The Natural Compound Cantharidin Induces Cancer Cell Death through Inhibition of Heat Shock Protein 70 (HSP70) and Bcl-2-associated Athanogene Domain 3 (BAG3) Expression by Blocking Heat Shock Factor 1 (HSF1) Binding to Promoters*

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**Background:** HSF1 is a transcription factor that enhances cancer formation and progression. Cantharidin inhibited the binding of HSF1 to the HSP70 promoter and subsequently blocked HSF1-dependent HSP70 expression.

**Results:** Cantharidin inhibited the binding of HSF1 to the HSP70 promoter and subsequently blocked HSF1-dependent HSP70 expression.

**Conclusion:** The HSF1-dependent expression of HSP70 and BAG3 is inhibited by cantharidin, causing the down-regulation of antiapoptotic BCL-2 family proteins, especially MCL-1.

**Significance:** This information provides a new target molecule and pathway of cantharidin.

Heat shock factor 1 (HSF1) enhances the survival of cancer cells under various stresses. The knock-out of HSF1 impairs cancer formation and progression, suggesting that HSF1 is a promising therapeutic target. To identify inhibitors of HSF1 activity, we performed cell-based screening with a library of marketed and experimental drugs and identified cantharidin as an HSF1 inhibitor. Cantharidin is a potent antitumor agent from traditional Chinese medicine. Cantharidin inhibited heat shock-induced luciferase activity with an IC50 of 4.2 μM. In contrast, cantharidin did not inhibit NF-κB luciferase reporter activity, demonstrating that cantharidin is not a general transcription inhibitor. When the HCT-116 colorectal cancer cells were exposed to heat shock in the presence of cantharidin, the induction of HSF1 downstream target proteins, such as HSP70 and BAG3 (Bcl-2-associated athanogene domain 3), was suppressed. HSP70 and its co-chaperone BAG3 have been reported to protect cells from apoptosis by stabilizing anti-apoptotic Bcl-2 family proteins. As expected, treating HCT-116 cancer cells with cantharidin significantly decreased the amounts of BCL-2, BCL-XL, and MCL-1 protein and induced apoptotic cell death. Chromatin immunoprecipitation analysis showed that cantharidin inhibited the binding of HSF1 to the HSP70 promoter and subsequently blocked HSF1-dependent p-TEFb recruitment. Therefore, the p-TEFb-dependent phosphorylation of the C-terminal domain of RNA polymerase II was blocked, arresting transcription at the elongation step. Protein phosphatase 2A inhibition with PP2CA siRNA or okadaic acid did not block HSF1 activity, suggesting that cantharidin inhibits HSF1 in a protein phosphatase 2A-independent manner. We show for the first time that cantharidin inhibits HSF1 transcriptional activity.

Cancer cells are characterized by genetic mutations in oncogenes and tumor suppressors. In many cases, cancer cells become dependent on oncogenes for tumor initiation and progression, which is termed oncogene addiction (1). Most anticancer drug-screening programs have focused on these oncogenes. However, cancer cells are still heavily dependent on specific signaling pathways that are independent of oncogenic pathways (2), such as the molecular chaperone pathway (3). Mutations in the cancer genome can result in suboptimal conformations for cellular proteins. In addition, cancer cells are exposed to high levels of reactive oxygen species (4), which makes them highly dependent on molecular chaperone activity; this is an example of nononcogene addiction.

The heat shock response is a protective mechanism against a wide range of stresses, including heat shock, oxidative stress, heavy metals, fever, or protein misfolding (5, 6). The heat shock response is largely mediated by heat shock transcription factor 1 (HSF1). Although heat shock proteins (HSPs) are only induced transiently upon stress, HSPs are often constitutively overexpressed in tumors. The expression of hsp70 is induced by several oncogenes such as H-ras12 (7), c-myc (8), c-mycb, SV40 large T antigen, and adenovirus E1α (9).

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3 The abbreviations used are: HSF1, heat shock factor 1; p-TEFb, positive transcription elongation factor b; PP2A, protein phosphatase 2A; BAG3, Bcl-2-associated athanogene domain 3; CHIP, Chromatin immunoprecipitation; pol II, RNA polymerase II; CTD, C-terminal domain; 17-AAG, 17-N-allylaminomethyl-17-demethoxygeldanamycin; HSP, heat shock protein.
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The down-regulation of HSP70 was found to inhibit cell proliferation and induce apoptosis (10). Similar results were reported when HSP27 was down-regulated (11). HSF1 knockdown inhibited the viability of malignant cancer cell lines but did not affect a normal cell line (12). In the case of HeLa cancer cells, viability was inhibited more than 90% by HSF1 shRNA.

Bcl-2 family proteins are key regulators of apoptosis and consist of both anti- and pro-apoptotic members. The oligomerization of pro-apoptotic members Bak and Bax causes mitochondrial outer membrane permeabilization, allowing the release of cytochrome c and the subsequent activation of caspases. Anti-apoptotic members, including Bcl-2, Bcl-xL, Bcl-w, and Mcl-1, bind to pro-apoptotic members and prevent their oligomerization (13). Anti-apoptotic Bcl-2 family proteins have emerged as key therapeutic targets, and small molecule Bcl-2/Bcl-xL inhibitors, such as ABT-236 and ABT-737, are now in early clinical trials (14). Although ABT-236 has clinical activity in Bcl-2-dependent tumors, many tumors are not dependent on Bcl-2 but depend instead on Mcl-1. The overexpression of Mcl-1 is a common mechanism of resistance against ABT-737 in cancer cells (15–17). Furthermore, the amplification of the Mcl-1 locus is one of the most frequent somatic genetic events in human cancer (18). These results suggest that the development of dual inhibitors against both Bcl-2/Bcl-xL and Mcl-1 is more promising than specific inhibitors that target one or the other.

