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Fisetin, a dietary flavonoid, induces apoptosis of cancer cells by inhibiting HSF1 activity throughblocking its binding to the hsp70 promoter

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There are no potential conflicts of interest.

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Abstract

Heat shock factor 1 (HSF1)is a transcription factorfor heat shock proteins (HSPs) expression thatenhances the survival of cancer cells exposed to various stresses. HSF1 knockout suppresses carcinogen-induced cancer induction in mice.Therefore, HSF1 is a promising therapeutic and chemo-preventive target. We performed cell-based screening with a natural compound collection and identified fisetin, a dietary flavonoid, as a HSF1 inhibitor. Fisetin abolished heat shock-induced luciferase activity with an IC_{50} of 14μM inHCT-116 cancer cells.The treatment of HCT-116 with fisetin inhibited proliferation with a GI_{50} of 23 μM. When the cells were exposed to heat shock in the presence of fisetin, the induction of HSF1 target proteins, such as HSP70, HSP27, and BAG3(Bcl-2-associated athanogene domain 3),were inhibited. HSP70/BAG3 complexes protect cancer cells from apoptosis by stabilizing anti-apoptotic Bcl-2 family proteins.The down-regulation of HSP70/BAG3 by fisetin significantly reduced the amounts of Bcl-2, Bcl-x_L, and Mcl-1 proteins, subsequently inducing apoptotic cell death. Chromatin immunoprecipitation assays showed that fisetin inhibited HSF1 activity by blocking the binding of HSF1 to the *hsp70* promoter.Intraperitoneal treatment of nude mice with fisetin at 30 mg/kg resulted in a 35.7% ($p<0.001$) inhibition of tumor growth.

Summary

HSF1-dependent HSP70/BAG3 expression protects cancer cells from apoptosis by stabilizing anti-apoptotic Bcl-2 family proteins. We identified fisetin as an HSF1 inhibitor. The down-regulation of HSP70/BAG3 by fisetin significantly reduced the amounts of Bcl-2, Bcl-x_L, and Mcl-1 proteins, thus inducing apoptosis of cancer cells. Fisetin inhibited HSF1 activity by blocking HSF1 binding to target gene promoters.

Abbreviations: HSF1, heat shock factor 1; HSE, heat shock element; HSP, heat shock protein; ChIP, Chromatin immunoprecipitation; p-TEFb, positive transcription elongation factor b; BAG3, Bcl-2-associated athanogene domain 3; PARP, poly (ADP-ribosyl) polymerase (PARP);

Introduction

Cancer cells have multiple oncogenes and a high degree of signal redundancy, which causes a single anticancer drugtends to have relatively weak therapeutic effects. Therefore, combination therapy or multi-targeted therapy is a way to solve these obstacles. Most natural compounds have multiple target molecules and thus are useful for treating cancers. A nutraceutical is any substance considered to be a food or part of a food that provides medical benefits(1,2).Intake of dietary foods, such as fruits or spices,has been reported to reduce the risk of cancer genesis.

Fisetin (3,7,3′,4′-tetrahydroxyflavone) is a flavonoid found in many edible fruits and vegetables, such as apples, grapes, kiwis, persimmons, strawberries, cucumbers, and onions that has multiple properties, such as anti-oxidant (3) and anti-inflammatory (4) activity. The highest levels of fisetin (160 μg/g wet food) are found in strawberries (5). Fisetin induced apoptosis in LNCaP human prostate cancer cells (6) and mice receiving fisetin at 45mg/kg inhibited tumor growth by 74.8% through androgen receptor inhibition (7). Similarly, fisetin also inhibited melanoma cell growth by disrupting Wnt/β-catenin signaling, and mice receiving fisetin at 45mg/kg suppressed tumor growth by 66.6% (8). Furthermore, fisetin has beenreported as an inhibitor of Aurora B kinase (9), NF-κB (10), mTOR (11), and PI3K/AKT and mTOR signaling (12). Recently Kang and colleagues reported that fisetin induces apoptosis in human non-small cell lung cancer by down-regulating Bcl-2 expression(13).

The heat shock response (HSR)protects cells from a wide range of stresses, including heat shock, oxidative stress, heavy metals, fever, or protein misfolding (Reviewed in (14,15)).The HSR is mainlymediated by heat shock transcription factor 1 (HSF1)that controls the transcription of heat shock proteins (HSPs), such as HSP70,HSP47, HSP27, and BAG3. Cells overexpressing HSP70 or HSP27 had an increased tumorigenicity when inoculated into mice (16,17).The expression of *hsp70* is

induced by several oncogenes, such as *H-ras*^{val12}(18), *c-myc*(19), *c-myb*, SV40 large T antigen, and adenovirus E1a (20).

