). It is of interest to note that the kinetics of HSF1 activation by celastrol and heat shock are similar (Fig. 3*A*). Because multiple phosphorylated states of HSF1 can exist with different activities (12, 30–32), we then examined the phosphorylation state of celastrol-induced HSF1. The different phosphorylated states of HSF1 are readily distinguished by their SDS-PAGE mobility; constitutively phosphorylated HSF1 migrates more rapidly than the stress-activated hyperphosphorylated HSF1. For both celastrol and heat shock treatments, the hyperphosphorylated HSF1 is detected by 30 min (Fig. 3*A*, *lower panel*). By 120 min, most of the HSF1 exists in the hyperphosphorylated state, as indicated by the band of higher mobility and the disappearance of the lower band. As has been observed previously, the time course of hyperphosphorylation lags behind the kinetics of HSF1 DNA binding (8).

To verify the biological significance of celastrol treatment on HSF1 DNA binding activity *in vivo*, we performed chromatin immunoprecipitation experiments to assess binding to the endogenous hsp70.1 promoter, a known downstream target. HeLa cells treated with either celastrol or heat shock for the indicated times were cross-linked and harvested, and chromatin was immunoprecipitated using a polyclonal antibody against human HSF1. PCR analysis using primers specific for the hsp70.1 promoter shows that both heat shock and celastrol induce HSF1 binding to the promoter *in vivo* with similar profiles over the time course of treatment (10–240 min; Fig. 3*B*). Specificity is indicated by the lack of HSF1 binding to the dihydrofolate reductase promoter by celastrol or heat shock treatment (data not shown).

Mechanism of HSF1 Activation by Celastrol—To further examine the effect of celastrol on HSF1 activation, we made use of a GAL4-HSF1 fusion construct to directly test for an affect on the transactivation function of DNA-bound HSF1. In this construct, the HSF1 DNA-binding domain is replaced by the DNA-binding domain of GAL4, resulting in a chimeric HSF1 that binds DNA constitutively but is not fully active until exposed to heat shock or other stresses (26). Consequently, when transfected together with a GAL4-luciferase reporter, GAL4-HSF1 is activated upon heat shock (Fig. 4). Celastrol treatment has a similar effect on activation of the hsp70 promoter reporter (4.4-fold for heat shock and 5.5-fold for celastrol). Therefore, in addition to the induction of HSF1 DNA binding and hyperphosphorylation (Fig. 3), celastrol treatment induces the transcriptional activity of a chimeric GAL4-HSF1 that is constitutively bound to DNA.

A feature of modulators of the heat shock response is that certain stressors can have combinatorial synergy with heat stress (3, 4, 33). Therefore, we examined whether suboptimal levels of heat shock and celastrol could have a synergistic effect on endogenous HSF1. Whereas separate exposure to either a 41 °C heat shock or 1.5 μM celastrol does not fully activate the hsp70.1pr-luc construct (relative to a 42 °C heat shock), simultaneous exposure to both 1.5 μM celastrol and 41 °C resulted in maximal levels of hsp70 promoter activity (Fig. 5). From these results, we conclude that celastrol treatment can lower the temperature threshold required for the heat shock response.

Celastrol Effects on Endogenous Heat Shock Gene Expression—We next wanted to test the effects of celastrol treatment on the expression of endogenous heat shock genes in both HeLa and the neuroblastoma cell line SH-SY5Y. In HeLa cells, the Hsp70 protein levels induced by treatment with 3 μM celastrol (8 h) are similar to levels achieved following 42 °C heat shock (1 h followed by 7 h of recovery; Fig. 6). The accumulation of Hsp70 corresponded to an increase in the levels of hsp70.1 mRNA. Because the heat shock response is generally not as strong in neuronal cells as in HeLa cells, we also tested the neuronal cell line SH-SY5Y. Induction of Hsp70 protein and