The Bcl-2-associated athanogene 1 (BAG1) family was identified as a Bcl-2-interacting protein and was found to enhance survival (19). Six BAG family members were reported to regulate HSP70/HSC70 function either positively or negatively. BAG-1 interacts with the proteasome and increases HSP70 client protein degradation (20). BAG3 inhibits the proteasomal degradation of HSP70 clients (21). Interestingly, BAG3 is an HSF1-inducible gene and has a role in enhancing cancer cell survival by stabilizing the Bcl-2 family proteins, such as Bcl-2, Bcl-xL, and Mcl-1 (22).

Cantharidin is a terpenoid isolated from blister beetles and other insects. The dried bodies of these beetles have been used in Chinese traditional medicine for the treatment of cancer for over 2000 years (23). Insects produce a large number of defensive molecules against predators, and these compounds have the potential to be used as medicinal drugs. Several groups reported that cantharidin induced apoptosis in hepatoma (24), multiple myeloma (25), pancreatic cancer cells (26, 27), and colon cancer (28). However, the clinical application of cantharidin is limited because of its toxicity. To reduce the toxicity of cantharidin, liposome-encapsulated cantharidin was synthesized and tested for its anticancer activity in vivo (29). PEG-liposomal cantharidin (5 mg/kg) significantly inhibited tumor growth in nude mice by ~75%, suggesting that cantharidin possesses highly effective antitumor activity. In addition, a diluted solution of cantharidin can be used as a topical medication to remove warts (30).

In this study, we identified cantharidin as an HSF1 inhibitor. Cantharidin down-regulates the levels of not only Bcl-2/Bcl-xL but also Mcl-1 by blocking HSF1-dependent HSP70/BAG3 expression. Furthermore, we demonstrate that the inhibition of HSF1 activity occurs by blocking HSF1 binding to target gene promoters. This is the first report that the anticancer activity of cantharidin involves HSF1 inhibition.

EXPERIMENTAL PROCEDURES

Reagents—The Spectrum Collection™ chemical library was purchased from MicroSource Discovery Systems, Inc. All chemicals used in the study, including cantharidin, norcantharidin, okadaic acid, 17-(allylamino)-17-demethoxydendamycin (17-AAG), DMSO, and monoclonal anti-α-actin antibody were purchased from Sigma. Antibodies against HSF1, HSP70, and HSP90α were purchased from Enzo Life Sciences. Antibodies against HSP27, poly(ADP-ribose) polymerase, ERK1/2, phospho-ERK1/2, p38, phospho-p38, acetylated lysine (Ac-K-103), and Bcl-2 were purchased from Cell Signaling Technology. Anti-BAG3 antibody was purchased from Abcam (Cambridge, UK). Phospho-Ser-2 RNA polymerase II antibody was purchased from Bethyl Laboratories Inc. Antibodies against RNA polymerase II (N-20), cyclin T1 (T-18), CDK9 (H-169), α-tubulin, histone H1 (FL-219), c-Myc (9E10), Bcl-xL (H-5), and Mcl-1 (S-19) were obtained from Santa Cruz Biotechnology. Antibodies against GAPDH, GSK3β (Ab-9), phospho-Ser-9 GSK3β, and phospho-Ser-303 HSF1 were obtained from Ab Frontier (Seoul, Korea).

Cell Culture—All cancer cell lines were originally obtained from ATCC. HCT-116 (human colon cancer) was maintained in McCoy’s 5A media (Invitrogen). A549 (small cell lung cancer cell), PC-3 (human prostate cancer), DU 145 (human prostate cancer), and MDA-MB-231 (human breast cancer) were cultured in RPMI 1640 media (Invitrogen). All culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen). Cell cultures were maintained at 37°C under 5% CO2 in an incubator.

Luciferase Reporter Construct and Dual-Luciferase™ Reporter Assay—p(HSE)4-TA-Luc plasmid was constructed as reported previously (31). The activity of the reporter was measured using a Dual-Luciferase™ reporter system (Promega, Madison, WI). HCT-116 cancer cells were seeded at a density of 2.5 × 10⁵ cells in 100 × 20-mm culture dishes. Cells were co-transfected with 9 μg of p(HSE)₄ TA-Luc vector and 1 μg of pRL-TK vector containing the Renilla luciferase gene as an internal control. The transfection was performed using Transfection (Roche Applied Science) according to the manufacturer’s protocol. Five hours after transfection, cells were trypsinized and seeded onto sterilized black-bottom 96-well plates at a density of 2 × 10⁴ cells per well. After incubation for 24 h, cells were pretreated with chemicals for 30 min, exposed to heat shock at 44°C for 20 min, and then incubated further at 37°C for 5 h. Firefly and Renilla luciferase activities were measured using a dual-light reporter gene assay kit (Promega).