HSF1 knock-out suppresses chemically induced skin cancer (21) and hepatocellular carcinoma induced by procarcinogen diethylnitrosamine(22) in mice. In addition, HSF1 knockdown has a minimal effect on normal primary human cells but significantly impairsthe proliferation of several human malignant cell lines (21).Similarly, the down-regulation of HSP70 or HSP27 was found to inhibit cell proliferation and induce apoptosis (23,24), suggesting that HSF1 is a highly potent target for cancer therapy.

BAG3 is a HSF1-inducible gene and protects cancer cellsfrom apoptosis by stabilizing the Bcl-2 family proteins, such as Bcl-2, Bcl-x_L, and Mcl-1 (25). The BAG1 (Bcl-2-associated athanogene 1) family was firstreported as a Bcl-2 interacting protein (26). Six BAG family members were reported to regulate HSP70 function either positively or negatively. Whereas BAG-1 interacts with the proteasome and increases the degradation of HSP70 client proteins(27), BAG3 inhibits their proteasomal degradation (28).

In this study, we identified fisetin as an HSF1 inhibitor. Fisetin decreased the levels of not only HSP70/BAG-3 but also Bcl-2 family proteins; these changes induced apoptosis of cancer cells. Furthermore, we demonstrated that the inhibition of HSF1 activity occurredby blocking HSF1 binding to target gene promoters. This study is the first report that the anticancer activity fisetin involves HSF1 inhibition.

Materials and Methods

Reagents

The natural chemical library has 716 compounds,some of them isolated from natural resources in our lab. Chemicals used in this study, including fisetin, quercetin, naringenin, kaempferol, baicalein,DMSO, and monoclonal anti-α-Actin antibody were purchased from Sigma (MO, USA). Antibodies against HSF1and HSP70 were purchased from Enzo Life Sciences (NY, USA). Antibodies against HSP27, PARP, Bcl-2, Caspase-3 (#9662), and Caspase-7 (#9492) were purchased from Cell Signaling Technology (MA, USA). Anti-BAG3 antibody was purchased from Abcam (Cambridge, UK). Antibodies against α-Tubulin, Histone H1(FL-219), c-Myc(9E10), Bcl-xL(H-5), and Mcl-1(S-19) were obtained from Santa Cruz Biotechnology (TX, USA). Antibodies against GAPDH and phospho-Ser303 HSF1 were obtained from Ab Frontier (Seoul, Korea).

Cell Culture and Cell Line Authentication

HCT-116 (colon carcinoma, ATCC® CCL-247™) in the year 2008, SW620 (colon carcinoma, ATCC® CCL-227™) in the year 2008,Mia PaCa-2 (pancreatic carcinoma ATCC® CCL-1420™) in the year 2008,MDA-MB-231 (breast adenocarcinoma ATCC®CRM-HTB-26™) in the year 2008, PC-3 (prostate adenocarcinoma,ATCC® CRL-1435™) in the year 2011, DU-145 (prostate carcinoma, ATCC® HTB-81™) in the year 2011, and LNCap (prostate carcinoma,ATCC® CRL-1740™) in the year 2011 were purchased from the American Type Culture Collection (Manassas, VA, USA). Because most of the results in this study were obtained using HCT-116 colon cancer cells, KCTC (Korean Collection for Type Cultures) authenticates HCT-116 cancer cells using short tandem repeat analysis.HCT-116 (human colon cancer) was maintained in McCoy's 5A media (GIBCO). SW620 (human colorectal cancer cell), Mia-PaCa-2 (human pancreatic cancer cells), PC-3 (human prostate cancer), DU 145 (human prostate cancer), LNCaP (human prostate cancer), MDA-MB-231 (human

breast cancer), and HFF (human foreskin fibroblast) were cultured in RPMI 1640 media (GIBCO). All culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO). Cell cultures were maintained at 37°C under 5% CO_2 in an incubator.