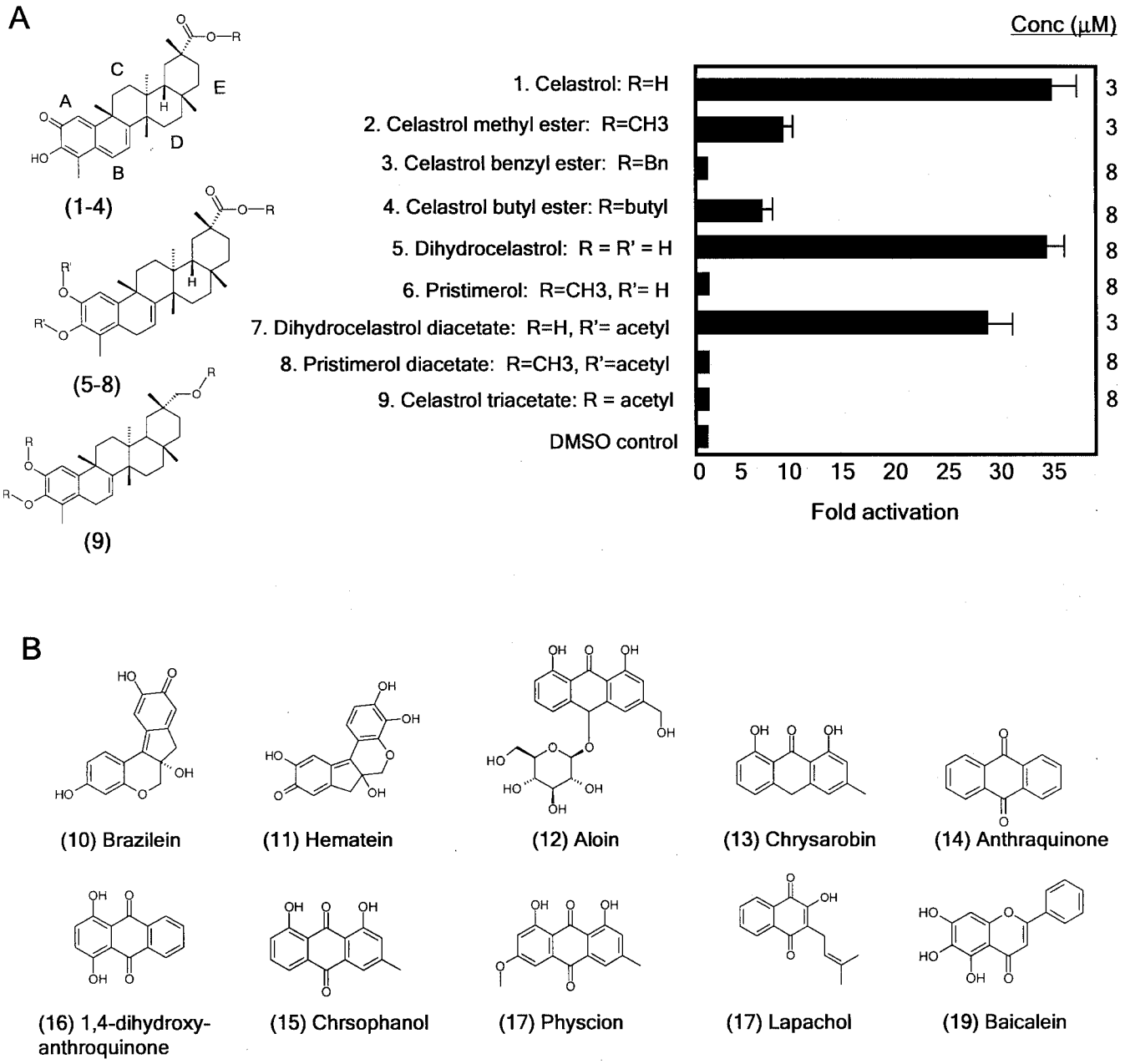

FIG. 2. **Celastrol activates a heat shock promoter reporter and this activity is specific to the structure of the molecule.** *A*, structure-function analysis of celastrol. HeLa hsp70.1pr-luc cells were treated with either 3 or 8 μM of the indicated celastrol derivatives for 24 h prior to luciferase analysis. *B*, other multi-ring compounds do not activate the reporter in HeLa hsp70.1pr-luc cells. A variety of quinone methides, anthrones, anthraquinones, and other multi-ring compounds were tested for activation of the hsp70.1pr-luc reporter at concentrations (*Conc*) up to 10 μM. None of the compounds shown here (*structures 10–19*) were active.

hsp70.1 mRNA is clearly observed in SH-SY5Y cells treated with either celastrol or heat shock, although the Hsp70 protein levels are lower than in HeLa cells (Fig. 6). Because Hsp40 can work together with Hsp70 as a cochaperone and Hsp27 has been implicated in protection from apoptosis, we tested whether these mRNAs were also induced. We found that celastrol, like heat shock, can induce these mRNAs as well (data not shown). Therefore, our results show that celastrol induces a "classical" heat shock response as indicated by increases in Hsp protein and mRNA expression levels.