Cell Proliferation Assay—Cells were seeded onto 96-well plates at a density of 6 × 10³ cells per well in McCoy’s 5A medium with 10% FBS. After 24 h, the medium was replenished with fresh complete medium containing cantharidin, norcantharidin, or 0.1% DMSO. After incubation for 48 h, the cell proliferation reagent WST-1 (Dojindo, Japan) was added to each well. The amount of WST-1 formazan produced was measured at 450 nm using an ELISA reader (Bio-Rad).
Chromatin Immunoprecipitation (ChIP) Assay—Cells were treated with either no heat or heat in the presence of different concentrations (1, 5, and 10 μM) of cantharidin. Cells were then fixed by adding formaldehyde (Sigma) to the medium to a final concentration of 1.5% for 15 min, after which glycine was added to a final concentration of 125 mM. The cells were then scraped and centrifuged for 5 min at 240 × g at room temperature. Pelleted cells were washed with ice-cold phosphate-buffered saline (PBS) containing protease inhibitors (1 mg phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). After centrifugation, cells were resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, and protease inhibitors) and incubated for 10 min on ice. After incubation, chromatin was sheared by sonication. After incubation, chromatin was sheared by sonication. After the removal of nuclear debris by centrifugation at 13,000 × g for 10 min at 4 °C, the lysates were diluted 10-fold with ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 12.7 mM EDTA, 16.7 mM Tris-HCL, pH 8.1, 167 mM NaCl) and then precleared for 30 min using 30 μl of Salmon Sperm DNA/protein A-agarase (Millipore). Immunoprecipitation was carried out at 4 °C overnight, and immune complexes were collected with Salmon Sperm DNA/protein A-agarase (Millipore). Antibodies used included anti-HSF1 (StressGen, SPA-901), anti-CDK9 (Santa Cruz Biotechnology, sc-8338 (H-169)), or preimmune rabbit serum as a control for nonspecific interaction. After washing three times with Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl Immune Complex Wash Buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and twice with TE Buffer (10 mM Tris-HCl, pH 8.1, 1 mM EDTA, pH 8.0), immunocomplexes were eluted with Elution Buffer (1% SDS, 0.1 M NaHCO3). Protein-DNA cross-links were reversed by incubating at 65 °C for 4 h. After proteinase K digestion, DNA was extracted with a PCR purification kit (Bioneer). Real time RT-PCR analysis was performed with the qRT-PCR Kit (Bioneer). Real time RT-PCR analysis was performed with the iQ™5 thermocycler (Bio-Rad), using FastStart SYBR Green PCR Master Mix (Roche Applied Science) to prepare the reaction mixtures. The primers used for real time PCR of human hsp70 genes were as follows: HSF1 ChIP assay, HSP70A forward primer, 5'-CCTCCCTCCCTTCTCCAG-3', and HSP70A reverse primer, 5'-TTCTCTCCTCTCTAGGAGAC-3'; and p-TfEb ChIP assay, HSP70A forward primer, 5'-GACTGCCAGGTCCTCGTCTAGC-3', and HSP70A reverse primer, 5'-GGTCCGTCCTCTGGAAGCACTGG-3'. The relative quantities of HSP70 promoter were normalized against the band intensity of the input DNA. Data were expressed as the mean ± S.D. of triplicate samples.

Quantitation of mRNA Using Quantitative Reverse Transcription-PCR—RNA was isolated using the RNeasy kit (Qiagen). Two micrograms of isolated RNA for each sample was reverse-transcribed with TOPscript™ RT DryMIX(dT18) kit (Enzymics™) according to the manufacturer’s instructions. Real time PCR was performed using IQ™ SYBR Green supermix (Bio-Rad) according to the manufacturer’s instructions using an iQ5 real time PCR detection system. The following primers were used for RT-PCR: HSP70 forward primer, 5'-ACCAGCGA-

GACGCAATCTTT-3', and HSP70 reverse primer, 5'-CCCTGCTACCTTTGAGAT-3'; HSP47 forward primer, 5'-GCGCATGTTCTCTACGCA-3', and HSP47 reverse primer, 5'-ATGGAACAGCACTGTTCGCT-3'; HSP27 forward primer, 5'-GGCATTTCTGAGTGGAG-3', and HSP27 reverse primer, 5'-AGGAGGCAGGACATGTTGC-3'; Mcl-1 forward primer, 5'-AGGCTGGATTGGTTTG-3', and Mcl-1 reverse primer, 5'-CACTTTCTGTGCGACCTCTTCTC-3'; Bcl-2 forward primer, 5'-CTGCACCTGACGCCCTAC-3', and Bcl-2 reverse primer, 5'-CACATCTGCTATGCCACCTTCTC-3'; and GAPDH forward primer, 5'-GGAGGACCTGAGAGCCAG-3', and GAPDH reverse primer, 5'-CCATGCCATGTGACCTCCC-3'. The relative quantities of HSP70 mRNA were normalized against GAPDH mRNA.

Western Blotting and Immunoprecipitation—Twenty micrograms of total protein was resolved by 7.5 or 12% SDS-PAGE and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% skim milk in TBS-T (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20). Proteins were detected with the indicated primary antibodies. The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit, anti-mouse, and anti-rat IgG from Jackson Immunoresearch. The antibodies were used at the dilution recommended by the manufacturers. The membrane was incubated with primary antibody for 2 h at room temperature, washed five times with TBS-T, and visualized with Lumina™ Forte Western HRP Substrate (Millipore). Blots were imaged using the ImageQuant LAS 4000, and protein band densities were analyzed using the MultiGauge version 3.0 software provided with the ImageQuant LAS 4000. For immunoprecipitation, 800 μg of lysates were incubated with primary antibody overnight at 4 °C with rotation, and then 50 μl of protein G magnetic beads (Millipore) was added. After 1 h, the lysates were removed, and beads were washed three times with RIPA buffer. Bead-bound proteins were resolved by SDS-PAGE and detected using specific antibodies.

FACS Analysis—HCT-116 cancer cells were treated with cantharidin at various concentrations for 48 h. Cells were then harvested by trypsinization, fixed with 70% chilled ethanol, and preserved at −20 °C before FACS analysis. Fixed cells were washed three times with PBS solution before being suspended in 500 μl of PBS and treated with 100 mg/ml RNase A at 37 °C for 30 min. Propidium iodide was added to a final concentration of 50 μg/ml for DNA staining, and 20,000 fixed cells were analyzed on a FACSCalibur system (BD Biosciences). The cell cycle distribution was analyzed using the ModFit program (BD Biosciences).
jected to SDS-PAGE and transferred to a PVDF membrane (Millipore). Proteins were detected with the indicated primary antibodies. The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit, anti-mouse, and anti-rat IgG (Jackson ImmunoResearch). The blots were developed with Luminata™ Forte Western HRP Substrate (Millipore).