Luciferase Reporter Construct and Dual-Luciferase™ Reporter Assay

p$(HSE)_4$-TA-Luc plasmid was constructed as reported previously (29). 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 $2.5x10^6$ cells in 100x20 mm culture dishes. Cells were co-transfected with 9μg of p$(HSE)_4$-TA-Luc vector and 1μg of pRL-TK vector containing the *Renilla* luciferase gene as an internal control. The transfection was performed using TransFection (Roche) according to the manufacturer's protocol. Five hours after transfection, cells were detached by trypsin with EDTA and seeded onto sterilized, black-bottom96-well plates at a density of $2x10^4$ cells per well. After incubation for 24 h, cells were pretreated with chemicals for 30 min, exposed to heat shock at 44°C for 15 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 $6x10^3$ cells per well in McCoy's 5A medium with 10% FBS. After 24 h, the medium was replenished with fresh complete medium containing fisetin, quercetin, 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 (25 and 50 μM) of fisetin. 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 125mM. The cells were then scraped and centrifuged for 5min at 240×g at room temperature. Pelleted cells were washed with ice-cold phosphate buffered saline (PBS) containing protease inhibitors (1mM phenylmethylsulfonyl fluoride(PMSF), 1μg/mL aprotinin, and 1μg/mL pepstatin A). After centrifugation, cells were resuspended in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1, and protease inhibitors) and incubated for 10 min on ice. After incubation, chromatin was sheared by sonication. After the removal of nuclear debris by centrifugation at 13000×g for 10min at 4°C, the lysates were diluted 10-fold with ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, 167mM NaCl) and then precleared for 30min using 30μl of Salmon Sperm DNA/Protein AAgarose (Millipore). Immunoprecipitation was carried out at 4°C overnight, and the immune complexes were collected with Salmon Sperm DNA/Protein A-Agarose (Millipore). The antibodies used included anti-HSF1(StressGen, SPA-901), or pre-immune rabbit serum as a control for non-specific interactions. After washing three timeswith Low Salt Immune Complex Wash Buffer(0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8.1, 150mM NaCl), High Salt Immune Complex Wash Buffer(0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8.1, 500mM NaCl), LiCl Immune Complex Wash Buffer(0.25M LiCl, 1% NP-40, 1% Sodium deoxycholate, 1mM EDTA, 10mM Tris-HCl pH8.1) and twice with TE Buffer(10mM Tris-HCl pH8.1, 1mM EDTA pH8.0), immunocomplexes were eluted with Elution Buffer(1% SDS, 0.1M $NaHCO_3$). Protein-DNA cross-links were reversed by incubating at 65°Cfor 4 h. After proteinase K digestion, DNA was extracted with a PCR-Purification Kit(Bioneer). Real-time PCR analysis was performed with the iQ^{TM}5 thermocycler (BIO-RAD), using FastStart SYBR Green Master(Roche) to prepare the reaction mixes. The primers used for real-time PCR of human *hsp70*genes were the followingfor the HSF1 ChIP assay:HSP70A forward primer, 5'-CACTCCCCCTTCCTCTCAG-3'; HSP70A reverse primer, 5'-TTCCCTTCTGAGCCAATCAC-3'. The relative quantities of *hsp70* were normalized to the input DNA. Data were expressed as the mean ± S.D. of triplicate samples.

Quantification of mRNA Using Quantitative Reverse Transcription PCR

RNA was isolated using an RNeasy kit (Qiagen). Two micrograms of isolated RNA for each sample was reverse-transcribed with TOPscript™ RT DryMIX(dT18) kit (EnzynoMics™) 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'-ACCAAGCAGACGCAGATCTTC-3'; HSP70 reverse primer, 5'-CGCCCTCGTACACCTGGAT-3'; HSP47 forward primer, 5'-CGCCATGTTCTTCAAGCCA-3'; HSP47 reverse primer, 5'-CATGAAGCCACGGTTGTCC-3'; HSP27 forward primer, 5'-GGCATTTCTGGATGTGAGCC-3'; HSP27 reverse primer, 5'-AGCAGGCAGGACATAGGTGC-3'; BAG3 forward primer, 5'-ATGCAGCGATTCCGAACTGAG-3'; BAG3 reverse primer, 5'-AGGATGAGCAGTCAGAGGCAG-3'; GAPDH forward primer, 5'-GGGAGCCAAAAGGGTCATCATCTC-3'; GAPDH reverse primer, 5'-CCATGCCAGTGAGCTTCCCGTTC-3'. The relative quantities of *hsp70* mRNA were normalized against the GAPDH mRNA.

Western Blotting and Immunoprecipitation

Twenty micrograms of lysate 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 dilutions recommended by the manufacturers. The membrane was incubated with the primary antibody for 2 h at room temperature, washed five times with TBS-T, and visualized with Luminata™ Forte Western HRP Substrate (Millipore). For immuno-precipitation, 800 μg of the lysates were incubated with

theprimary antibody overnight at 4°Cwith rotation, and then 50 µl of protein G magnetic beads (Millipore) were 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 fisetin at various concentrations for 48 h. Cells were then harvested, fixed with 70% chilled ethanol, and preserved at -20°Cbefore FACS analysis. Fixed cells were washed three times with phosphate-buffered saline (PBS) solution before being suspended in 500µl PBS and treated with 100mg/ml RNase A at 37°Cfor 30 min. Propidium iodide was added to a final concentration of 50 mg/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).

Nuclear and Cytoplasmic Extraction

Cells were seeded onto 100 x 20 mm plates at a density of $2x10^6$ cells in McCoy's 5A medium with 10% FBS. After 24 h, cytoplasmic and nuclear protein extracts were isolated using the NE-PER kit (Thermo Scientific) according to the manufacturer's instructions. A volume of samples corresponding to 20 µg was subjected 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).

