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Note

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The Conjugated Double Bond of Coniferyl Aldehyde Is Essential for Heat Shock Factor 1 Mediated Cytotoprotection

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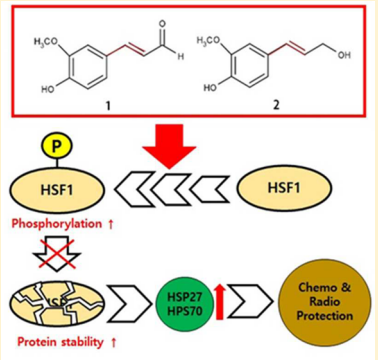
Supporting Information

ABSTRACT: Coniferyl aldehyde (**1**) is previously reported as a potent inducer of heat shock factor 1 (HSF1). Here, we further examined the active pharmacophore of **1** for activation of HSF1 using the derivatives coniferyl alcohol (**2**), 4-hydroxy-3-methoxyphenylpropanal (**3**), and 4-hydroxy-3-methoxyphenylpropanol (**4**). Both **1** and **2** resulted in increased survival days after a lethal radiation (IR) dose. The decrease in bone marrow (BM) cellularity and Ki67-positive BM cells by IR was also significantly restored by **1** or **2** in mice. These results suggested that the vinyl moiety of **1** and **2** is necessary for inducing HSF1, which may be useful for developing small molecules for cytoprotection of normal cells against damage by cytotoxic drugs and radiation.



Heat shock factor 1 (HSF1) is a master regulator of the heat shock response and enables cell development, survival, and proliferation in mammals. HSF1 exists in an inactive condition in the cytoplasm. After exposure to proteotoxic stresses, heat shock proteins (HSPs) are expressed by HSF1 and facilitate chaperone activities and protein refolding. HSF1 trimerizes and translocates to the nucleus to bind heat shock elements in promoters of target genes and hyperphosphorylation.[1] Expression of HSPs increases with HSF1 activation, which occurs when cells are exposed to hyperthermia or biological stresses such as heat, high pressure, or toxic compounds. HSPs protect cells by influencing a variety of cellular processes that determine cell fate.[2] Thus, HSF1 may be a pharmacological target for cytoprotection.[3−5]

Pharmacological regulators of the heat shock response are small molecules that enhance expression or function of HSPs and are promising for chronic or acute treatment of human disease. An important feature of HSPs is cytoprotection and repair of cells and tissues against deleterious effects of stress and trauma. Overexpression of one or more HSP genes is sufficient to protect against lethal exposure to heat, cytotoxic drugs, and tumor necrosis factor. The protective effect of HSPs is related to interference with apoptotic pathways.[2]

Radiation therapy is an important modality for treating cancer. Almost 40% of cancer patients require radiation therapy for disease management. Although radiation therapy improves the survival of a significant number of cancer patients, toxicity and injury of radiotherapy to normal tissues and organs is substantial. Moreover, recent clinical anticancer therapy has been targeting a decrease in normal cell damage.[6,7] Several reports have attempted to develop new mitigators or protectors from nontoxic phytochemicals[8,9] or natural products,[10] to protect normal tissues against radiation or cytotoxic drug-mediated injury during cancer therapy or exposure to radiation hazards.[11] Furthermore, after the Chernobyl disaster (1986) and Fukushima nuclear disaster (2011) caused radiation damage, such hazards have awakened the need to develop radiation protectors.[12] Work on radioprotective chemicals started a considerable time ago in the United States, at the inception of the Manhattan Project.[12,13] However, many of the chemicals had severe side effects such as nausea, vomiting, and hypotension.[13] The search for safe and nontoxic radioprotectors has yielded a great deal of information and several clinical trials.[14,15]

Many previous phytochemical investigations have resulted in the isolation of lignans, flavonoids, iridoids, and terpenoids. Among them, coniferyl aldehyde (**1**), a phenolic compound isolated from the bark of *Eucommia ulmoides* Oliv. (Eucommiaceae), showed cytoprotective effects against radiation and cytotoxic drugs by inducing HSPs, especially HSP27 and HSP70, through activation of HSF1.[16] Therefore, this study investigated the active sites of **1** for activation of HSF1 using its derivatives coniferyl alcohol (**2**), 4-hydroxy-3-methoxyphenylpropanal (**3**), and 4-hydroxy-3-methoxyphenylpropanol (**4**).

