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2,4-Bis(4-hydroxybenzyl)phenol Inhibits Heat Shock Transcription Factor 1 and Sensitizes Lung Cancer Cells to Conventional Anticancer Modalities

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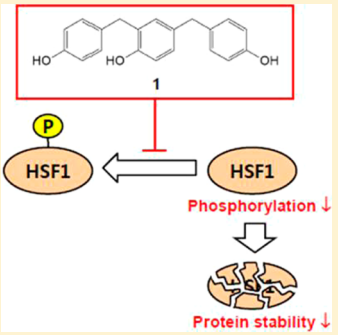
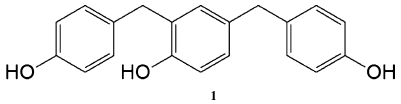
Supporting Information

ABSTRACT: Heat shock factor 1 (HSF1) is a transcription factor that regulates expression of heat shock protein (HSP) genes in response to stress. HSPs are expressed at high levels in a wide range of tumors. It has been reported that HSF1 and HSPs are associated closely in tumorigenesis. In the present study, a screen was performed using a luciferase reporter under the control of a heat shock element to find inhibitors of HSF1 activity, and 2,4-bis(4-hydroxybenzyl)phenol (**1**), isolated from the rhizomes of *Gastrodia elata*, was identified as an active compound. This substance effectively inhibited HSF1 activity and decreased levels of HSP27 and HSP70. Compound **1** induced the degradation of HSF1 protein through dephosphorylation of HSF1 on S326, which decreases HSF1 protein stability. In addition, **1** also induced growth arrest and apoptosis of NCI-H460 human lung cancer cells. Markers of apoptosis, such as cleaved PARP and cleaved caspase-3, were detected after treatment with **1**. Furthermore, cotreatment with **1** and conventional anticancer modalities such as paclitaxel, cisplatin, or ionizing radiation potentiated their effects on lung cancer cells. These results suggest that inhibition of HSF1 by **1** may help overcome resistance to conventional anticancer modalities in HSF1-overexpressed cancer cells.



Heat shock factor 1 (HSF1) is a master regulator of the heat shock response and a transcription factor that regulates the expression of heat shock proteins (HSPs), which are conserved evolutionarily in eukaryotes.[1] HSF1 activates HSP transcription by binding to heat shock elements of the promoter regions of HSP genes.[2,3] HSPs, so-called stress proteins, are upregulated in response to various types of stress, caused by inflammation, toxins, hypoxia, ischemia, starvation, oxidative stress, and heavy metals.[4,5] However, HSPs are also expressed at high levels in a wide range of tumors and are associated with a poor prognosis and resistance to therapy.[6] Many reports have shown that HSF1 and HSPs play important roles in the development of tumors, including malignant transformation, cancer cell survival, and proliferation.[7,8] For example, studies have shown that HSF1 is able to facilitate initial oncogenic transformation and maintain the malignant phenotype of established cancer cell lines driven by diverse mutations.[9,10] In cell cultures and mice, the genetic ablation of *hsf1* effectively impairs cellular transformation and tumorigenesis driven by oncogene activation or tumor suppressor loss.[11,12] During malignant progression, HSPs become expressed at high levels to facilitate tumor cell growth and survival. Tumor cell death has been shown to be inhibited by the ability of HSP27 and HSP70 to block programmed cell death, including the intrinsic and extrinsic pathways of caspase-mediated apoptosis.[6] HSP70 also inhibits senescence by blocking the pro-senescence effects of wild-type p53.[13] HSP90 delays the onset of replicative senescence by binding telomerase.[14] Moreover, HSP27 and HSP70 have been closely associated with resistance to cancer therapy.[15,16]

In the present study, a new inhibitor of HSF1 activity was found by screening natural products that were isolated from several herbal medicines, including the rhizomes of *Gastrodia elata* Blume (Orchidaceae). From this species, 2,4-bis(4-hydroxybenzyl)phenol (**1**) was found to be as an inhibitor of HSF1 activity. The rhizomes of *G. elata* have been used as a traditional oriental medicine for treating headaches, dizziness, rheumatism, convulsions, and epilepsy. Previous phytochemical work on these rhizomes has resulted in the isolation of various types of phenolic compounds including compound **1**.[17,18] However, the biological activity and mechanisms of action of **1** have not yet been reported except for a relaxant effect on smooth muscle.[19] The present study demonstrates that 2,4-bis(4-hydoxybenzyl)phenol exerts a potential anticancer effect via the suppression of HSPs, and this effect is involved in the dephosphorylation and degradation of HSF1 protein.