Celastrol Is Cytoprotective against Severe Stress—Molecular chaperones have been shown to have wide ranging abilities to protect stressed cells from the acute or chronic consequences of protein damage; this cellular phenomenon is known as cytoprotection (34–37). To determine whether celastrol treatment can have a cytoprotective effect through the induction of heat shock proteins, we tested the ability of celastrol to protect cells from severe stress. For the cytoprotection assay, we employed protection from a 45 °C heat stress, a standard condition used in stress cytoprotection. HeLa cells (Fig. 7*A*) and SH-SY5Y cells (data not shown) were pretreated with either 3 μM celastrol or a 42 °C heat shock for 1 h, recovered for 5 h, and then given a 20- or 40-min 45 °C heat treatment. The fraction of cell death, measured by trypan blue uptake, was measured 24 h later. Pretreatment with either celastrol or 42 °C heat shock were both able to significantly protect cells from cell death induced by 20 or 40 min at 45 °C.

To further assess the potential of celastrol to protect cells from stress-induced apoptosis, similarly pretreated cells were heat-shocked at 45 °C for 35 min and assayed 24 h later for cytosolic nucleosomes, an indicator of apoptotic cell death, using the Cell Death enzyme-linked immunosorbent assay (Roche

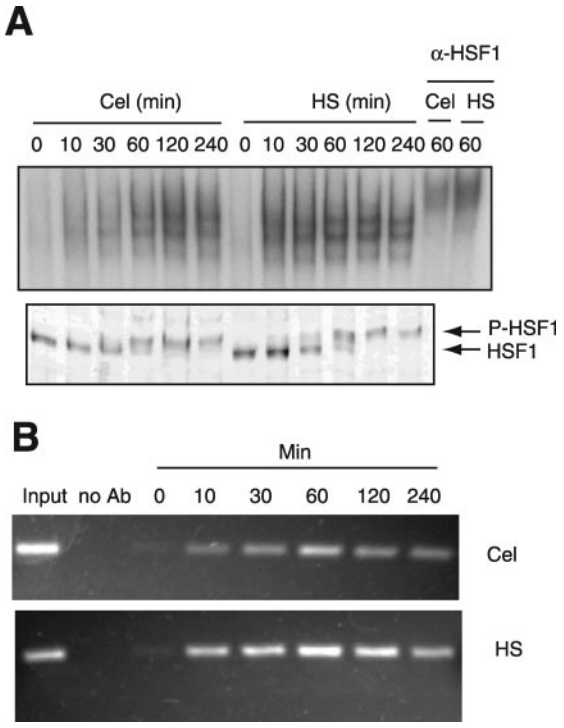

FIG. 3. **Celastrol induces HSF1 DNA binding and hyperphosphorylation.** *A*, *upper panel*, gel mobility shift analysis of whole cell extracts from HeLa cells treated with 3 μM celastrol or 42 °C heat shock for the indicated periods of time shows that both celastrol and heat shock can induce DNA binding. Supershift analysis shows that the induced DNA-protein complexes contain HSF1. *Lower panel*, 3 μM celastrol and 42 °C heat shock induce hyperphosphorylation of HSF1 in HeLa cells. The same whole cell extracts used for the gel shifts were analyzed by Western analysis for HSF1 hyperphosphorylation, as indicated by the more slowly migrating band. *B*, chromatin immunoprecipitation experiments performed at the indicated time points show that both 3 μM celastrol and 42 °C heat shock induce HSF1 binding to the hsp70.1 promoter *in vivo*. Chromatin was cross-linked, harvested, and immunoprecipitated with an antibody specific for HSF1. The samples were then analyzed by PCR with primers specific for the hsp70.1 promoter. The controls include input DNA before immunoprecipitation and a no antibody control.

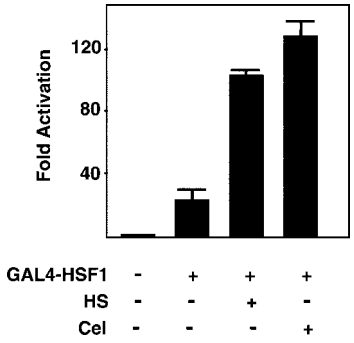

FIG. 4. **Celastrol functions through the activation domain of HSF1.** 293T cells were transfected with a GAL4-luc reporter and a GAL4-HSF1 fusion construct. 24 h after transfection, the cells were treated with 3 μM of celastrol (*Cel*) for 24 h or a 42 °C heat shock (*HS*) for 1 h followed by a 23-h recovery. The data are plotted as fold luciferase activation over the no treatment control. The experiments were performed in triplicate.