Nude Mouse Xenograft Assay

Xenograft experiment with HCT-116 cellswas carried out in accordance with the guidelines and under

the approval of the Institutional Review Committee for Animal Care and Use, Korea Research Institute of Bioscience and Biotechnology. Seven-week-old female inbred specific-pathogen-free (SPF) Balb/c nude mice were housed under sterile conditions with 12 hour light/dark cycles, and fed food and water *ad libitum*. For the evaluation of the *in vivo* anti-tumor activity of fisetin, HCT-116 cells (0.3 ml of 4×10^7 cells/ml) were implanted subcutaneously into the right flank of the mice on day 0. Fisetin was dissolved in 10% DMAC, 10% Tween80, and 80% [20% 2-Hydroxypropyl-β-cyclodextrin(in distilled water)]. Vehicle control and fisetin (30 mg/kg, intraperitoneal injection)were administered three times per weekfor 25 days. Tumor volumes were estimated by using the formula length (mm) × width (mm) × height (mm)/2. To determine the toxicity of the compound, the body weight of tumor-bearing animals was recorded. On day 25, the mice were sacrificed and the tumors were weighed.

Statistical Analysis

The data are expressed as the means ± standard deviations (S.D.s), and the degree of significance was analyzed using Student's t-test. P-values below 0.05 were considered to indicate statistical significance.

Results

Screening for inhibitors of HSF1 activity

A heat shock-dependent luciferase reporter plasmid, p$(HSE)_4$-TA-Luc, was used for screening of HSF1 inhibitors as described previously (29).716 structurally diversenatural compounds were screened to identify HSF1-inhibitory chemicals. For this, HCT-116 cancer cells were cotransfected with a p$(HSE)_4$-TA-Luc reporter and aninternal control vector constitutively expressing pRL-TK to normalize for transfection efficiency. There was 75-fold induction of the luciferase activity in HCT-116 cancer cells by heat shock stress (**Fig. 1A**). Then, cells were treated with compounds at 10 μM for 30 min and then exposed to heat shock at 44°C for 15 min. After 5 h of recovery at 37°C, luciferase activity was measured.From these experiments, we identified fisetin as a HSF1 inhibitor.

Surprisingly fisetin (3,7,3′,4′-tetrahydroxyflavone) but not quercetin (3,3′,4′,5,6-pentahydroxyflavone)was identified in our screening even though the latter was previously reported as a HSF1 inhibitor (30-32). We measured the inhibitory activity of fisetin and quercetin on the HSF1 reporter in several cancer cell lines (**Fig. 1A-D**). Fisetin inhibited HSF1 reporter activity in a concentration-dependent manner, with 50% inhibition at14 μMfor HCT-116cancer cells. In addition, fisetin inhibited HSF1 reporter activity in all cancer cell lines and had stronger activity compared with quercetin. We tested 4 structural analogs of fisetin, quercetin, naringenin, kaempferol, and baicalein (**Fig. 1E**).Quercetin and kaempferol showed 54% and 66% inhibitory activity at 100 μM, respectively. Naringenin and baicalein did not inhibit reporter activity, even at 100 μM.

Inhibition of heat shock-induced HSP mRNAs and proteins by fisetin

Because fisetindecreased HSF1-dependent reporter activity, the inhibitory effect of fisetin on the

endogenous *hsp70*, *hsp47*, and*hsp27*gene expression was investigated.HCT-116 cells were exposed to heat shock at 43°C for 1 h in the absence orpresence of fisetin. Then, the cells were incubated at37°C for 30 min to allow for recovery, and the total RNAs were isolated. The expression of*hsp70* mRNA was evaluated by quantitative real-time reverse transcription-PCR.As shown in **Fig. 2A**, heat shock treatmentcaused an11-fold increase in*hsp70* mRNA expression relative to the non-heat shock condition. Pretreatment of HCT-116 cells with fisetin blocked heat shock-induced *hsp70* mRNA expression in a concentration-dependent manner, with 50% inhibition at 17μM.Similarly, fisetin inhibited *hsp47*,*hsp27*,and *BAG3* mRNA expression in a concentration-dependent manner (**Figs. 2B-D**).The IC_{50} for each of the HSP's mRNA expression is very similar to the IC_{50} for the HSF1 reporter assay (**Fig. 1** and **Fig. 2**).