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RESULTS AND DISCUSSION

To identify novel inhibitors of HSF1 activity, a luciferase reporter under the control of a heat shock element was used as an assay to screen several natural compounds isolated from selected herbal medicines. 2,4-Bis(4-hydroxybenzyl)phenol (**1**),

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isolated from the rhizomes of *G. elata*, was shown to be an inhibitor of HSF1 activitiy. To evaluate whether **1** really regulates HSF1 activity, human lung cancer NCI-H460 cells were treated with **1** of the indicated concentration from 0 to 100 μM for 12 h prior to conducting a luciferase reporter assay. The luciferase was placed under the control of a heat shock element of *hsp25*, which is the murine form of human *hsp27*, or *hsp70*. As shown in Figure 1, compound **1** inhibited *hsp25* and *hsp70* promoters in a dose-dependent manner. Since HSF1 is usually overexpressed in cancer cells,[20] it was examined whether inhibition of HSF1 functions by **1** is specific to cancer cells. For this purpose, normal cells of HSF1 knockout mouse embryonic fibroblast (HSF1+/+ and HSF1−/− MEF) cells were used and examined for the effects of **1**. Protein levels in HSF1, HSP25 (or HSP27), and HSP70 were dramatically lower in normal MEF cells than those in NCI-H460 cancer cells (Figure S1A, Supporting Information). Luciferase activities of *hsp25* or *hsp70* were also lower in normal MEF cells than in cancer cells (Figure S1B, Supporting Information). Moreover, the promoter activity of HSF1 inhibited by **1** in cancer cells was not detected in normal cells with a low expression of HSF1 (Figure S1C, Supporting Information). From the results obtained, it may be suggested that inhibition of HSF1 function by **1** was shown only in HSF1-overexpressed cancer cells, but not in normal cells with a lower expression of HSF1.

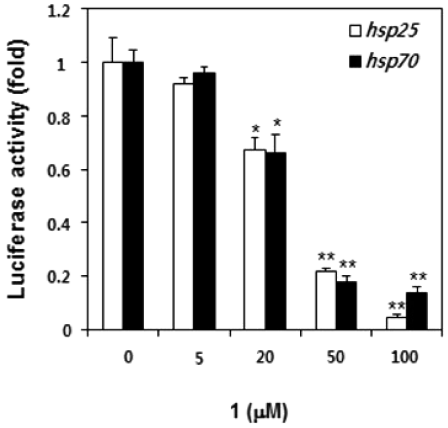

Figure 1. Inhibitory effect of **1** on *hsp25* and *hsp70* promoter activity. NCI-H460 cells were treated with the indicated concentration (0, 5, 20, 50, or 100 μM) of **1** for 12 h prior to assay using a luciferase reporter under the control of a heat shock element. Values are expressed as the fold relative to a negative control (DMSO). Each data point represents the mean ± SE of three experiments vs control (*p < 0.05, **p < 0.01).

To investigate the change in mRNA and protein expression levels of HSP27, HSP70, and HSF1 caused by **1**, quantitative RT-PCR and Western blot analysis were performed using RNA and protein extracted from NCI-H460 cells. Cells were treated with 0, 1, 3, 5, 10, or 20 μM **1** for 12 h or with 20 μM **1** for 0, 1, 3, 6, 12, or 24 h. Figure 2A shows that treatment with **1** decreased HSP27 and HSP70 cellular mRNA expression, but not that of HSF1. Compound **1** also decreased HSF1, HSP27, and HSP70 protein expression in a dose- and time-dependent manner in NCI-H460 cancer cells (Figure 2B and C).

Compound **1** induced a decrease in the HSF1 protein levels without a change in the mRNA levels. Consequently, **1** was tested to see if it influenced the stability of the HSF1 protein. When NCI-H460 cells were treated with the protein synthesis inhibitor cyclohexamide (10 μg/mL) with or without 10 μM **1**,