In a previous study, **1**, isolated from the bark of *E. ulmoides*, increased HSF1 protein and its transcriptional targets *hsp25* (murine form, *hsp27* for human form) and *hsp70*.[17] Compound **1** time-dependently and dose-dependently induced HSF1 protein, accompanied by increased HSP27 and HSP70 protein. Compound **1** did not show any discernible cytotoxicity in L132

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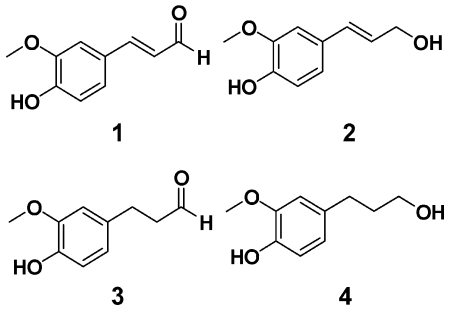
normal lung cells. However, paclitaxel and cisplatin, which are effective anticancer drugs, showed significant cytotoxicity effects. Coniferyl aldehyde (**1**) regulates HSF1 protein stability, and this function is mediated by phosphorylation of HSF1. Compound **1** phosphorylates HSF1 on Ser326; however, other serine residues such as Ser216 and Ser303/307 are not changed by **1**. These data indicated that **1** is responsible for increased protein stability and transcriptional activity by HSF1.[16,17]

In the present investigation, the active pharmacophore of **1** for activation of HSF1 was examined. For this purpose, **1** and compounds with similar structures (**2**–**4**), which all have guaiacol (GU, also called 2-methoxyphenol) moieties in their basic structure, were compared for HSF1 activation. Compounds **3** and **4** were obtained through the general reductive hydrogenation for **1** using H_2 gas at atmospheric pressure under 10%-Pd/C catalytic conditions in methanol. Stable pGL4-*hsp70* or -*hsp25* NCI-H460 cells were plated, and after 24 h, luciferase activity was assayed from cell lysates using luciferase assay kits and detected by a luminometer. Compounds **1** and **2** at 10 or 30 μM resulted in increased promoter activity, while **3** and **4** did not (Figure S1A, Supporting Information). Expression of HSPs such as HSP27 and HSP70 after treatment for 12 h with 10 or 30 μM compounds was monitored by Western blots. HSF1 and HSP proteins were upregulated by **1** and **2**. However, with **3** and **4**, which do not have a vinyl moiety, the HSF1, HSP27, and HSP70 proteins did not increase, even at the higher dose of 30 μM (Figure S1B, Supporting Information). Celastrol (1 μM), an HSP inducer, was used as the positive control. These data indicated that only **1** and **2**, a hydroxy derivative of **1**, induced promoter activity of *hsp25* and *hsp70* and the HSF1 and HSP proteins. Therefore, it is hypothesized that for the activation of HSF1 the benzene vinyl moiety of **1** or **2** is essential.



Indeed, both **1** and **2** increased the promoter activity of *hsp25* and *hsp70* (Figure 1A). mRNA of *hsp27* and *hsp70* and their proteins were also induced by 3 μM **1** and **2** (Figure 1B and C). When cell viability was measured in an MTT assays, **1** and **2** did not show any cytotoxicity, even at 20 μM (Figure 1D), while celastrol showed strong cytotoxicity. Compound **1** was more cytotoxic than **2** based on normal cell viability. Since **1** is reported to be phosphorylated at Ser326 of HSF1,[16] it was determined if **2** also phosphorylates HSF1 at Ser326, similar to **1**. Both **1** and **2** at 3 μM phosphorylated HSF1 at Ser326, which might be the main pathway for HSF1 activation (Figure 1E).

In a previous study, HSF1 and HSPs were upregulated by **1** in a time- and dose-dependent manner, with maximal induction at 3 μM and 12 h.[16] Compound **2** also induced an increase in HSF1 and HSPs, similar to **1**. HSF1 induction was more prominent in **1**-treated cells. Time-dependent expression of HSP27 was