Knockdown of PP2CA Proteins Using siRNA — Human PP2CA small interfering RNAs were synthesized as duplexes (Bioneer Inc., Korea) with the sequence 5'—AUGGAACUUGACGAUCUCUA—3' (Millipore). The negative control siRNA, 5'—CCUACGCCACCAAUUUCGU—3', was purchased from Bioneer Inc. (Korea).

HCT-116 cancer cells were seeded at a density of 5×10⁵ cells per well on 60-mm plates and incubated for 18 h. Cells were then transfected with 10 nM PP2CA siRNA and the control siRNA duplexes, described above, after incubation for 20 min with Oligofectamine RNAi Max (Invitrogen) in serum-free Opti-MEM (Invitrogen). Twenty four hours after transfection, the medium was replaced with fresh medium containing 10% FBS and incubated for an additional 24 h.

RESULTS

Screening for Inhibitors of HSF1 Activity — A structurally diverse library of 2,000 compounds was screened to identify HSF1 inhibitory chemicals. This chemical library is composed of marketed and experimental drugs and natural products. We used the p(HSE)₄-TA-Luc reporter cell-based reporter assay system previously reported (31). Cantharidin ((3aR,7aS)-3a,7a-dimethylhexahydro-4,7-epoxyisobenzofuran-1,3-dione) was identified as a hit compound (Fig. 1B) and was therefore characterized further. Cantharidin is a potent anticancer agent from traditional Chinese medicine.

The effect of cantharidin on heat shock-induced luciferase activity in HCT-116 cancer cells transfected with p(HSE)₄-TA-Luc was investigated. There was an ~42-fold induction of luciferase activity in response to heat shock in HCT-116 cancer cells (Fig. 1A). Cantharidin inhibited heat shock-induced luciferase activity in a concentration-dependent manner, with 50% inhibition at 4.2 μM. Norcantharidin (7-oxabicyclo [2.2.1] heptane-2,3-dicarboxylic anhydride) (Fig. 1B) is a structural analog of cantharidin, which showed very weak inhibitory activity compared with cantharidin.

To exclude the possible nonspecific transcriptional inhibitory activity of cantharidin, its effect on NF-κB activity was tested. A pNF-κB-Luc plasmid for an NF-κB luciferase reporter assay was obtained from Stratagene (La Jolla, CA) and used as described previously (32). As shown in Fig. 1C, NF-κB reporter activity was stimulated by treating cells with 20 ng/ml TNF-α. However, pretreating cells with cantharidin did not inhibit TNF-α-dependent NF-κB reporter activity even at high concentrations. This result suggests that cantharidin is not a general transcription inhibitor.

Inhibitory Activity of Heat Shock-induced HSP mRNAs and Proteins by Cantharidin — Because cantharidin inhibited HSF1-dependent reporter activity, the inhibitory effect of cantharidin on the endogenous HSP70, HSP47, and HSP27 promoter activities was investigated. For this experiment, HCT-116 cancer cells were exposed to heat shock stress at 43°C for 1 h in the presence or absence of cantharidin. The cells were then incubated at 37°C for 30 min to allow recovery. After the isolation of total RNA, HSP70 mRNA expression was evaluated by quantitative reverse transcription-PCR. As shown in Fig. 2A, heat
Cantharidin caused a 12-fold increase in HSP70 mRNA expression relative to the nonheat shock condition. The pre-treatment of HCT-116 cancer cells with cantharidin blocked heat shock-induced HSP70 expression in a concentration-dependent manner, with 50% inhibition at 5 μM. Similarly, cantharidin inhibited HSP47 and HSP27 mRNA expression in a concentration-dependent manner (Fig. 2A–C). In contrast, norcantharidin weakly inhibited HSP70 and HSP47 mRNA expression at high concentration (Fig. 2D–F). In accordance with its effect on mRNA expression, cantharidin also significantly down-regulated heat shock-induced HSP70 and HSP27 expression at high concentrations (Fig. 2G, H). These results proved that cantharidin is an inhibitor of HSF1 transcription activity.

Effects of Cantharidin on BAG3, BCL-2, BCL-xL, and MCL-1 Expression—BAG3 is HSF1-inducible and facilitates cancer cell survival by stabilizing the Bcl-2 family proteins (22). We therefore analyzed the effect of cantharidin on the expression of BAG3. For this experiment, HCT-116 cancer cells were exposed to heat shock stress at 43 °C for 1 h, and then incubated at 37 °C for 30 min; the quantitative analysis of mRNA levels of HSP70, HSP47, and HSP27 was performed using real-time PCR. The expression of each mRNA was normalized against the GAPDH gene. Each experiment was repeated three times, and each value is the mean ± S.D. Statistical significance (p value) was determined with an unpaired t test. #, p < 0.01 versus no heat control; *, p < 0.05; **, p < 0.01 versus heat control. G and H, cantharidin inhibited heat-induced HSP70 and HSP27 expression in HCT-116 cancer cells. HCT-116 cancer cells were treated with the indicated concentrations of cantharidin or norcantharidin for 30 min, exposed to heat shock at 43 °C for 1 h, and then incubated at 37 °C for 5 h. Whole cell lysates were analyzed by Western blotting as described under “Experimental Procedures.”