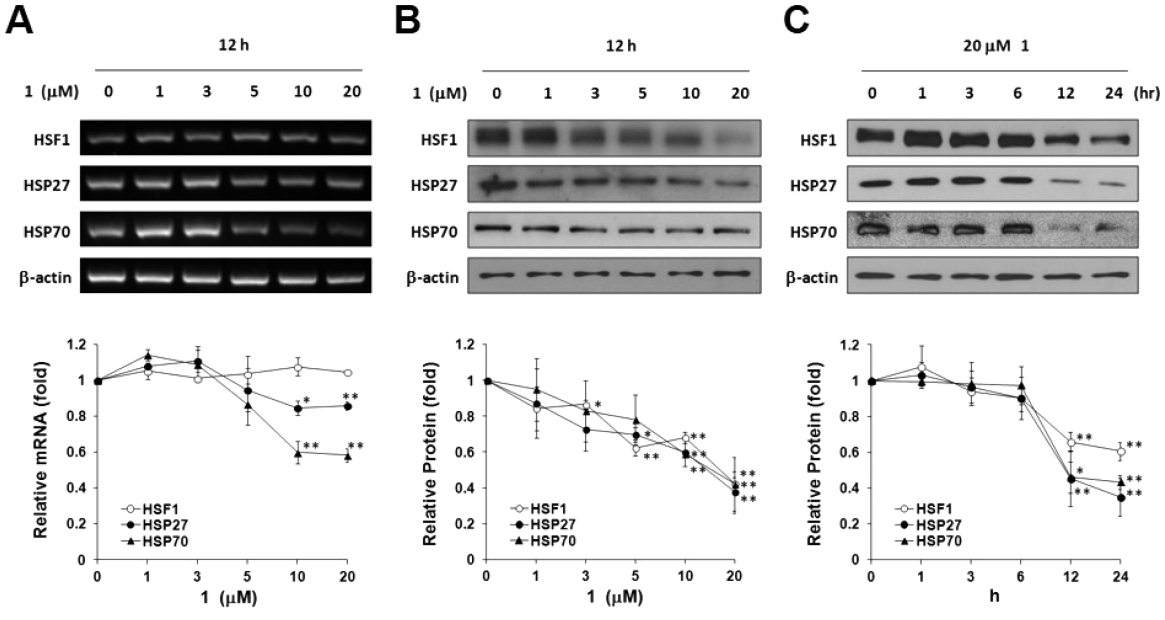

Figure 2. (A) Inhibitory effect of **1** on mRNA expression of HSP27 and HSP70, but not HSF1. NCI-H460 cells were treated with **1** at the indicated concentration (0, 1, 3, 5, 10, or 20 μM) for 12 h prior to RNA isolation. qRT-PCR was performed with HSF1, HSP27, and HSP70 primers. mRNA band intensity was normalized to the loading control band (β-actin) using the NIH ImageJ program. (B and C) Dose- and time-dependent inhibitory effects of **1** on HSF1, HSP27, and HSP70 protein levels. NCI-H460 cells were treated with **1** at the indicated concentration (0, 1, 3, 5, 10, or 20 μM) for 12 h or with 20 μM **1** for the indicated time (0, 1, 3, 6, 12, or 24 h) prior to protein extraction. Western blot analysis was performed with anti-HSF1, anti-HSP27, and anti-HSP70 antibodies. Protein band intensity was normalized to the loading control band (β-actin) using the NIH ImageJ program. Values are expressed as the fold relative to negative control (DMSO). Each data point represents the mean ± SE of three experiments vs control (*p < 0.05, **p < 0.01).

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cells treated with a combination of cyclohexamide and **1** showed a significantly faster degradation of HSF1 protein than cells treated with cyclohexamide alone (Figure 3). This suggested that **1** decreases the stability of HSF1 protein.

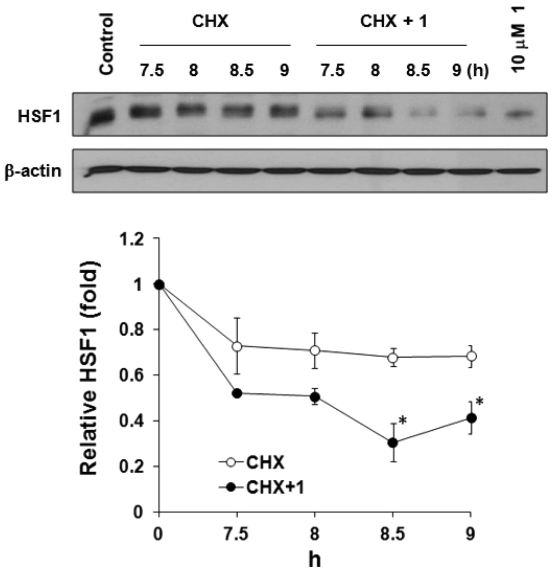

Figure 3. Effect of **1** on HSF1 stability in cyclohexamide (CHX)-treated NCI-H460 cells. Cells were incubated for the indicated time period (7.5, 8, 8.5, or 9 h) in the presence of CHX (10 μg/mL) with or without 10 μM **1**. Western blotting analysis was performed for the detection of HSF1 protein. Typical results are shown from three independent analyses. The intensity of the HSF1 protein band was measured and normalized to the loading control band (β-actin) using the NIH ImageJ program. Values are expressed as the fold relative to the negative control (DMSO). Each data point represents the mean ± SE of three experiments vs control (without **1**) (*p < 0.05, **p < 0.01).

Since post-translational modification of HSF1 by **1** has been shown to affect the stability of HSF1,[21] the changes of HSF1 phosphorylation induced by this compound **1** were evaluated. As shown in Figure 4A, **1** dephosphorylated HSF1 at S230, S303/307, and S326 in a dose-dependent manner. Moreover, the HSF1 band shifted as it was dephosphorylated by **1**, an observation in agreement with previous findings.[22] The dephosphorylation induced by **1** of HSF1 was the most dramatic at S326.