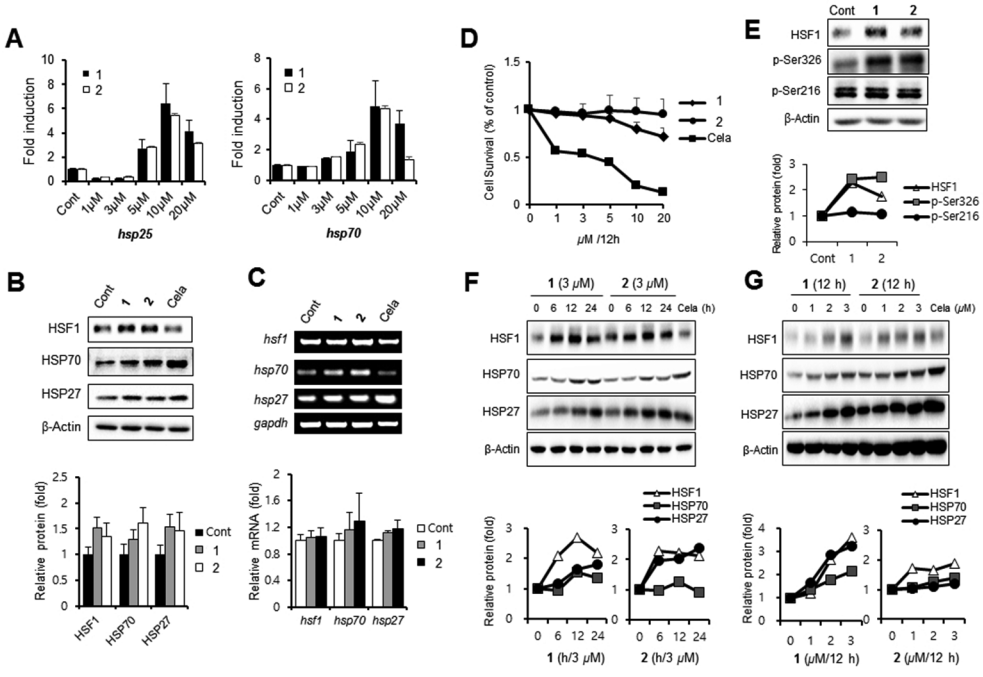


Figure 1. Compound **2** is a hydroxy derivative of **1** with similar effects to **1** for inducing HSF1 and HSPs. (A) HSP promoter activity after treatment with **1** and **2**. NCI-H460 cells were transfected with luciferase reporter construct with the *hsp25* or *hsp70* promoter. Values are expressed as the means ± SD of at least three independent experiments in the form of fold-change relative to the negative control. (B) Immunoblotting was performed 12 h after treatment with 3 μM **1** or **2** in L132 human lung fibroblast cells. (C) RT-PCR with the same conditions as immunoblotting. (D) MTT assays were performed 24 h after treatment with the indicated doses of **1** and **2**. (E) Phosphorylation patterns of HSF1 after 12 h of treatment of **1** or **2** were detected by immunoblotting. (F) Immunoblotting was performed 12 h after **1** or **2** treatment or with (G) 3 μM **1** and **2** at the indicated times. Band intensities were measured and normalized to loading control bands. Experiments were performed for at least three independent experiments. Values are presented as means ± SD. Celastrol (Cela, 1 μM) was used as the positive control.

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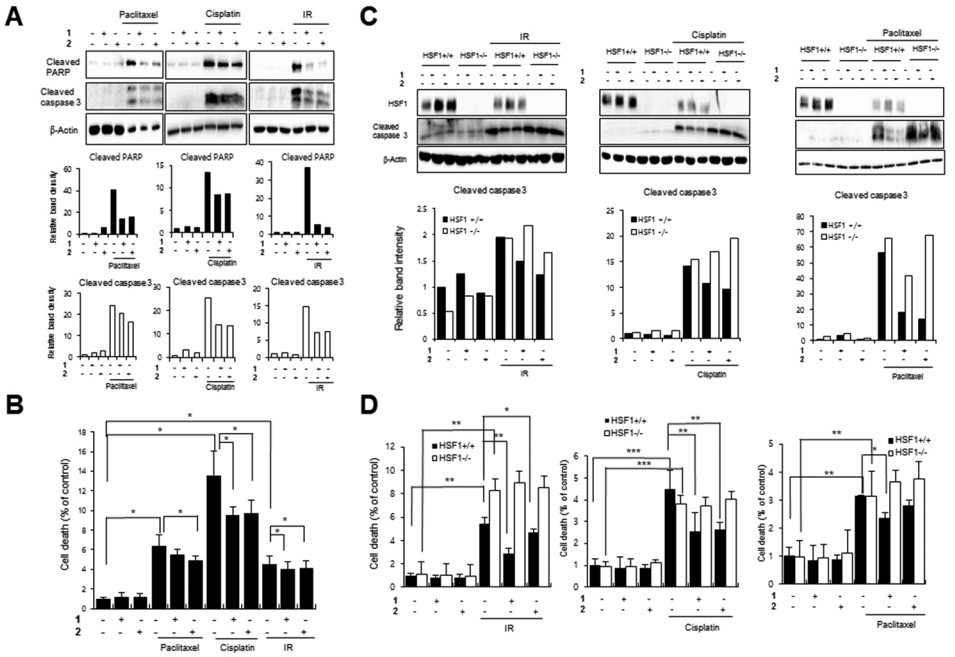