Applied Science). HeLa or SH-SY5Y cells, which were treated with a 45 °C heat shock alone, showed between 3- and 4-fold enrichment of cytosolic nucleosomes. As expected, pretreatment of HeLa cells with a 42 °C heat shock protected from apoptotic cell death as measured by a decrease in cytosolic nucleosomes. Likewise, celastrol pretreatment also protected HeLa cells from apoptosis (1.7-fold), and similar results were

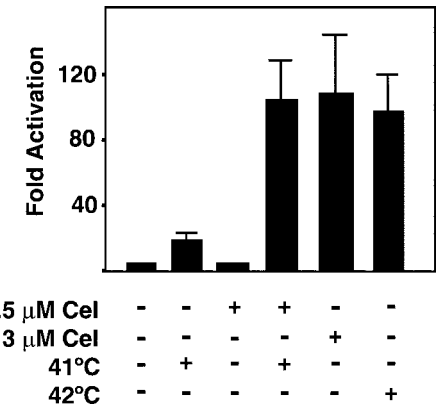

FIG. 5. **Celastrol can synergize with heat stress.** HeLa hsp70.1pr-luc cells were treated with a 1-h heat shock at the indicated temperatures or with celastrol (*Cel*) at the indicated concentrations. Luciferase activity was measured 24 h later.

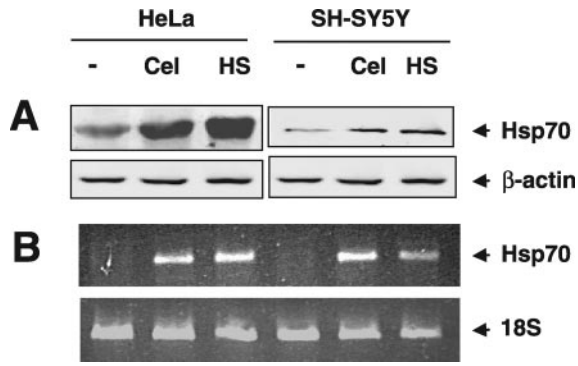

FIG. 6. **Celastrol induces Hsp70 protein and mRNA levels.** *A*, Western analysis shows that treatment of HeLa and SH-SY5Y cells with 3 μM celastrol (*Cel*) induces Hsp70 protein expression to a similar extent as heat shock (*HS*). The cells were treated with 3 μM celastrol for 8 h prior to analysis. Heat shock was performed at 42 °C for 1 h, followed by recovery for 7 h. β-Actin was used as a loading control. *B*, reverse transcription-PCR shows induction of hsp70.1 mRNA upon treatment with 3 μM celastrol or 42 °C heat shock. The extracts used to make the RNA were from the same samples as in *A*. 18 S rRNA primers were used as a control for equal RNA levels.

obtained for SH-SY5Y neuroblastoma cells (Fig. 7*B*). Therefore, celastrol pretreatment can protect cells from a subsequent lethal heat stress, causing a decrease in total cell death and a corresponding decrease in apoptotic cell death. These results demonstrate that celastrol is effective as a cytoprotective agent against stress-induced cell death.

DISCUSSION

The studies presented here identify celastrol as a founding member of a new class of molecules of the triterpene family with pharmacological activity to induce the human heat shock response in neuronal and non-neuronal tissue cells. Celastrol exhibits kinetics of induction similar to that observed for heat shock. Our data shows that the effect of celastrol treatment is on the activity of HSF1 at multiple levels, including activation of DNA binding, hyperphosphorylation of HSF1, and transcriptional activation of heat shock genes. Increased levels of HSF1 DNA binding to the heat shock element in the Hsp70 promoter are observed both *in vitro* by electromobility shift assay and in *in vivo* experiments using chromatin immunoprecipitation, suggesting that all downstream events regulated by HSF1 are affected.

Activators of the heat shock response have often been linked to the generation of unfolded proteins or to the inactivation of molecular chaperones that participate in feedback inhibition of the HSF1 transcriptional response. To address whether celas-