FIGURE 2. Cantharidin inhibits heat shock-induced transcription of HSP genes and protein expression of HSPs. A–F, heat-induced transcription of HSP70, HSP47, and HSP27 was repressed by cantharidin (canth) (A–C) in a dose-dependent manner but not by norcantharidin (D–F). HCT-116 cancer cells were treated with cantharidin (1–30 μM) for 30 min at 37 °C, exposed to heat shock at 43 °C for 1 h, and then incubated at 37 °C for 30 min; the quantitative analysis of mRNA levels of HSP70, HSP47, and HSP27 was performed using real-time PCR. The expression of each mRNA was normalized against the GAPDH gene. Each experiment was repeated three times, and each value is the mean ± S.D. Statistical significance (p value) was determined with an unpaired t test. #, p < 0.01 versus no heat control; *, p < 0.05; **, p < 0.01 versus heat control. G and H, cantharidin inhibited heat-induced HSP70 and HSP27 expression in HCT-116 cancer cells. HCT-116 cancer cells were treated with the indicated concentrations of cantharidin or norcantharidin for 30 min, exposed to heat shock at 43 °C for 1 h, and then incubated at 37 °C for 5 h. Whole cell lysates were analyzed by Western blotting as described under “Experimental Procedures.”

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result indicated that BAG1 is not a downstream effector of HSF1.

It is worth noting that treating cells with cantharidin induced slower migration of the BAG3 protein. Currently, we do not know the reason, but it is possible that PP2A inhibition by cantharidin induces the hyperphosphorylation of BAG3. Because HSP70 and BAG3 are important for the stability of anti-apoptotic Bcl-2 family proteins, we analyzed the effects of cantharidin on the levels of BCL-2, BCL-xL, and MCL-1 proteins. As shown in Fig. 3C, cantharidin caused a decrease in the expres-
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FIGURE 4. Cantharidin inhibits proliferation of HCT-116 cancer cells and arrests the cell cycle at the G2/M phase. A, HCT-116 cancer cells were treated with 0.1% DMSO or different concentrations of cantharidin or norcantharidin. After incubation for 48 h, a cell proliferation assay was performed as described under “Experimental Procedures.” Proliferation is expressed as the percentage of cantharidin-treated cells compared with the 0.1% DMSO-treated cells. Each value is the mean ± S.D. B, HCT-116 cancer cells were treated with the indicated concentrations of cantharidin or vehicle solvent (0.1% DMSO) for 48 h. After incubation, cells were subjected to FACS analysis. The relative percentages of cells in the sub-G1 (<2N), G2, M, and G0/G1 phases were determined by using the ModFit program (BD Biosciences).

Cantharidin Induces Apoptosis—Blocking HSF1 by cantharidin decreased the expression of HSF1 and p53. Cantharidin caused an increase in the proportion of cells in the sub-G1 population in cantharidin-treated cells compared with control levels. These results strongly supported that HSF1 inhibition by cantharidin is important for the antiproliferative activity of the compound. The effect of cantharidin on the proliferation of various other tumor cell lines was also analyzed; these cell lines and the GI50 value for each are as follows: MDA-MB-231 (2.2 μM), DU145 (2.4 μM), PC3 (2.8 μM), and A549 (2.9 μM). Both p53 wild-type (HCT116) and p53-deficient (MDA-MB-231) cancer cell lines were sensitive to cantharidin, indicating that cantharidin could exert its inhibitory activity in a p53-independent pathway.

Cantharidin Inhibits Heat Shock-induced Recruitment of HSF1 and p-TEFb to the HSP70 Promoter—Upon heat shock, HSF1 is recruited to the promoters of heat shock genes. Because cantharidin reduced the HSF1 transcription activity, the effect of cantharidin on the recruitment of HSF1 to the HSP70 promoter was assessed by ChIP analysis of HCT-116 cell extracts prepared from heat-shocked cells treated with cantharidin. As shown in Fig. 5, A and B, HSF1 association with the HSP70 promoter was significantly increased by heat shock. However, treating cells with cantharidin inhibited heat shock-induced HSF1 binding to the HSP70 promoter.

To analyze the temporal pattern of protein expression by cantharidin, HCT-116 cancer cells were treated with 5 μM cantharidin for different times. As shown in Fig. 3H, treating HCT-116 cancer cells with cantharidin for 3 h decreased the expression of BAG3 and MCL-1 and MCL-1 degradation could be rescued by proteasome inhibitor MG132. As shown in Fig. 3G, pretreating HCT-116 cells with MG132 could effectively block cantharidin-induced BCL-2, BCL-xL, and MCL-1 degradation. However, because cantharidin inhibited synthesis of HSP70 and BAG3, MG132 could not rescue their cantharidin-induced down-regulation (Fig. 3G).

To analyze the temporal pattern of protein expression by cantharidin, HCT-116 cancer cells were treated with 5 μM cantharidin for different times. As shown in Fig. 3H, treating HCT-116 cancer cells with cantharidin for 3 h decreased the expression of BAG3 and MCL-1, followed by the down-regulation of MCL-1 after 6 h of treatment. The down-regulation of BCL-2 and BCL-xL was detected 12 h after treating cells with cantharidin. The down-regulation of BCL-2 most likely required more time than that of MCL-1 because of its longer half-life (33).