Figure 2. Cytoprotective effect of **1** and **2** against paclitaxel, cisplatin, and IR damage. Cell death by immunoblotting of apoptosis-related cleavage of PARP and caspase-3 (A, C) or FACS (B, D) after 24 h treatment with 0.5 μM paclitaxel or 20 μM cisplatin or 48 h of 10 Gy IR in L132 cells (A, B) or HSF1+/+ and HSF1−/− MEF cells (C, D) with or without pretreatment with **1** and **2** (*$p < 0.05$; **$p < 0.01$). Band intensities were measured and normalized to loading control bands. Values are expressed as means ± SD of at least three independent experiments in the form of fold-change relative to the negative control.

more prominent in **2**-treated cells, while the dose-dependence of HSP27 expression was more obvious with **1**-treated cells. For HSP70 expression, neither **1** nor **2** had strong effects. The results indicated that both **1** and **2** increased expression of HSF1 and HSPs (Figure 1F and G).

To examine if HSF1 induction by **1** and **2** affected cell damage induced by paclitaxel, cisplatin, or IR, **1** and **2** were used at 3 μM for pretreatment followed by incubation with or without 0.5 μM paclitaxel, 20 μM cisplatin, or IR 10 Gy. To determine induction of apoptosis, cleavage of PARP and caspase-3 was examined. To determine cell survival, FACS was performed. Treatment of L132 cells with paclitaxel and cisplatin for 24 h or IR for 48 h increased cleavage of PARP and caspase-3. One-hour pretreatment with **1** or **2** before paclitaxel, cisplatin, or IR reduced the cleavage (Figure 2A). Paclitaxel, cisplatin, or IR was cytotoxic to L132 cells, but a 1 h pretreatment with 3 μM **1** or **2** inhibited paclitaxel-, cisplatin-, or IR-mediated cytotoxicity. These data indicated that pretreatment with **1** or **2** protected cells against damage from toxic drugs or IR (Figure 2B). To determine whether **1**- and **2**-mediated cytoprotective effects depended on HSF1 expression, HSF1−/− and HSF1+/+ cells were tested. One-hour pretreatment with **1** or **2** reduced paclitaxel, cisplatin, or IR cytotoxicity and apoptosis in HSF1+/+ cells at 24 h after paclitaxel and cisplatin treatment and 48 h after IR exposure. In HSF1−/− cells, **1** and **2** did not inhibit paclitaxel, cisplatin, or IR cytotoxicity and apoptosis (Figure 2C and D). These results suggested that **1**- and **2**-mediated cyto- or radioprotection was dependent on HSF1 activation.

Compound **1** increases survival after lethal doses of IR.[16] Thus, it was determined whether the cytoprotective effects by **2** would be conserved in an animal model like **1** by investigating the survival after exposure to a lethal dose of IR (7 Gy). IR alone resulted in signs of radiation sickness such as reduced intake of food and water, irritability, and lethargy at 10 days with complete mortality within 22 days. A single treatment of **1** or **2** before IR or three treatments with **1** and **2** after IR at 5 or 10 mg/kg increased mean survival days and reduced radiation sickness characteristics compared to mice given IR alone (Figure 3A). Normal mice treated with **1** or **2** at 10 mg/kg did not exhibit any noticeable signs of toxicity within 30 days.

Bone marrow (BM) damage after a sublethal dose of IR (4.5 Gy) was also examined. At 9 days, IR significantly induced BM damage; however, **1** or **2**, at 10 mg/kg, inhibited IR-induced BM damage (Figure 3B). IR significantly reduced circulating white blood cells (WBCs), neutrophils, and lymphocytes after 9 days, and recovery of this parameter was observed following combined treatment with **1** or **2** (Figure 3C). Effects were more obvious in **2**-treated mice than **1**-treated mice except for neutrophils. Cellular proliferation was also examined using immunohistochemistry of Ki67 with BM tissues, and combined treatment with **1** or **2** reduced IR-mediated inhibition of Ki67-positive cells in BM (Figure 3D). When the weight of spleens was examined, reduced spleen weights by IR were mitigated by combined treatment with **1** or **2** (Figure 3E).