Next, we analyzed effect of fisetin of the expressions of HSP proteins. For this, HCT-116 cells were subjected to heat shock at 43°C for 1h in the presence or absence of fisetin and incubated at 37°C for 6h to allow recovery.Consistent with its effect on HSPs mRNA expression, fisetin also significantly down-regulated HSF1 downstream target proteins, such as HSP70, HSP27, and BAG3, in a concentration-dependent manner (**Fig. 2E**).Interestingly, amounts of HSF1 were decreasedby treating HCT-116 cells with heat shock and further decreased when the cells were treated with heat shock and fisetin (**Fig. 2E**). However, when HCT-116 cells were subjected to heat shock at 43°C for 1h in the absence or presence of fisetinwithout recovery at 37°C, amounts of HSF1 was not decreased by fisetin (**Fig. 5D**), suggesting that HSF1 was degraded during the recovery time at 37°C. In addition, HSF1 migrated slowly on SDS-PAGEby hyper-phosphorylation of HSF1(**Fig. 2E**).

Fisetin induces apoptosis through the down-regulation of HSP70/BAG3/Bcl-2

BAG3 was reported to be induced by HSF1 and enhance cancer cell survival by stabilizing the anti-apoptotic Bcl-2 family proteins (25). As shown in Fig. 2D, heat shock treatment increased BAG3

mRNA expression more than 4-foldrelative to the non-heat shock condition. The pretreatment of HCT-116 cancer cells with fisetin inhibited heat shock-induced BAG3 expression in a concentration dependent manner (Fig. 2D). Fisetin also decreased heat shock-induced BAG3 protein expression (Fig. 2E).

Because HSP70 and BAG3 are molecular chaperonesthat stabilize of anti-apoptotic Bcl-2 family proteins, we analyzed the effects of fisetin on the levels of Bcl-2, Bcl-x_L, and Mcl-1 proteins. HCT-116 cells were treated with fisetin at various concentrations (0-50 μM) for 48 h. As shown in **Fig. 3A**, fisetin caused a decrease in the expression of HSP70, BAG3, Bcl-2, Bcl-x_L, and Mcl-1in a concentration-dependent manner. As the amounts of Bcl-2, Bcl-x_L, and Mcl-1 were decreased by treating cells with fisetin, PARP cleavage appeared, indicating apoptotic cell deathbrought on by the fisetin treatment.

To analyze the temporal changes in protein expression caused by fisetin, HCT-116 cancer cells were treated with 50μM fisetin for different amounts of time. As shown in **Fig. 3B**, treating HCT-116 cancer cells with fisetin for 12 h decreased the expression of HSP70 and BAG3 followed by the down-regulation of Bcl-2 after 24 h of treatment. Significant down-regulation of HSP70/BAG3/Bcl-2 is coincident with PARP cleavage, indicating apoptosis of the HCT-116 cancer cells. Unexpectedly, the down-regulation of Mcl-1 and Bcl-x_L was detected 3 h after treating cells with fisetin. Because Mcl-1 and Bcl-x_L were down-regulated earlier than HSP70/BAG3 by fisetin, their expressionmight be mediated by another target molecule and not by HSF1 inhibition. However, the down-expression of Mcl-1 and Bcl-x_L could not induce PARP cleavage (**Fig. 3B**). These results suggested that HSP70/BAG3/Bcl-2 down-expression is important to induce HCT-116 apoptosis.Because, PARP cleavage was strongly induced by treating HCT-116 cancer cells with fisetin for 24 h (Fig. 3B), we analyzed whether fisetin activated executive caspases. As shown in Fig. 3C, active caspase-3 and caspase-7 was induced by fisetin in a concentration-dependent manner and thiscaspases activation is coincident with PARP cleavage.

To assess the sensitivity of HCT-116 cancer cells to fisetin,the cells were treated with fisetin or quercetin at different concentrations (0 - 100 μM) for 48h (**Fig. 4A**). Fisetin exhibited a dose-dependent inhibition of HCT-116 cell growth over a broad range of concentrations, with a GI_{50} of 23μM, where $GI_{50}$is the inhibitor concentration at which a 50% inhibition of cell growth is observed. However, the GI_{50} of quercetin was more than 100μM, suggesting a 4-fold weaker activity compared to fisetin. The relative inhibitory activity of fisetin and quercetin on the proliferation of HCT-116 cancer cells (**Fig. 4A**) is very similar to the inhibitory activity results from the HSF1-dependent reporter assay (**Fig. 1**). These results strongly suggest that HSF1 inhibition by fisetin is important for the anti-proliferative activity of the compound.The effect of fisetin on the proliferation of various other tumor cell lines was also analyzed; these cell lines and the GI_{50} value for each are as follows: Mia-PaCa-2 (29μM), SW620 (37μM), MDA-MB-231 (38μM), DU-145 (17μM), PC3 (41μM), and LNCaP (41μM). When immortalized human foreskin fibroblast cells (HFF) were used for proliferation, GI_{50} was 145μM, indicating that cancer cells were more sensitive to fisetin compared to the immortalized normal cells.