Cantharidin Arrests the Cell Cycle at G2/M Phase and Induces Apoptosis—Blocking HSF1 by cantharidin decreased the expression of HSF70 and BAG3 and subsequently destabilized pro-survival Bcl-2 family proteins, leading to cancer cell death. To assess the sensitivity of HCT-116 cancer cells to cantharidin, the cells were treated with cantharidin or norcantharidin at different concentrations (0–100 μM) for 48 h (Fig. 4A). Cantharidin exhibited a dose-dependent inhibition of HCT-116 cell growth over a broad range of concentrations, with an IC50 of 5 μM, where IC50 is the concentration at which 50% inhibition of cell growth is observed. However, the IC50 of norcantharidin is 40 μM, suggesting 8-fold weaker activity compared with cantharidin. The relative inhibitory activity of cantharidin and norcantharidin on proliferation (Fig. 4A) is very similar to the inhibitory activity on HSF1-dependent transcription (Fig. 1A). These results strongly supported that HSF1 inhibition by cantharidin is important for the antiproliferative activity of the compound. The effect of cantharidin on the proliferation of various other tumor cell lines was also analyzed; these cell lines and the IC50 value for each are as follows: MDA-MB-231 (2.2 μM), DU145 (2.4 μM), PC3 (2.8 μM), and A549 (2.9 μM). Both p53 wild-type (HCT116) and p53-deficient (MDA-MB-231) cancer cell lines were sensitive to cantharidin, indicating that cantharidin could exert its inhibitory activity in a p53-independent pathway.

Because cantharidin inhibited cancer cell proliferation, the phase of the cycle affected by the compound was evaluated. HCT-116 cancer cells were treated with cantharidin at different concentrations for 48 h and subjected to FACS analysis. Cantharidin caused an increase in the proportion of G2/M phase cells in a concentration-dependent manner, indicating a cell cycle arrest at the G2/M phase (Fig. 4B). The increase in the proportion of the sub-G1 population in cantharidin-treated cells suggested that cantharidin induced apoptosis.

Cantharidin Inhibits Heat Shock-induced Recruitment of HSF1 and p-TEFb to the HSP70 Promoter—Upon heat shock, HSF1 is recruited to the promoters of heat shock genes. Because cantharidin reduced the HSF1 transcription activity, the effect of cantharidin on the recruitment of HSF1 to the HSP70 promoter was assessed by ChIP analysis of HCT-116 cell extracts prepared from heat-shocked cells treated with cantharidin. As shown in Fig. 5, A and B, HSF1 association with the HSP70 promoter was significantly increased by heat shock. However, treating cells with cantharidin inhibited heat shock-induced HSF1 binding to the HSP70 promoter.
Like HSF1, p-TEFb is rapidly recruited to the HSP70 promoter upon heat shock. p-TEFb is a heterodimer of CDK9 and cyclin T. HSF1 plays an essential role in the recruitment of p-TEFb (34). p-TEFb is essential for the induction of heat shock genes and HSF1 functions by recruiting p-TEFb to the promoters of these genes. Therefore, we wanted to confirm that cantharidin blocks HSF1-dependent p-TEFb recruitment to the HSP70 promoter using ChIP. As shown in Fig. 5, C and D, p-TEFb association with the HSP70 promoter was significantly enhanced by heat stress. However, treating cells with cantharidin inhibited p-TEFb recruitment to the HSP70 promoter in a concentration-dependent manner.

Because the CDK9 subunit of p-TEFb phosphorylates Ser-2 of the RNA polymerase II (pol II) C-terminal domain (CTD) and cantharidin inhibits p-TEFb recruitment to the HSP70 promoter, it was important to test whether cantharidin could inhibit the phosphorylation of pol II CTD Ser-2. As shown in Fig. 5E, the phosphorylation of CTD Ser-2 was significantly increased by heat shock, and the heat shock-induced phosphorylation of CTD Ser-2 was significantly inhibited by treating cells with cantharidin.
Cantharidin inhibits HSF1 binding to HSP70 promoter with cantharidin. This result is consistent with the inhibitory effect of cantharidin on the recruitment of p-TEFb to the HSP70 promoter. Treating cells with cantharidin did not change the amounts of cyclin T1 or CDK9 expression (Fig. 5F), excluding the possibility that cantharidin decreased pol II CTD Ser-2 phosphorylation by down-regulating p-TEFb expression.

Cantharidin Did Not Block Nuclear Localization of HSF1 upon Heat Shock—HSF1 is primarily localized in the cytosol under normal growth conditions. Upon stress, it translocates into the nucleus where it binds to the promoters of HSPs. Because ChIP analysis showed that cantharidin blocked HSF1 binding to the HSP70 promoter, we tested the possibility that cantharidin inhibited HSF1 translocation into the nucleus by performing a subcellular fractionation analysis. As shown in Fig. 5G, under normal conditions, HSF1 localized primarily in the cytoplasm and translocated into the nucleus upon heat stress. In addition, this nuclear translocation was not affected by cantharidin treatment. This result suggested that cantharidin did not inhibit the translocation step but inhibited the binding of HSF1 to the promoters of HSPs. Interestingly, HSP70 and HSP27 partially translocated into the nucleus upon heat shock stress.

HSP70 has many clients, most notably misfolded or unfolded proteins. Then it is possible that down-regulation of HSP70 by cantharidin can cause proteotoxic stress. We performed a subcellular localization of HSF1 in the presence of cantharidin or MG132 as a proteotoxic stress inducer. As shown in Fig. 5H, treating HCT-116 cells with MG132 significantly enhanced nuclear localization of HSF1. However, when HCT-116 cells were treated with cantharidin, it could not enhance nuclear translocation of HSF1. This result suggested that cantharidin did not induce proteotoxic stress. Misfolded proteins by cantharidin may be degraded by proteasome, releasing proteotoxic stress accumulation.