HSF1 activation by treatment with **1** or **2** may be an obstacle for radio- and chemotherapy of tumors. However, this possibility can be possibly excluded because a previous study has indicated that treatment of **1** did not affect IR-induced cell death in lung tumor tissues in an orthotopic lung tumor model.[16] Although **1** and **2** had similar effects, **1** exhibited more cytotoxicity than **2**, based on cell viability, suggesting a greater clinical promise of **2** rather than **1**.

In summary, the present study identified an active pharmacophore for activating HSF1, which may be useful for developing small molecules for cytoprotection against damage by drugs such as paclitaxel and cisplatin and by radiation of normal cells.

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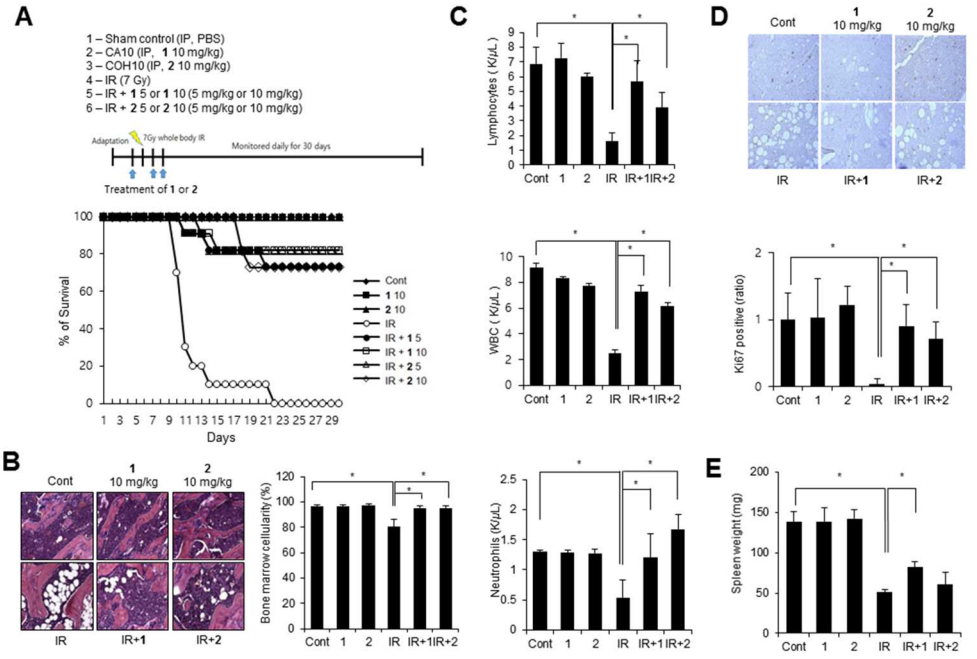

Figure 3. Effect of **1** and **2** on survival days after 7 Gy lethal IR or 4.5 Gy IR-induced bone marrow (BM) damage in ICR mice. (A) Mice underwent whole-body irradiation with 7 Gy. Compound **1** or **2** (5 and 10 mg/kg, once before and three times after IR) was injected intraperitoneally (ip). The number of days survival was recorded. Each group contained 10 mice. (B) Mice received 4.5 Gy whole-body radiation. Compound **1** or **2** (10 mg/kg, once before and three times after IR) was injected ip. Nine days after IR, mice were sacrificed. Each group contained five mice. After H&E staining of BM, quantification of BM cellularity using an image analyzer was performed. The graph indicates the percentage (means ± SD) of hematopoietic cells compared to unit area (200 μm × 200 μm, original magnification 200×) of BM. WBCs, lymphocytes, and neutrophils in the peripheral blood (C) and Ki67-positive cells of BM (D) were measured. The number of Ki67-positive cells in five fields per femur was counted at an original magnification of 1000×. (E) Relative spleen weights were calculated, with values of control spleens set to 1.0. Data are presented as means ± SD of five mice (*$p < 0.05$).