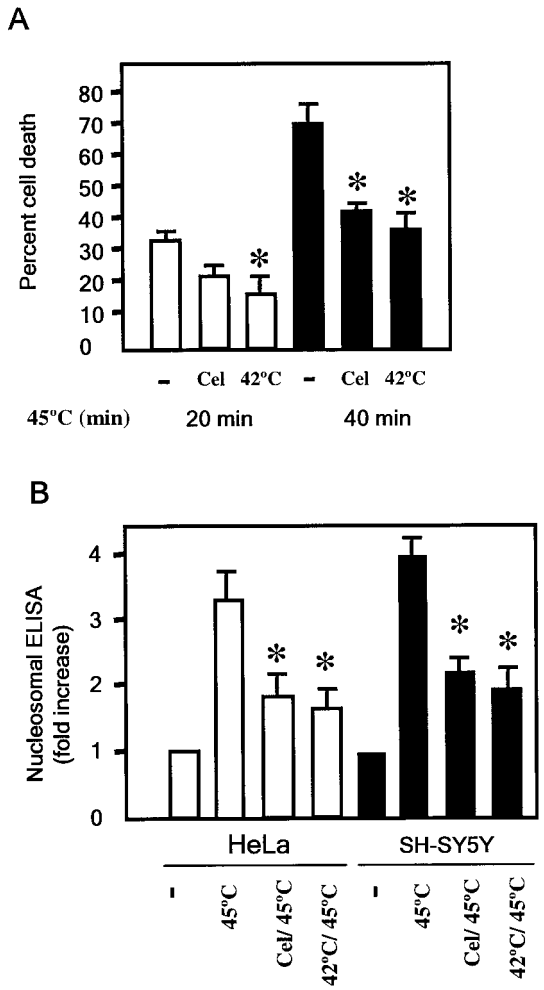

FIG. 7. **Celastrol protects cells from severe stress.** *A*, celastrol (*Cel*) protects cells from 45 °C heat-induced cell death. HeLa cells were pretreated with 3 μM celastrol for 1 h, washed three times and given fresh medium, and then recovered for 5 h prior to a 20 or 40 min 45 °C heat treatment. 24 h later, the percentage of cell death was determined by trypan blue uptake. As controls, the cells either received no pretreatment or a 1-h 42 °C pretreatment. The differences in group means were compared by the Student's *t* test. A *p* value <0.05 (*) was considered statistically significant. *B*, celastrol pretreatment protects cells from 45 °C heat-induced apoptosis. HeLa and SH-SY5Y cells were either treated or not treated with 3 μM celastrol for 1 h, followed by washing to remove the celastrol and a 5-h recovery at 37 °C. The cells were then treated for 35 min with a 45 °C lethal heat treatment, returned to 37 °C, and allowed to grow for 24 h prior to analysis of cytoplasmic nucleosomes using the Cell Death Elisa Plus method (Roche Applied Science). The results are plotted as fold enriched nucleosomes as compared with the no treatment control. The differences in group means were compared by the Student's *t* test. A *p* value <0.05 (*) was considered statistically significant. *ELISA*, enzyme-linked immunosorbent assay

trol interfered with the chaperone function of Hsp70, we employed *in vitro* assays in which the folding of a chemically denatured substrate could be monitored as a function of Hsp70 activity. Our results show that celastrol has no effect on Hsp70 (Supplemental Fig. 2*A*). We also tested celastrol to see whether it directly causes global protein denaturation using luciferase, a thermolabile enzyme that can be monitored in assays for chaperone activity. Incubation of luciferase with celastrol over a 30-min time course did not affect luciferase activity *in vitro* (Supplemental Fig. 2*B*). Likewise, celastrol had no effect on luciferase activity expressed in mammalian cells (Supplemental Fig. 2*C*). Therefore, we conclude that celastrol does not have global effects on protein folding or Hsp70 chaperone activity. The recent identification of nearly 200 genes that control protein folding quality control (38) offers other potential targets for celastrol.

Celastrol is a member of the triterpenoid family of compounds that are known for their anti-inflammatory and anti-tumor properties (39, 40). Triterpenoids inhibit the activity of DNA polymerase α and β, polymerases involved in DNA replication and repair, respectively (41, 42). Triterpenoids also inhibit DNA topoisomerase I and II, molecules that catalyze the breaking and rejoining of DNA and that are required for DNA replication, repair, and recombination (41). Because many anti-cancer drugs work by interfering with DNA polymerases and topoisomerases, this could account for the anti-tumor effects of triterpenoids. Triterpenoids also have been implicated in altering signaling pathways, including the up-regulation of transforming growth factor β/Smad signaling and the down-regulation of nuclear factor κB (NF-κB) signaling, both resulting in anti-inflammatory consequences (43, 44). The down-regulation of NF-κB by celastrol is especially interesting because many compounds that activate the heat shock response also inhibit NF-κB, indicating a potential link between the two systems (1). In support of this, the kinases GSK3, extracellular signal-regulated kinase, protein kinase C, and c-Jun N-terminal kinase have all been implicated in both NF-κB and HSF1 regulation, with opposite effects in each system (32, 45). Direct phosphorylation of HSF1 by these kinases has been implicated in transcriptional repression, whereas direct phosphorylation of the RelA subunit of NF-κB by these kinases has been implicated in increased transcriptional activity. Calcium/calmodulin-dependent protein kinase II is an exception to this rule, with a positive transcriptional effect on both HSF1 and NF-κB (31, 46). However, because several kinases do have opposite transcriptional effects in the two systems and NF-κB activation has been linked to HSF1 inhibition and vice versa, it is possible that celastrol alters a kinase/phosphatase balance that is in common to both signaling systems.