Because fisetin inhibited cancer cell proliferation, the phase of the cell cycle affected by the compound was evaluated. HCT-116 cancer cells were treated with fisetin at different concentrations for 48 h and subjected to FACS analysis. Fisetin caused an increase in the proportion of the sub-G_1 population in a concentration-dependent manner, indicating fisetin-inducedapoptosis (**Fig. 4B**).

Fisetin did not block nuclear localization of HSF1 upon heat shock

HSF1 is primarily localized in the cytosol under normal growth condition. Upon stress, it translocates into the nucleus where it binds to the promoters of HSPs. Because fisetin blocked HSF1-dependent HSPs expression, we examined the possibility that fisetin inhibited HSF1 translocation into the nucleus by performing a subcellular fractionation analysis. As shown in **Fig. 5A**, under normal conditions, HSF1 was localized mainly in the cytoplasm and was translocated into the nucleus upon

heat shock stress. In addition, nuclear translocation was not inhibited by fisetin treatment. This result suggested that fisetin did not block the translocation stage but inhibited the step(s)of HSF1 action in the nucleus, such as binding step to the promoters of HSPs.

Fisetin inhibits heat shock-induced recruitment of HSF1 to the hsp70 promoter

Upon heat shock, HSF1 is recruited to the promoters of heat shock genes.Because fisetin reduced transcription activation by HSF1, the effect of fisetin on the recruitment of HSF1 to the *hsp70* promoter was analyzed by ChIP analysis. For this, HCT-116 cells were subjected to heat shock at 43°C for 1h in the presence or absence of fisetinwithout recovery time at 37°C.As shown in **Fig. 5B and 5C**, HSF1 binding to the *hsp70* promoter was increased by heat shock. However, treating cells with fisetin significantly inhibited heat shock-induced HSF1 association with the *hsp70* promoter.Next, we tested whether decreased binding of HSF1 by fisetin was caused by HSF1 degradation.Treating cells with fisetin using the same conditions as for ChIP assays (pretreatment of fisetin at 25μM or 50 μM for 30 min followed by heat shock at43°Cfor 1h without recovery time at 37°C)did not decreased the amount of HSF1 (**Fig. 5D)**, excluding the possibility that fisetin decreased HSF1 binding to the *hsp70* promoter by down-regulating HSF1 expression.

Fisetin inhibits the growth of HCT-116 cells in BALB/c nude mice

HCT-116 tumor xenografts in nude mice were used to investigate the inhibitory activity of fisetin on tumor growth *in vivo*.HCT-116 cells were implanted subcutaneously into the right flank of nude mice on day 0.Vehicle control and fisetin (30 mg/kg, intraperitoneal injection)were administered three times per week for 24 days. To determine the toxicity of the compound, the body weight of tumor-bearing mice was measured. On day 25, the mice were sacrificed and the tumors were removed and weighed. Mice treated with fisetin showed a 35.7% ($p<0.05$) decrease in tumor volume and a 34.3% ($p<0.001$) decrease in tumor weight compared with control mice (**Fig. 6**).These results suggest that fisetin could block growth of tumor in an animal model.

Discussion

HSF1 is activated in a broad range of cancer specimens taken directly from cancer patients (33). HSF1 activation is an indicator of poor prognosis in breast, colon, and lung cancers. HSF1-knockout mice have significantly reduced tumor incidence induced by chemical carcinogens on the skin or on the liver by the mutation of the p53 tumor suppressor (21,22). Therefore, HSF1 is a promising target for the chemo-preventive purposesafter surgery and fortherapeutic treatment of cancer patients.

Mice that received fisetin at 45mg/kg inhibited prostate tumor growth by 74.8% (7) and melanoma tumor growth by 66.6% (8). Mice treated with fisetinat 30mg/kg showed a 35.7% ($p<0.05$) decrease in colon tumor growth compared with control mice (**Fig. 6**). These results suggested that fisetin can be effective in reducing tumor growth in an animal model.

Previously we reported a novel compound KRIBB11 as an HSF1 inhibitor(29). Using affinity chromatography with biotinyl-KRIBB11, we found that KRIBB11 associated with HSF1 *in vitro*. Chromatin immunoprecipitation analysis showed that KRIBB11 did not inhibit HSF1 binding to the *hsp70* promoter. Instead, KRIBB11 decreased HSF1-dependent recruitment of p-TEFb to the *hsp70* promoter. Unlike KRIBB11, fisetininhibited HSF1 itself binding to the *hsp70* promoter (**Fig. 5Band 5C**). Currently we do not know whether fisetin directly associates with HSF1. It is important to identify direct target of fisetin in order to understand its mode of action in cells.