EXPERIMENTAL SECTION

General Experimental Procedures. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian NMR AS 400 MHz instrument. Chemical shifts (δ) are in parts per million (ppm) relative to tetramethylsilane as an internal standard, and coupling constants (J values) are in hertz. Mass spectrometric investigations were performed on an AB SCIX API 4000 equipped with an electrospray ionization (ESI) source. The solvents and reagents used were of the highest commercial grade available and used as received.

Test Compounds and Chemicals. Coniferyl aldehyde (**1**; 98% purity), coniferyl alcohol (**2**; 98% purity), paclitaxel (purity ≥99%), and cisplatin (purity ≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Synthesis of Compounds 3 and 4. A reaction mixture of **1** (0.20 g, 1.12 mmol) and 10%-Pd/C (catalytic amount) was stirred under a H_2 atmosphere (equipped with a balloon) for 2 h and then filtered. After washing with methanol, the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (eluant: ethyl acetate−*n*-hexane, 1:2 → 1:1) to give compounds **3** (0.024 g, 11.9%) and **4** (0.12 g, 70.4%).

*4-Hydroxy-3-methoxyphenylpropanal (**3**):* colorless oil; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.65 (t, J = 7.6 Hz, 2H), 2.79 (t, J = 7.6 Hz, 2H), 3.77 (s, 3H), 6.54 (dd, J = 8.0, 2.0 Hz, 1H), 6.61 (d, J = 2.0 Hz, 1H), 6.70 (d, J = 8.0 Hz, 1H), 7.56 (s, 1H), 9.72 (t, J = 1.2 Hz, 1); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 27.4, 45.1, 55.5, 111.4, 114.9, 120.2, 131.3, 144.3, 147.0, 201.7; ESIMS m/z 181.09 $[M + H]^+$ (calcd for $C_{10}H_{13}O_3^+$, 181.09).

*4-Hydroxy-3-methoxyphenylpropanol (**4**):* colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.87 (tt, J = 8.0, 6.4 H, 2H), 2.64 (t, J = 8.0 Hz, 2H), 3.68 (t, J = 6.4 Hz, 2H), 3.87 (s, 3H), 7.69 (dd, J = 8.4, 1.6 Hz, 1H), 6.70 (d, J = 1.6 Hz, 1H), 6.83 (d, J = 8.4 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 31.2, 34.6, 55.5, 60.2, 112.5, 115.2, 120.3, 132.9, 144.3, 147.3; ESIMS m/z 183.10 $[M + H]^+$ (calcd for $C_{10}H_{15}O_3^+$, 183.10).

Antibodies. Monoclonal antibodies against HSF1 were from Abcam (diluted 1:1000; Cambridge, MA, USA). Anti-HSP70, HSP27, and β-actin were from Santa Cruz Biotechnology (diluted 1:1000; Santa Cruz, CA, USA). Anticleaved PARP and caspase-3 were from Cell Signaling Technology (diluted 1:1000; Danvers, MA, USA).

Plasmids and Cell Culture. Human embryonic lung L132 cells were from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin (Gibco BRL) at 37 °C in a humidified 5% CO_2 incubator. HSF1 knockout mouse embryonic fibroblasts (HSF1+/+ and HSF1−/− MEF) cells were kindly provided by Dr. Ivor Benjamin (University of Utah, Salt Lake City, UT, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Thermo Scientific, Rockford, IL, USA), supplemented with heat-inactivated 10% FBS (Thermo Scientific), 0.1 mmol/L non-essential amino acids, glutamine, HEPES, and antibiotics at 37 °C in a 5% CO_2 humidified incubator.

Promoter Assay. Promoter activities of *hsp25* or *hsp70* were evaluated using luciferase reporter constructs that stably expressed *hsp25* or *hsp70*. Human lung cancer NCI-H460 cells were plated at 1×10^5 cells in 60 mm dishes and 2×10^4 cells in a 96-well plate. After 24 h, cells were treated with test chemicals and harvested 12 h later. Luciferase activity was assayed from cell lysates using a luciferase assay system (Promega, Madison, WI, USA), and the results were normalized to β-galactosidase expression (Promega). All results were the mean of three independent experiments.

Irradiation. Cells in 60 mm Petri dishes were exposed to γ-radiation (7 or 10 Gy as a single dose) generated by a ^{137}Cs gamma-ray source (Elan 3000, Atomic Energy of Canada, Mississauga, Canada) at a dose rate of 3.81 Gy/min. Radiation workers annually received radiation safety management training provided by the Korea Foundation of Nuclear Safety (KoFONS).