Although anti-inflammatory and anti-tumor effects seem to be general properties of the triterpenoid family of compounds, our analysis of celastrol and 19 chemically related compounds reveals that the chemical structure of celastrol appears highly specific for activation of the human heat shock response. We show that only celastrol, celastrol methyl ester, and dihydrocelastrol diacetate are similarly active. Celastrol butyl ester and dihydrocelastrol also have effects on heat shock activity, although at higher concentrations. Included in our assays were other quinone methides, known to be generally reactive in oxidative pathways (47). Neither brazilein nor hematein could activate the heat shock response, indicating that simply having an active quinone methide is insufficient. Moreover, dihydrocelastrol diacetate is not a quinone methide, yet it is highly active. Activation of the heat shock response, therefore, must be related to specific structural features of celastrol and not because of a generic ring structure, because celastrol benzyl ester does not have an effect on the heat shock reporter (up to 10 μM concentration), whereas celastrol methyl ester is active. The topology of the A/B rings (Fig. 2) of celastrol is different from that of dihydrocelastrol diacetate, which has a reduced ring structure, yet both compounds are active. Although relatively small changes in structure appear to have large activity effects, it is not yet clear whether the differences in activity are due to changes in binding to a putative protein target or due to differences in stability, absorption, distribution, or metabolism (*i.e.* pharmacokinetics) of the various compounds in our whole cell assay. Interestingly, when the same set of compounds was tested for inhibition of an NF-κB-driven reporter, an identical

chemical specificity was observed.[2]

Celastrol has several potential advantages relative to other known small molecule modulators of the heat shock response. First, the kinetics of activation of the heat shock response by celastrol shares many of the same kinetic features of heat shock, such as rapid activation within minutes and to the same magnitude of induction. Other chemical modulators of the heat shock response, such as nonsteroidal anti-inflammatory drugs, hemin, proteasome inhibitors, and serine protease inhibitors, exhibit a delayed activation of HSF1 and heat shock genes similar to the 2–4-h kinetics observed for heavy metals (3, 7, 48–51). Second, celastrol has a relatively low EC_{50} value of 3 μM, compared with millimolar levels for other compounds. This EC_{50} value is in a range that could potentially be lowered to the nanomolar level through further compound optimization. Third, previously reported *in vivo* effects of celastrol are promising. Extracts containing celastrol have been given to Chinese patients for many years without published reports of carcinogenicity or other limiting side effects (22, 23). Additionally, in rat models for Alzheimer's disease, celastrol at 7 μg/kg (equivalent to about a 0.25-mg dose for a human adult) was found to improve memory, learning, and psychomotor activity (39). Perhaps some of the pharmacological effects of celastrol in this rodent model for neurodegenerative disease could also be due to effects on heat shock gene expression.

The induction of molecular chaperones has been shown to be cytoprotective against a variety of stresses, including DNA damage, UV irradiation, serum withdrawal, chemotherapeutic agents, and lethal heat stress (52). This may be due in part to the known anti-apoptotic functions of the chaperones Hsp70, Hsp40, Hsp27, and Hsp90 (53–55). Heat shock proteins have been demonstrated to intervene at multiple points in the apoptotic pathway, including inhibition of c-Jun N-terminal kinase activation, prevention of cytochrome *c* release and disruption of apoptosome formation (37, 56–60). The transcriptional up-regulation of molecular chaperones caused by an intermediate heat shock therefore has cytoprotective benefits against subsequent stresses such as a lethal 45 °C heat stress. In our experiments, we observe that celastrol can induce cytoprotection against a lethal heat stress in both HeLa cells and the neuroblastoma cell line SH-SY5Y to a similar extent as a 42 °C heat shock. These results show that celastrol may have broadly protective effects in a wide range of cellular pathologies associated with cell damage and proteotoxicity through the up-regulation of the human heat shock response and induction of molecular chaperones.

In addition to their therapeutic uses, small molecules are often useful tools to dissect molecular pathways. Studies with the drug sodium salicylate, for instance, were the first to show that HSF1 activation involves a multi-step process (7, 8). Like sodium salicylate, celastrol may also prove to be a valuable tool for furthering our knowledge of the activation of the heat shock response. Further studies with celastrol may also lead to additional therapeutic targets for modulation of the heat shock response.

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[2] S. D. Westerheide, J. P. Devlin, R. B. Silverman, and R. I. Morimoto, unpublished data.