Cantharidin Did Not Increase Acetylation of HSF1—HSF1 is acetylated in a stress-inducible manner (35). Several lysine residues on HSF1 are acetylated. Among them, the acetylation of Lys-80, which makes a direct contact with the DNA phosphate backbone, was shown to reduce HSF1 DNA binding activity (35). The inhibition of SIRT1 by nicotinamide, a well known histone deacetylase inhibitor, significantly reduced HSF1 binding at the HSP70 promoter through the elevation of HSF1 acetylation (35). We therefore tested whether cantharidin blocked HSF1 DNA binding through enhanced HSF1 acetylation. HCT-116 cancer cells were transfected with plasmids encoding HSF1-myc and p300-HA and treated with DMSO, cantharidin, or positive control nicotinamide and then exposed to heat shock stress. Immunoprecipitated HSF1 was analyzed by Western blotting with acetylated lysine-specific antibodies. As shown in Fig. 5I, treating cells with cantharidin did not increase the acetylation of HSF1. However, nicotinamide enhanced the acetylation of HSF1. This result suggested that the inhibition of HSF1 binding to the HSP70 promoter is not through HSF1 acetylation.

Cantharidin Does Not Inhibit HSF1 Activity through PP2A Inhibition—Cantharidin has inhibitory activity against PP2A (36). It is possible that cantharidin inhibits HSF1 activity by blocking PP2A. We therefore measured HSF1 activity in the presence of okadaic acid, another PP2A inhibitor isolated from the marine sponge Halichondria okadai. The IC50 values of cantharidin and okadaic acid against PP2A are 50 and 12 nM, respectively (36), suggesting okadaic acid is four times more active than cantharidin. The GI50 values of cantharidin and okadaic acid against HCT116 cancer cells were 5 μM and 10 nM, respectively. Thus, we treated cells with up to 100 nM okadaic acid for this assay. As shown in Fig. 6A, treating cells with okadaic acid could not inhibit heat shock-induced HSF1 reporter activity. Similarly, okadaic acid could not inhibit heat shock-dependent HSP70 mRNA induction or HSP70 protein expression (Fig. 6, B and C). To confirm that PP2A phosphatase activity was inhibited by okadaic acid in our assay, we analyzed the phosphorylation of GSK-3β (37) and ERK1/2 (38), known substrates of PP2A phosphatase. As shown in Fig. 6D, the phosphorylation of GSK3β and ERK1/2 was increased by 30 nM okadaic acid treatment, indicating that okadaic acid could inhibit PP2A activity in cells.

The IC50 values of cantharidin and okadaic acid against PP2A are 50 and 12 nM, respectively (36). However, cantharidin’s GI50 value (5 μM) is significantly higher than okadaic acid’s value (10 nM). Therefore, we analyzed inhibitory activity of these compounds on PP2A using cell-based assay. We used the phospho-GSK-3β and ERK1/2 proteins as PP2A substrates. As shown in Fig. 6E, the phosphorylation of GSK3β and ERK1/2 was significantly reduced by cantharidin.
increased by treating HCT-116 cells with okadaic acid or cantharidin. We measured band intensity of phospho-GSK3β protein using the ImageQuant LAS 4000, and protein band densities were analyzed using the MultiGauge version 3.0 software provided with the ImageQuant LAS 4000. For phospho-GSK3β, the IC50 values from cell-based assay of cantharidin inhibition of PP2A activity were determined using the ImageQuant LAS 4000.
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FIGURE 7. Cantharidin inhibits the induction of HSPs by an HSP90 inhibitor or a proteasome inhibitor. HCT-116 cancer cells were pretreated with the indicated concentrations of cantharidin or vehicle solvent (0.1% DMSO) for 30 min and exposed to 1 μM 17-AAG (A) or 10 μM MG 132 (B) for 5 h at 37 °C, and whole cell lysates were analyzed by Western blotting as described under “Experimental Procedures.” HCT-116 cancer cells were treated with 0.1% DMSO, 2 μM cantharidin, 0.2 μM 17-AAG (C), or 0.2 μM MG132 (D). After incubation for 48 h, proliferation assay was performed as described under “Experimental Procedures.” Proliferation is expressed as the percentage of compound-treated cells compared with the 0.1% DMSO-treated cells. Each value is the mean ± S.D.

DISCUSSION

Because of the extremely high cost of drug development from screening to approval, it is highly desirable to find existing drugs that can be repurposed for cancer therapy. This approach also accelerates the drug discovery process. For example, the diabetic drug metformin is being explored for its use in cancer therapy. If we can find an existing drug that inhibits HSF1 activity, it may be useful for further development as cancer therapy. To identify new HSF1 inhibitors, we performed cell-based screening with a library of marketed and experimental drugs. From this screening, we identified cantharidin as a hit compound. Cantharidin is an active compound from dried blister beetles, which have been used in Chinese traditional medicine for over 2000 years (23). Cantharidin is known as an inhibitor of protein phosphatase 2A (PP2A) (36). Therefore, we first hypothesized that cantharidin might inhibit HSF1 activity by
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Many research groups have reported on the anticancer activity of cantharidin, focusing on its PP2A inhibitory activity. However, PP2A is a known tumor suppressor (43). The tumor suppressive function of PP2A was first suggested when PP2A was established as a target of the tumor-promoting compound okadaic acid (44–46). In addition, the genetic mutations of the PP2A genes have been reported in several human cancers (45, 46). Oncogenic viral proteins, including SV40 T antigen, bind to PP2A and inhibit its activity (47). Furthermore, FTY720, known as Gilenya (Novartis), is an activator of PP2A (48). FTY720 induced the apoptosis of cancer cell lines derived from various tissues, such as breast cancer, hepatocarcinoma, leukemia, and melanoma (49). FTY720 also inhibits tumor growth in xenograft models. These results strongly support the consensus that PP2A has a tumor suppressor function.

However, PP2A is an essential gene because the knock-out of the α isof orm of the catalytic subunit is lethal in mice (50). In addition, PP2A inhibition by okadaic acid can induce apoptosis (51, 52). Furthermore, PP2A regulates proliferation by modulating the initiation of DNA replication (53). Therefore, the complete inhibition of PP2A activity can block cell growth and survival. These contradictory observations suggest that PP2A has inhibitory or stimulatory activity on cell growth depending on cellular context and warrant caution in interpreting results.