A previous report has provided evidence that phosphorylation of S326 contributes to activation of HSF1, but its significance as a regulatory mechanism was not explored.[23] To investigate whether phosphorylation of S326 in HSF1 is critical to protein stability, NCI-H460 cells were incubated in the presence of cyclohexamide (10 μg/mL) with transfection of constructs expressing either the phosphorylated form (S326E) or the dephosphorylated form (S326A) of HSF1. The comparison between protein levels of FLAG-tagged HSF1 mutants showed that the dephosphorylated form (S326A) of HSF1 was less stable and more subject to degradation than the phosphorylated form (S326E) (Figure 4B). This suggests for the first time that the dephosphorylation of HSF1 at S326 by **1** decreases the stability of HSF1 protein. Similar experiments focusing on S230 failed to show changes in protein half-life (data not shown).

Since the inhibition of HSF1 activity by **1** might be toxic to cancer cells, NCI-H460 cancer cell viability was measured after 24 h of treatment with various concentrations of **1** from 0 to 100 μM. As shown in Figure 5A, **1** induced cancer cell death. Furthermore, Figure 5B and C show that there was a dose- and time-dependent increase in apoptotic markers (cleaved PARP and cleaved caspase-3) and decrease in proliferative markers (cyclin D and cyclin E) in cancer cells after treatment with **1**. This result confirms that compound **1** induces cancer cell apoptosis.

To elucidate the role of HSF1 in **1**-induced cancer cell apoptosis, the viability of NCI-H460 cancer cells in which HSF1 had been silenced using siRNA transfection (siHSF1) was investigated. These cells were treated with various concentrations of **1** from 0 to 100 μM for 24 h. siControl was used as negative control for siHSF1. A comparison of survival between HSF1-suppressed cells and negative control cells showed that the ablation of HSF1 abolished the apoptotic effect of **1** (Figure 6A). Although **1** also displayed HSF1-independent cytotoxicity at high doses because the inhibition potential of HSP27 and HSP70 by **1** seems to be different; inhibition of HSP27 requires HSF1, while inhibition of HSP70 is independent of HSF1 (Figure 6B). Furthermore, protein levels of cleaved PARP and cleaved caspase3 did not markedly change in siHSF1-treated groups, indicating that induction of apoptosis by compound **1** is also independent of HSF1. However, Figure 6B confirmed that **1** induced more dramatic apoptosis of cancer cells in the presence of HSF1 than without it. This suggests that HSF1 plays a role in the cytotoxic effect of **1**. Moreover, when the cytotoxicity of **1** was examined in normal HSF1+/+ and HSF1−/− cells with a low expression of HSF1, **1** did not show any differential cytotoxicity between HSF1+/+ and HSF1−/− cells with a low expression of HSF1 (Figure S2, Supporting Information). From the results, it might be suggested that **1**-mediated cytotoxic effects might be specific for HSF1-overexpressed cancer cells.

Cancer cells are often resistant to treatment with conventional anticancer modalities such as paclitaxel, cisplatin, and radiation. Resistance to treatment has been shown to be associated with overexpression of HSPs.[15,16] Accordingly, whether or not HSF1 inhibition by **1** may be useful in combination with conventional anticancer modalities was evaluated. As shown in Figure 7A, B, and C, cotreatment with **1** and paclitaxel, cisplatin, or ionizing radiation (IR) was more effective than each treatment alone. While the combination of paclitaxel and **1** showed a synergistic increase of apoptosis, cisplatin and IR exhibited only additive increases of apoptosis on cotreatment with **1**. This result suggests that **1** can enhance the anticancer effect of conventional anticancer modalities.

In summary, the present study has demonstrated that 2,4-bis(4-hydroxybenzyl)phenol (**1**), a new inhibitor of HSF1, usually overexpressed in cancer cells, induces dephosphorylation and subsequent degradation of HSF1 and results in decreased levels of HSP27 and HSP70. Evidence that **1**-induced dephosphorylation of HSF1 on S326, in particular, contributes to the decrease of HSF1 protein stability was provided in the present study for the first time. Compound **1** induced growth arrest and apoptosis of human lung cancer NCI-H460 cells and moreover potentiated the effects of conventional anticancer modalities including paclitaxel, cisplatin, and radiation. Taken together, these results suggest that the ability of **1** to inhibit HSF1 may be a useful adjunct to conventional cancer treatments.