Immunoblotting. Cells were plated at 2×10^5 mm in cell culture dishes and grown to 30% confluence. Equal amounts of protein were dissolved in lysis buffer, and lysates were centrifuged at 13 000 rpm for 30 min at 4 °C. Protein concentration was determined using Bradford (Bio-Rad, CA, USA) assays with bovine serum albumin for standard curves. The optical density of standards and samples was read by a

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microplate reader at 595 nm. Prepared samples were mixed with sample buffer and boiled at 100 °C for 5 min, and proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% or 12%). Separated protein bands were transferred to nitrocellulose membranes. After blocking with 5% skimmed milk in phosphate-buffered saline with Tween 20 (PBST), membranes were incubated with antibodies for 18 h at 4 °C, washed and incubated with horseradish peroxidase, and visualized via enhanced chemiluminescence. Membranes were detected by ChemiDoc MP Systems (Bio-Rad).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted using TRIzol (Invitrogen, Grand Island, NY, USA), and cDNA was synthesized using ReverTra Ace RT-PCR kits (Toyobo, Osaka, Japan). *Hsf1*, *hsp70*, and *hsp27* transcripts were measured by RT-PCR (GenDEPOT, Barker, TX, USA) with *gapdh* as the internal control gene. Change in relative gene expression was normalized to *gapdh* mRNA using the NIH ImageJ program.

MTT Assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assays were used as an indirect measure of death. Medium was removed and replaced with 100 μL of MTT reagent (5 mg/mL) in PBS. Plates were incubated for 4 h at 37 °C, and 100 μL of DMSO was added to dissolve formazan crystals. Multiwell plates were shaken for 15 min, and signals detected using ELISA at 540 nm. MTT assay results were expressed as cell numbers by conversion of absorbance into cell numbers using a calibration curve. Calibration curves were prepared by seeding an assigned number of 1×10^6 cells into 96-well plates for 24 h before adding MTT solution.

Flow Cytometry. Cells were plated in 100 × 20 mm cell culture dishes and treated with drugs. Cells were washed two times with 1× PBS, separated using trypsin-EDTA (Gibco-Invitrogen, Paisley, Scotland, UK), and centrifuged at 13 000 rpm for 3 min at 4 °C twice. After adding 1 mL of 1× PBS and 10 μL of propidium iodide 5 mM (Sigma-Aldrich) to polystyrene round-bottom tubes, samples were detected by a FACs flow cytometer (BD Bioscience San Jose, CA, USA).

Survival Day Detection and Bone Marrow Damage Experiments after Irradiation in ICR Mice. Female ICR mice (5−6 weeks old, 25−30 g) were from Central Lab Animal, Inc. (Seoul, South Korea). Mice received 7 Gy of whole-body IR. After IR, animals were housed under normal laboratory conditions and checked twice daily. A stock solution of **1** and **2** was prepared by dissolving in DMSO. The solution was diluted with modified PBS to the desired concentration for injection into mice. A fresh solution of **1** was prepared before each injection. For BM damage experiments, female ICR mice (5−6 weeks old) received 4.5 Gy of whole-body IR.[18] Compounds **1** and **2** (10 mg/kg, one pretreatment of **1** or **2** before IR and three post-treatments of **1** or **2** after IR) were administered by intraperitoneal (ip) injection. Each group contained five mice. All protocols involving mice were approved by the Institutional Animal Care and Use Committee (IACUC No. 15-080) of Laboratory Animal Genomic Center (Ewha Womans University, South Korea).

Immunohistochemistry. Immunohistochemistry was performed on sections of paraffin-embedded BM tissue using monoclonal mouse anti-human Ki67 antibody (Dako, M7240). Briefly, after deparaffinization and hydration, the sections were treated with heat-mediated antigen retrieval using 10 μM citrate buffer (pH 6.0) for 20 min. Then the endogenous peroxidase activity was quenched by 3% hydrogen peroxide solution for 10 min. Nonspecific binding was prevented by incubation with 5% normal rabbit serum for 1 h. After that, the sections were incubated with anti-human Ki67 antibody (1:100 dilutions) as the primary antibody overnight at 4 °C. Secondary antibody incubation and staining were performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA).

Statistical Analysis. All results represent means ± SD of three independent experiments. Statistical significance of differences between untreated controls and treated groups was compared by Student's *t* test.

■ ASSOCIATED CONTENT

Supporting Information

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Additional information (PDF)

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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