REFERENCES

1. Morimoto, R. I., and Santoro, M. G. (1998) *Nat. Biotechnol.* **16,** 833–838
2. Hughes, R. E., and Olson, J. M. (2001) *Nat. Med.* **7,** 419–423
3. Lee, B. S., Chen, J., Angelidis, C., Jurivich, D. A., and Morimoto, R. I. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7207–7211
4. Vigh, L., Literati, P. N., Horvath, I., Torok, Z., Balogh, G., Glatz, A., Kovacs, E., Boros, I., Ferdinandy, P., Farkas, B., Jaszlits, L., Jednakovits, A., Koranyi, L., and Maresca, B. (1997) *Nat. Med.* **3,** 1150–1154
5. Morley, J. F., and Morimoto, R. I. (2004) *Mol. Biol. Cell* **15,** 657–664
6. Hsu, A. L., Murphy, C. T., and Kenyon, C. (2003) *Science* **300,** 1142–1145
7. Jurivich, D. A., Sistonen, L., Kroes, R. A., and Morimoto, R. I. (1992) *Science* **255,** 1243–1245
8. Cotto, J. J., Kline, M., and Morimoto, R. I. (1996) *J. Biol. Chem.* **271,** 3355–3358
9. Ali, A., Bharadwaj, S., O'Carroll, R., and Ovsenek, N. (1998) *Mol. Cell. Biol.* **18,** 4949–4960
10. Zou, J., Guo, Y., Guettouche, T., Smith, D. F., and Voellmy, R. (1998) *Cell* **94,** 471–480
11. Bagatell, R., Paine-Murrieta, G. D., Taylor, C. W., Pulcini, E. J., Akinaga, S., Benjamin, I. J., and Whitesell, L. (2000) *Clin. Cancer Res.* **6,** 3312–3318
12. Larson, J. S., Schuetz, T. J., and Kingston, R. E. (1988) *Nature* **335,** 372–375
13. Mosser, D. D., Kotzbauer, P. T., Sarge, K. D., and Morimoto, R. I. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3748–3752
14. Zimarino, V., Tsai, C., and Wu, C. (1990) *Mol. Cell. Biol.* **10,** 752–759
15. Westwood, J. T., Clos, J., and Wu, C. (1991) *Nature* **353,** 822–827
16. Wu, C. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 441–469
17. Pirkkala, L., Nykanen, P., and Sistonen, L. (2001) *FASEB J.* **15,** 1118–1131
18. Abbott, A. (2002) *Nature* **417,** 109
19. Heemskerk, J., Tobin, A. J., and Bain, L. J. (2002) *Trends Neurosci.* **25,** 494–496
20. Chen, B., Duan, H., and Takaishi, Y. (1999) *Phytochemistry* **51,** 683–697
21. Ngassapa, O., Soejarto, D. D., Pezzuto, J. M., and Farnsworth, N. R. (1994) *J. Nat. Prod.* **57,** 1–8
22. Li, R. L., and Shu, D. F. (1989) *Investigations and Clinical Applications of Trysterygium wilfordü hook F*, China Science and Technology Press, Beijing, China
23. Gunatilaka, A. A. L., Herz, W., Kirby, G. W., Moore, R. E., Steglich, W., and Tamm, C. (1996) *Triterpenoid Quinonemethides and Related Compounds* (*Celastroids*) Springer, Vienna
24. Williams, G. T., McClanahan, T. K., and Morimoto, R. I. (1989) *Mol. Cell. Biol.* **9,** 2574–2587
25. Michels, A. A., Nguyen, V. T., Konings, A. W., Kampinga, H. H., and Bensaude, O. (1995) *Eur. J. Biochem.* **234,** 382–389
26. Shi, Y., Kroeger, P. E., and Morimoto, R. I. (1995) *Mol. Cell. Biol.* **15,** 4309–4318
27. Sarge, K. D., Murphy, S. P., and Morimoto, R. I. (1993) *Mol. Cell. Biol.* **13,** 1392–1407
28. Mosser, D. D., Theodorakis, N. G., and Morimoto, R. I. (1988) *Mol. Cell. Biol.* **8,** 4736–4744
29. Beresford, G. W., and Boss, J. M. (2001) *Nat. Immunol.* **2,** 652–657
30. Sorger, P. K. (1990) *Cell* **62,** 793–805
31. Holmberg, C. I., Hietakangas, V., Mikhailov, A., Rantanen, J. O., Kallio, M., Meinander, A., Hellman, J., Morrice, N., MacKintosh, C., Morimoto, R. I., Eriksson, J. E., and Sistonen, L. (2001) *EMBO J.* **20,** 3800–3810
32. Holmberg, C. I., Tran, S. E., Eriksson, J. E., and Sistonen, L. (2002) *Trends Biochem. Sci.* **27,** 619–627
33. Jurivich, D. A., Sistonen, L., Sarge, K. D., and Morimoto, R. I. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2280–2284
34. Solomon, J. M., Rossi, J. M., Golic, K., McGarry, T., and Lindquist, S. (1991) *New Biol.* **3,** 1106–1120
35. Elia, G., Amici, C., Rossi, A., and Santoro, M. G. (1996) *Cancer Res.* **56,** 210–217
36. Wissing, D., and Jaattela, M. (1996) *Int. J. Hyperthermia* **12,** 125–138
37. Mosser, D. D., Caron, A. W., Bourget, L., Denis-Larose, C., and Massie, B. (1997) *Mol. Cell. Biol.* **17,** 5317–5327
38. Nollen, E. A., Garcia, S. M., van Haaften, G., Kim, S., Chavez, A., Morimoto, R. I., and Plasterk, R. H. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6403–6408
39. Allison, A. C., Cacabelos, R., Lombardi, V. R., Alvarez, X. A., and Vigo, C. (2001) *Prog. Neuropsychopharmacol. Biol. Psychiatry* **25,** 1341–1357
40. Chang, F. R., Hayashi, K., Chen, I. H., Liaw, C. C., Bastow, K. F., Nakanishi, Y., Nozaki, H., Cragg, G. M., Wu, Y. C., and Lee, K. H. (2003) *J. Nat. Prod.* **66,** 1416–1420
41. Mizushina, Y., Iida, A., Ohta, K., Sugawara, F., and Sakaguchi, K. (2000) *Biochem. J.* **350,** 757–763
42. Deng, J. Z., Starck, S. R., and Hecht, S. M. (2000) *Bioorg. Med. Chem.* **8,** 247–250
43. Jin, H. Z., Hwang, B. Y., Kim, H. S., Lee, J. H., Kim, Y. H., and Lee, J. J. (2002) *J. Nat. Prod.* **65,** 89–91
44. Suh, N., Roberts, A. B., Birkey Reffey, S., Miyazono, K., Itoh, S., ten Dijke, P., Heiss, E. H., Place, A. E., Risingsong, R., Williams, C. R., Honda, T., Gribble, G. W., and Sporn, M. B. (2003) *Cancer Res.* **63,** 1371–1376
45. Chen, L. F., and Greene, W. C. (2004) *Nat Rev Mol. Cell. Biol.* **5,** 392–401
46. Meffert, M. K., Chang, J. M., Wiltgen, B. J., Fanselow, M. S., and Baltimore, D. (2003) *Nat. Neurosci.* **6,** 1072–1078
47. Thompson, D. C., Thompson, J. A., Sugumaran, M., and Moldeus, P. (1993) *Chem. Biol. Interact.* **86,** 129–162
48. Liu, R. Y., Corry, P. M., and Lee, Y. J. (1994) *J. Cell Sci.* **107,** 2209–2214
49. Holmberg, C. I., Illman, S. A., Kallio, M., Mikhailov, A., and Sistonen, L. (2000) *Cell Stress Chaperones* **5,** 219–228
50. Sistonen, L., Sarge, K. D., and Morimoto, R. I. (1994) *Mol. Cell. Biol.* **14,** 2087–2099
51. Rossi, A., Elia, G., and Santoro, M. G. (1998) *J. Biol. Chem.* **273,** 16446–16452