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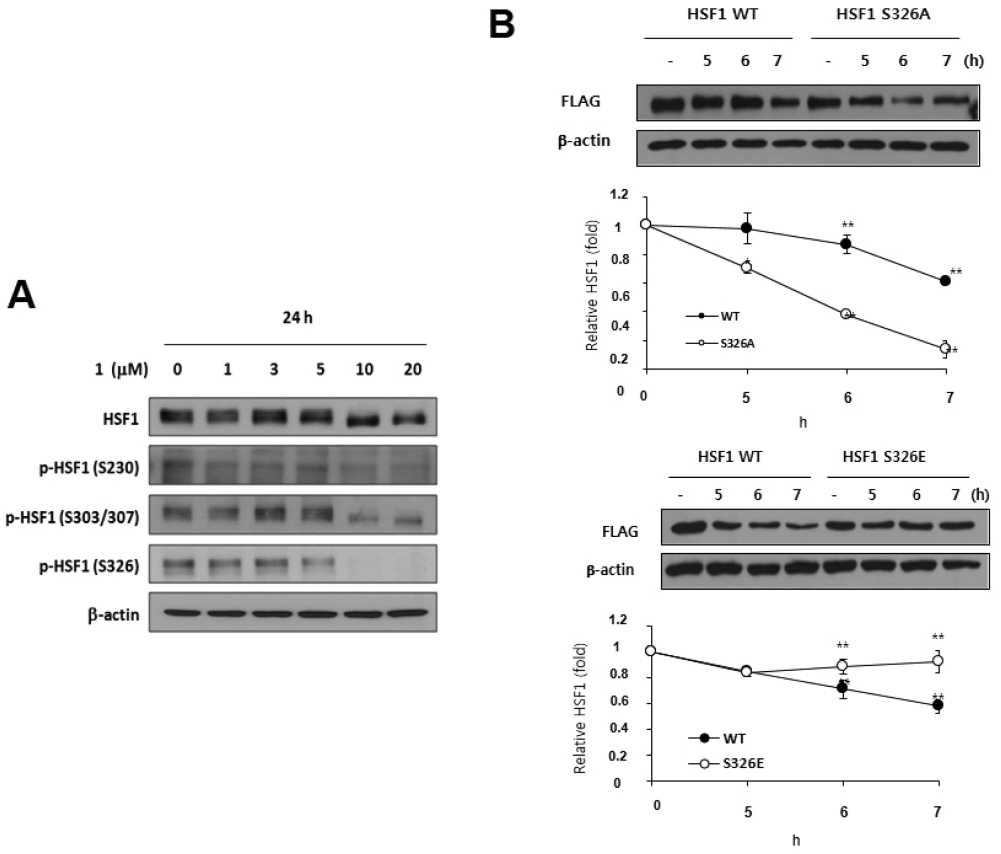

Figure 4. (A) Effect of **1** on phosphorylation of HSF1. After NCI-H460 cells were treated with **1** at the indicated concentration (0, 1, 3, 5, 10, or 20 μM) for 24 h, phosphorylation of HSF1 was detected by Western blotting with specific phospho-HSF1 antibodies. β-Actin was used as a loading control. (B) Effect of phosphorylation at S326 on HSF1 stability in CHX-treated NCI-H460 cells. Cells were incubated for the indicated time period (0, 5.5, 6, 6.5, 7, or 7.5 h) in the presence of CHX (10 μg/mL) with transfection of the wild type (WT), the phosphorylated form (S326E), or the dephosphorylated form (S326A) construct of HSF1. Western blotting analysis was performed for detection of the transfected HSF1 protein. Typical results are shown from three independent analyses. The intensity of FLAG-tagged HSF1 protein was measured and normalized to the loading control band (β-actin) using the NIH ImageJ program. Values are expressed as the fold relative to negative control (DMSO). Each data point represents the mean ± SE of three experiments vs control (with S326E) (*p < 0.05, **p < 0.01).

EXPERIMENTAL SECTION

Test Compound, Chemicals, and Reagents. 2,4-Bis(4-hydroxybenzyl)phenol (**1**) was isolated from the rhizomes of *G. elata*, as described previously.[18] The purity evaluation of **1** was determined by analytical HPLC (0 min, methanol–water, 1:1; 20 min, 1:0; flow rate 1 mL/min, detector UV 270 nm). The t_R of **1** was 9.98 min, and its purity was 98%. Analytical HPLC was carried out on a Waters instrument composed of a 1525 binary HPLC pump and a 2487 dual-wavelength absorbance detector, with a Phenomenex Luna 5 μm $C_{18}(2)$ column (250 mm × 4.60 mm i.d.). Paclitaxel was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cisplatin and cyclohexamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). These compounds were dissolved in DMSO and diluted in cell culture medium. All other chemicals were procured from Sigma-Aldrich. The following primers were used for quantitative RT-PCR: HSF1 (5′-GGTCAAGCCAGAGAGAGACG-3′, 5′-CTCATGCTTCATGGCCAGGA-3′), HSP27 (5′-CCTGGATGT-CAACCACTTC-3′, 5′-CTGGGATGGTGATCTCGTTG-3′), HSP70 (5′-ACAAGTCCGAGAACGTGCAG-3′, 5′-GCATCGATGTCGAAGGTCAC-3′), and β-actin (5′-GCCATGTACGTAGCCATCCA-3′, 5′-GAACCGCTCAT-TGCCGATAG-3′). The following sequences were used for siRNA experiments: HSF1 (5′-GAGAUCUAUAAACAGACAG(dTdT)-3′, 5′-CUGUCUGUUUAUAGAUCUC(dTdT)-3′), negative control (5′-CCUACGCCACCAAUUUCGU(dTdT)-3′, 5′-ACGAAAUUGGUGGCGUAGG(dTdT)-3′).