When HCT-116 cells were subjected to heat shock at 43°C for 1h in the absence or presence of fisetin and incubated at 37°C for 6h to allow recovery, amounts of HSF1 were decreased by heat shock and by heat shock with fisetin compared to the untreated cells (**Fig. 2E**). However, when HCT-116 cells were exposed to heat shock at 43°C for 1h in the absence or presence of fisetin without recovery time at 37°C, HSF1 was not decreased by fisetin (**Fig. 5D**). Because we prepared ChIP samples from the cells exposed to heat shock at 43°C for 1h in the absence or presence of fisetin

without recovery time at 37°C, decreased binding of HSF1 to the*hsp70* promoter was not by HSF1 degradation but by reduced association between HSF1 and the hsp70 promoter.

Reviewers made important comments. One of them was little HSF1-mediated luciferase activity at normal temperatures (**Fig. 1**). If this is true, how does the inhibition of HSF1 lead to cell death under non-stressed (non-heat shock conditions)?As shown in **Fig. 1A**, there was 75-fold induction of the luciferase activity in HCT-116 cancer cells by heat shock stress. However, as shown in **Fig. 2**, there is only 2.5-fold induction of the *hsp27* mRNA to 11-fold induction of the *hsp70* mRNA by heat shock stress. Human*hsp27* promoter has one HSE (HSF1 element)(34) and *hsp70* promoter has two HSE (35).In contrast, HSF1 reporter plasmid used in this study has 4 copies of HSE. Therefore, it is possible that assays using our reporter plasmid significantlyamplified difference of HSF1 activities between normal and stressed conditions.

To estimate HSPsexpressions at normal temperature compared to those at heat shock stress, it is better to use amounts of HSPs mRNA and protein in the HCT-116 cancer cells. As shown in **Fig2**, depending on HSPs genes, there is 2.5-fold to 11-fold difference of mRNA between normal and heat shock stress cultures (**Fig. 2A-D**). In addition, there is 1.4-fold to 2-fold difference of HSP proteins between normal and heat shock stress (**Fig. 2E**). These results suggested that basal level of HSPs protein is at least 50% of heat shock-induced HSPs level in HCT-116 cancer cells. These basal expressions of HSPs proteins wereefficiently down-regulated by treating HCT-116 cancer cells with fisetin 50 μM for 24h, inducing PARP cleavage (**Fig. 3B**).

Many HSF1 inhibitors (for review, see ref.(36)) have been reported, including quercetin (30,31), QC12 (37), KNK437 (38), Stresgenin (39), triptolide (40), Emunin (41), NZ28 (41), KRIBB11 (29), phenyl isothiocyanate (42), rocaglates (43), and cantharidin (44).Strong efficacy and low toxicity are important factors to consider when developing a cancer therapeutic. Triptolide shows potent activity against HSF1, and it was used to show the antitumor effect of HSF1 inhibition*in vivo*(45). However,

triptolide inhibits NF-κB and AP-1 as well as HSF1. Similarly, quercetin is a naturallyoccurring flavonoid that inhibits multiple target proteins, including HSF1, NF-κB, several kinases, and CYP3A4.

Surprisingly, even though quercetin wasincluded in our study, it was not initially identified as having inhibitory effects. Our reporter plasmid has 4 copies of HSF1 binding elementsupstream from the luciferase promoterto induce luciferase expression only in response to HSF1 activation. In the case of quercetin, it inhibited HSF1 reporter activity at high concentration (13.1% inhibition at 20 μM and 23.2% inhibition at 50 μM in HCT-116 cancer cells) (**Fig. 1**). Because we screened compounds at 10 μM, quercetin was not selected as a hit.Recently, quercetin target proteins were identified by affinity chromatography with biotinylated quercetin (46). Biotinyl-quercetin binds directly to HSP70 and HSP90.Therefore, it is likely that quercetin inhibits HSP70 and HSP90 functionsby directlybinding to them.When HCT-116 cancer cells were treated with fisetin or quercetin at 20 μM, HSF1 luciferase reporter activity was inhibited by 74.1% and 13.1%, respectively (**Fig. 1A**). Similarly fisetin and quercetin exhibited a dose-dependent inhibition of HCT-116 cell growth over a broad range of concentrations, with a GI_{50} of 23μM and 100μM, respectively (**Fig. 4A**). These results indicate that fisetin has stronger HSF1 inhibitory and anti-proliferation activities than quercetin.

Fisetin was reported to inhibit AR (6,7). Therefore, we tested whether fisetin inhibited prostate cancer in an AR-dependent manner. AR-negative DU145 and PC3 prostate cancer cells and AR-positive LNCaP prostate cancer cells were treated with fisetin at various concentrations (0-300 μM) and the inhibition of proliferation was analyzed. As described in the results, we obtained a $GI_{50}$of 17 μM, 41 μM, and 41 μM for DU145, PC3, and LNCaP, respectively. This result was somewhat unexpected because if fisetin inhibited AR, it significantly inhibited growth of AR-positive LNCaP prostate cancer compared with AR-negative DU-145 and PC3 prostate cancer cells. However, fisetin inhibited the proliferation of both AR-negative and AR-positive prostate cancer cells, indicating that

fisetin has other target molecule besides AR.