52. Samali, A., and Orrenius, S. (1998) *Cell Stress Chaperones* **3,** 228–236
53. Beere, H. M. (2001) *Science's STKE* http://stke.sciencemag.org/cgi/content/full/sigtrans;2001/re1
54. Mosser, D. D., and Morimoto, R. I. (2004) *Oncogene* **23,** 2907–2918
55. Sreedhar, A. S., and Csermely, P. (2004) *Pharmacol. Ther.* **101,** 227–257
56. Gabai, V. L., Meriin, A. B., Mosser, D. D., Caron, A. W., Rits, S., Shifrin, V. I., and Sherman, M. Y. (1997) *J. Biol. Chem.* **272,** 18033–18037
57. Creagh, E. M., Carmody, R. J., and Cotter, T. G. (2000) *Exp. Cell Res.* **257,** 58–66
58. Beere, H. M., Wolf, B. B., Cain, K., Mosser, D. D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R. I., Cohen, G. M., and Green, D. R. (2000) *Nat. Cell Biol.* **2,** 469–475
59. Saleh, A., Srinivasula, S. M., Balkir, L., Robbins, P. D., and Alnemri, E. S. (2000) *Nat. Cell Biol.* **2,** 476–483
60. Mosser, D. D., Caron, A. W., Bourget, L., Meriin, A. B., Sherman, M. Y., Morimoto, R. I., and Massie, B. (2000) *Mol. Cell. Biol.* **20,** 7146–7159