Plasmids and Cell Culture. The promoter region of wild-type mouse HSP25 and wild-type human HSP70 was cloned into the pGL3 luciferase reporter vector (Promega, Madison, WI, USA), as previously described.[24] Wild-type human HSF1, the phosphorylation mutant of HSF1 (S326E), and the dephosphorylation mutant of HSF1(S326A) were cloned into the p3xFLAG-Myc-CMV-26 expression vector (Sigma-Aldrich). The cells were transfected using Lipofectamine reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's recommended protocols. Human lung cancer NCI-H460 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Gibco BRL, Rockville, MD, USA) at 37 °C in a humidified 5% CO_2 incubator.

HSP Promoter Assay. The promoter activities of HSP25 and HSP70 were evaluated using luciferase reporter constructs, as previously described.[24] Briefly, human lung cancer NCI-H460 cells were plated at 1 × 10^5 cells in 35 mm dishes and cotransfected with 400 ng of luciferase reporter construct and 400 ng of β-galactosidase expression vector. After 24 h of incubation, cells were treated with chemical and harvested 12 h later. Luciferase activity was assayed from cell lysates using Luciferase Assay Systems (Promega), and the results were normalized to the β-galactosidase expression (Promega).

Quantitative Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was extracted using TRIzol (Invitrogen). cDNA was synthesized using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan), as described by the manufacturer. HSF1, HSP27, HSP70, and β-actin transcript levels were measured by

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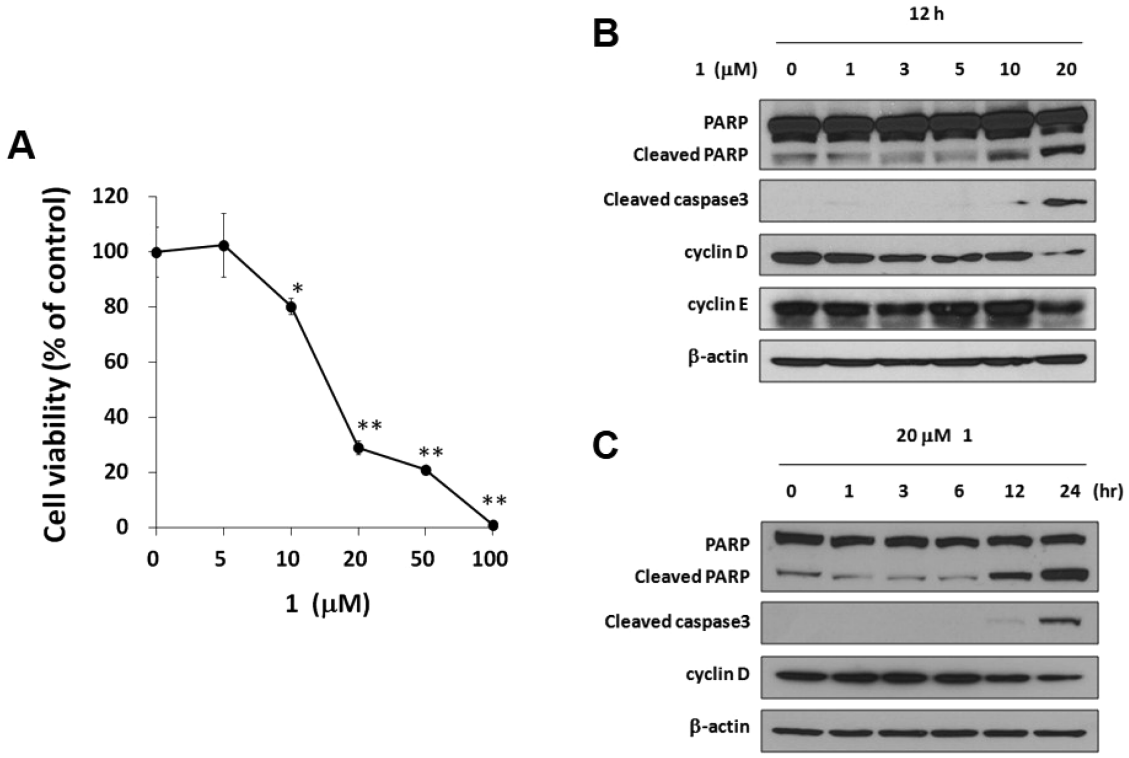