In contrast to PP2A’s controversial role in cell proliferation, HSF1 enhances the survival of cancer cells against various stresses, and knock-out of HSF1 has been reported to impair cancer formation and progression. HSF1 has been considered a promising therapeutic target in several cancers. Therefore, a significant portion of the antiproliferative activity of cantharidin is likely to be associated with HSF1 inhibition. In addition, it is important to determine whether the PP2A inhibitory activity of cantharidin has an additive effect with its anticancer activity.

Several groups synthesized more than a thousand analogs of cantharidin to study its PP2A inhibition (54), as these studies were performed when the HSF1 inhibitory activity of cantharidin was unknown. It is important to analyze cantharidin and its analogs for HSF1 inhibitory activity in addition to its PP2A inhibition. This report will be very helpful for researchers who develop cantharidin or its analogs for cancer therapeutics.

In mice, HSF1 is dispensable for growth and survival under controlled laboratory conditions (55). The Hsf1-deficient mouse displays no overt organ system abnormalities and, in the absence of acute stress, lives to late adulthood. However, under stress conditions, HSF1 is essential for survival (55). Because HSF1 is important for both cancer and nontransformed cells under stress conditions, inhibition of HSF1 can exert not only anticancer activity but also toxicity. Therefore, it is important to identify nonstressful conditions to minimize the cytotoxic effects of HSF1 inhibitor for its further use.

In summary, we identified cantharidin as an HSF1 inhibitor and characterized its mechanistic activity. Cantharidin induces cancer cell death through the inhibition of HSP70 and BAG3 expression by blocking HSF1 binding to the promoters of HSP70 and, presumably, BAG3. The down-regulation of HSP70/BAG3 chaperones reduced the stability of anti-apoptotic Bcl-2 family proteins, such as BCL-2, BCL-xL, and MCL-1, inducing cancer cell death (Fig. 8). In particular, blocking PP2A activity. We tested this possibility by analyzing HSF1 transcription activity after PP2A inhibition by siRNA or okadaic acid. Unexpectedly, the knockdown of PP2A with siRNA or the inhibition of PP2A activity by okadaic acid could not block heat shock-induced HSF1 transcriptional activity (Fig. 6). These results suggest that PP2A inhibition is not sufficient for or is not involved in HSF1 inhibition by cantharidin.

Cantharidin inhibited HSF1 reporter activity with an IC50 of 4.2 μM. Similarly, it decreased the HSF1-dependent transcription of HSP70, HSP47, HSP27, and BAG3 in a concentration-dependent manner. In addition, cantharidin exhibited a dose-dependent inhibition of HCT-116 cancer cell proliferation over a broad range of concentrations, with a GI50 of 5 μM. In contrast, the structural analog norcantharidin had an ~10-fold weaker inhibitory activity against HSF1 compared with cantharidin (Fig. 1A) and an 8-fold weaker inhibitory activity toward HCT-116 cancer cell proliferation (Fig. 4A). These results strongly suggest that HSF1 is a target for the antiproliferative activity of cantharidin. However, we can not exclude other possible targets besides PP2A and HSF1 for cantharidin.

If the inhibition of HSF1 activity by cantharidin is important for its anticancer activity, how does it work? Bcl-2 family members are important anti-apoptotic proteins in human cancer cells, and their stability is highly dependent on HSP70/BAG3 chaperones (22). Therefore, the down-regulation of HSP70/BAG3 by cantharidin caused a dramatic reduction in BCL-2, BCL-xL, and MCL-1 stability, leading to the induction of apoptosis (Fig. 3, C and H). This conclusion is supported by the fact that cantharidin did not decrease the levels of BCL-2, Bcl-xL, or MCL-1 mRNA (Fig. 3, D–F) but diminished their protein levels. These data are consistent with a prior report with the siRNA-targeted knockdown of BAG3 (22). Jacobs and Marnett (22) reported that the silencing of HSF1 or BAG3 expression with siRNA caused a dramatic reduction in BCL-2, BCL-xL, and MCL-1 protein levels.

Because Bcl-2 family proteins are important for apoptosis, Bcl-2 antagonists, such as ABT-263 (Navitoclax) and ABT-737, have been developed. However, ABT-263 and ABT-737 are unable to bind Mcl-1. Therefore, Mcl-1 is not inhibited by ABT-263 or ABT-737 and is considered to be a major factor in resistance to ABT-737 (15, 41, 42). Furthermore, the amplification of the Mcl-1 locus is the most frequent somatic genetic event in human cancer (18). These results suggest that dual inhibitors against both Bcl-2/Bcl-xL and Mcl-1 would be more useful than individual specific inhibitors. Interestingly, cantharidin decreased not only BCL-2 and BCL-xL but also MCL-1 (Fig. 3C), which may make it more effective for cancer therapy.

To determine the anticancer mechanism of cantharidin, we analyzed the binding of HSF1 to the HSP70 promoter by using ChIP. As shown in Fig. 5, A and B, we found that HSF1 binding to the promoter was inhibited when HCT-116 cancer cells were treated with cantharidin. The acetylation of HSF1 Lys-80 was reported to reduce HSF1 DNA binding activity (35). However, cantharidin did not increase HSF1 acetylation. Currently, we do not know how cantharidin blocks HSF1 binding to the HSP70 promoter. It is important to identify the direct molecular targets of cantharidin other than PP2A to explain its working mechanism.
Cantharidin Inhibits HSF1 Binding to HSP70 Promoter

MCL-1 overexpression in ABT-737 drug-resistant cancer can be effectively overcome by HSF1 inhibitors.

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