Figure 5. (A) Cytotoxic effect of **1** in human lung cancer NCI-H460 cells. Cell viability was measured using the MTT assay after treatment with various concentrations of **1** from 0 to 100 μM for 24 h in NCI-H460 cells. Values are expressed as percentages relative to the negative control (DMSO). Data represent the means ± SE of three independent experiments (*p < 0.05, **p < 0.01 vs control). (B and C) Dose- and time-dependent apoptotic effect of **1** in human lung cancer NCI-H460 cells. Cells were treated with **1** at the indicated concentration (0, 1, 3, 5, 10, or 20 μM) for 12 h or with 20 μM **1** for the indicated time period (0, 1, 3, 6, 12, or 24 h) prior to protein extraction. Apoptotic markers (cleaved PARP and cleaved caspase 3) and proliferative markers (cyclin D and cyclin E) were detected by Western blotting. β-Actin was used as a loading control.

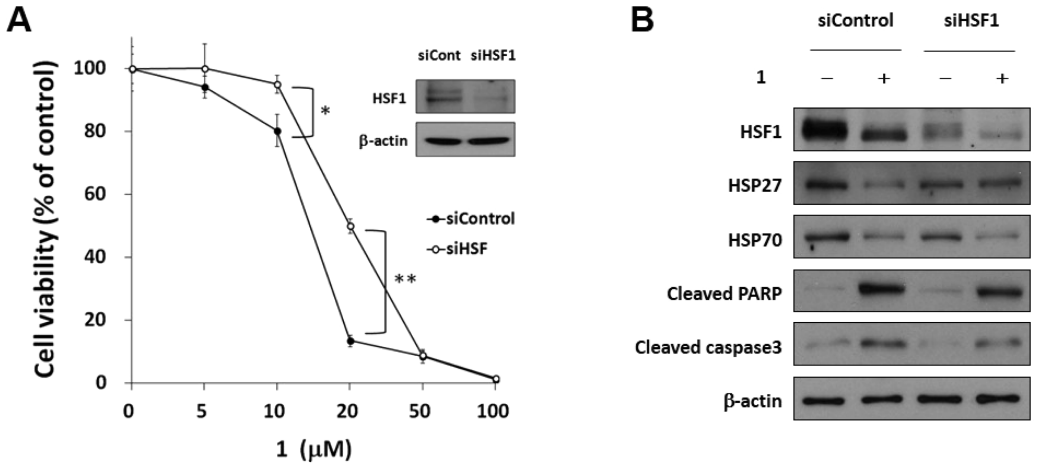

Figure 6. (A) Loss of the cytotoxic effect of **1** in HSF1-suppressed NCI-H460 cells. Cell viability was measured using an MTT assay after treatment with various concentrations of **1** from 0 to 100 μM for 24 h in NCI-H460 cells previously transfected with siHSF1. An siControl was used as negative control for siHSF1 transfection. Values are expressed as percentage relative to negative control (DMSO). Data point represents the mean ± SE of three experiments vs control (*p < 0.05, **p < 0.01). (B) Loss of the apoptotic effect of **1** in HSF1-suppressed NCI-H460 cells. Cells transfected with siHSF1 to suppress HSF1 expression were treated with 15 μM **1** for 24 h prior to protein extraction. siControl was used as a negative control for siHSF1 transfection. Apoptotic markers (cleaved PARP and cleaved caspase3), HSF1, HSP27, and HSP70 were detected by Western blotting. β-Actin was used as a loading control.

qRT-PCR (GenDEPOT, Barker, TX, USA). β-Actin was used as an internal control gene. The change in relative gene expression was normalized to β-actin mRNA levels using the NIH ImageJ program.

Western Blot Analysis. Protein expression was evaluated as previously described.[2] NCI-H460 cells were treated with the test compound at varying concentrations for the indicated time periods. Cell extracts were obtained by lysis in a modified RIPA buffer. Proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose membrane (GE Healthcare, Little Chalfont, UK), and incubated with the specified primary antibody for 18 h at 4 °C. Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized with the ECL detection system (Thermo Scientific, Rockford, IL, USA). β-Actin was used as an internal control. The change in relative protein

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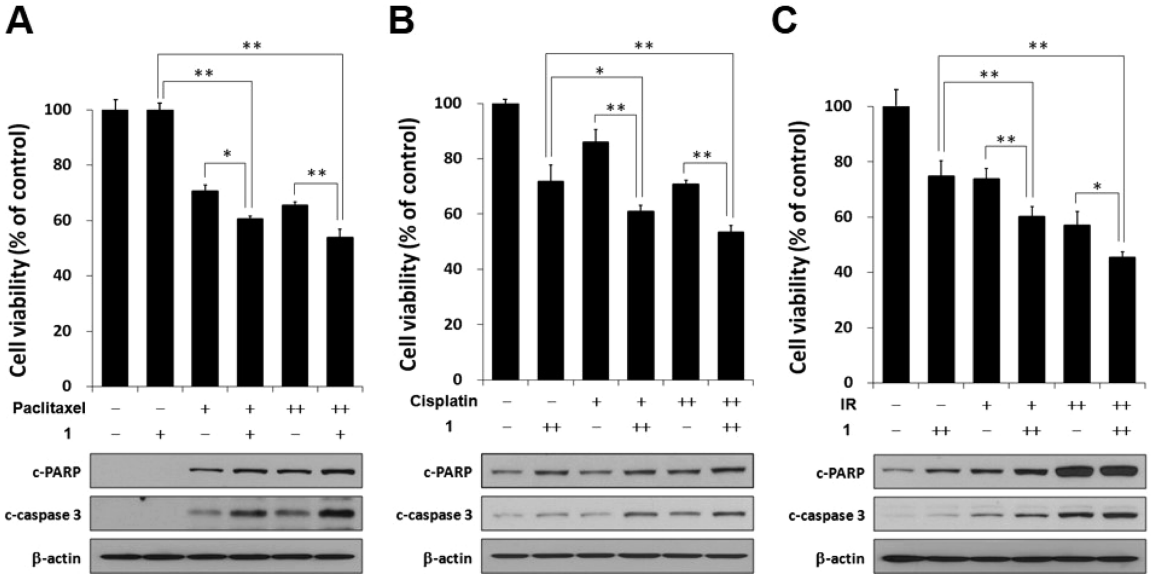

Figure 7. (A) Treatment with a combination of **1** and paclitaxel compared with **1** or paclitaxel alone. (B) Combination of **1** and cisplatin compared with **1** or cisplatin alone. (C) Combination of **1** and IR compared with **1** or IR alone. NCI-H460 cells were treated with **1** (5 or 10 μM) and paclitaxel (0.05 or 0.1 μM), cisplatin (20 or 50 μM), or IR (5 or 10 Gy) for 24 h. Cell survival was measured by MTT assay. Values are expressed as percentage relative to negative control (DMSO). Data point represents the mean ± SE of three experiments vs control (*$p < 0.05$, **$p < 0.01$). The apoptotic markers, cleaved PARP and caspase3, were detected by Western blotting using cell lysates. β-Actin was used as a loading control, with **1** −, 0 μM; **1** +, 5 μM; **1** ++, 10 μM; paclitaxel −, 0 μM; paclitaxel +, 0.05 μM; paclitaxel ++, 0.1 μM; cisplatin −, 0 μM; cisplatin +, 20 μM; cisplatin ++, 50 μM; IR −, 0 Gy; IR +, 5 Gy; IR ++, 10 Gy.

expression was normalized to β-actin using the NIH ImageJ program. Anti-HSF1, HSP27, HSP70, cleaved PARP, cleaved caspase-3, cyclin D, cyclin E, FLAG, and β-actin antibodies were purchased from Santa Cruz Biotechnology or Cell Signaling Technologies (Danvers, MA, USA).

Protein Stability Analysis. Cells were incubated with 10 μg/mL cyclohexamide (CHX), a protein synthesis inhibitor, for the time period indicated. Cell extracts were obtained by lysis in a modified RIPA buffer. Proteins were analyzed by SDS-PAGE electrophoresis and immunoblotting.

Cell Viability Assay. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Sigma-Aldrich) assay was used as an indirect measure of death, as previously described.[25] Briefly, the medium was removed and replaced with MTT reagent (0.5 mg/mL) in PBS. The plates were incubated for 1 h at 37 °C, and 100 μL of DMSO was added to dissolve the formazan crystals. The absorbance was detected at a wavelength of 540 nm. A calibration curve was used to convert absorbance to numbers of cells. Cell viability was determined as a percentage of the untreated control.

siRNA Inhibition of HSF1. Specific HSF1 siRNA or negative control siRNA was transfected into NCI-H460 cells using Lipofectamine 2000 reagent (Invitrogen), as described by the manufacturer. After 48 h, the transfected cells were assayed for suppression of targeted genes and used in subsequent experiments.

Ionizing Radiation Treatment. Cells in 60 mm Petri dishes were exposed to γ radiation (5 or 10 Gy as a single dose), which was generated by a ^{137}Cs gamma-ray source (Elan 3000, Atomic Energy of Canada, Ottawa, ON, Canada) at a dose rate of 3.81 Gy/min.

Statistical Analysis. All results represent the means ± SE of three independent experiments. The statistical significance of differences between groups was determined by analysis of variance (ANOVA) or Student's *t* test. In all cases, the level for a statistically significant difference was set at $p < 0.05$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Author Contributions

#T. Yoon and G.-Y. Kang contributed equally to this work.

Notes

The authors declare no competing financial interest.

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