Cancer cells have multiple mutations and oncogenes. In addition, signaling has a high degree of pathway redundancy. Therefore, disruption of a single target function hasfrequently weak or little anticancer effect. Therefore, combination therapy with target-specific drugs is important approach to get therapeutic effect. However, multi-targeted therapy can be an alternative approach for the same purpose. In general, flavonoids have multiple target molecules in cells and fisetin might inhibit not only HSF1 but also other target proteins including NF-κB, β-catenin, and AR.Therefore, anti-tumor effects of fisetin could be a sum of these multi-targets inhibitions. Even though we mentioned beneficial effects of multi-targeted natural compounds, they have detrimental properties, too. Because flavonoids have multiple targets, it can inhibit normal cell, producing off-target toxicity. Therefore, in the case of multi-targeted therapy, we have to carefully evaluate beneficial and detrimental effects for clinical use.

Bcl-2 family members are key regulators of cancer cell survival and consist of both anti- and pro-apoptotic proteins. Pro-survival Bcl-2 family proteins have beenemerging as promising therapeutic targets, and small Bcl-2/Bcl-x_L inhibitors, such as ABT-737 and ABT-236, are being developed (for review, (47,48)). ABT-236 has clinical activity in Bcl-2-dependent tumors. However, many tumors are not dependent on Bcl-2 but depend instead on Mcl-1. In addition, the overexpression of Mcl-1 causes resistance against ABT-737 in cancer cells (49-51). Furthermore the amplification of the Mcl-1 locus was reported as one of the most frequent somatic genetic events in human cancer (52). These reports suggest that atriple inhibitor against both Bcl-2/Bcl-x_L and Mcl-1 is more effective than specific inhibitors that target one or the other.This concept was consistent with our result (**Fig. 3B**). It is worth noting thateven though Mcl-1 and Bcl-x_L were decreased 3 h after fisetin treatment, there was no PARP cleavage, suggesting thatthe down-regulation of Mcl-1 and Bcl-x_L was insufficient to induce HCT-116 apoptosis (**Fig. 3B**). HSP70 expression was decreased 12 h after treatment, and the significant down-regulation of HSP70/BAG3 and Bcl-2 family proteinswere observed 24 h after

treatment. This temporal pattern correlated with PARP cleavage in HCT-116 cancer cells. Our HSF1 inhibitor fisetinacted as a triple inhibitor by simultaneouslydecreasing the expression of Bcl-2, Bcl-x_L, and Mcl-1 through the down-regulation of their chaperones, HSP70 and BAG3. Therefore, fisetin can be useful to overcome to the single agent induced resistance problem.

Generallychemo-preventive use of a drug requires long term treatment; hence, it is necessary that the drug be less toxic than common therapeutic anticancer drugs. Interestingly, fisetin is an ingredient of food, such as apples, grapes, kiwis, persimmons, strawberries, cucumbers, and onions. The amount of fisetin is 160 μg in 1g of strawberries. In addition, fisetin was reported to have onlya minimal effect on the growth of epithelial cells (6). Therefore, it is interesting to test whetherfisetin can be a candidate compound to use as a chemo-preventive agentfor long term administration after surgery.

The present study is the first report thatfisetin, a dietary flavonoid,can inhibit HSF1 activity, interfere with cancer cellproliferation, and induce apoptosis. In addition, we determined the inhibitory mechanism of fisetin;it blocked HSF1 binding to the promoters of the *hsp70*gene (and presumably the*bag3* gene), down-regulating the expression of HSP70 and BAG-3. Low levels of HSP70/BAG3 might destabilize anti-apoptotic Bcl-2 family proteins, inducing apoptotic cancer cell death.

Acknowledgements

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Figure Legends

Fig. 1. Fisetin inhibits HSF1 reporter activity in a dose-dependent manner.

(A-D) HCT-116, SW-620, Mia PaCa-2 and MDA-MB-231 cancer cells were transfected with the p(HSE)$_4$-TA-Luc reporter plasmid and treated with or without heat in the presence of different concentrations (0, 10, 20, or 50 μM) of fisetin or quercetin. The reporter assay was performed as described under "Experimental Procedures". **(E)**HCT-116 cancer cells expressing the p(HSE)$_4$-TA-Luc reporter were pretreated for 30 min with the indicated concentrations of fisetin, quercetin, naringenin, kaempferol and baicalein and then exposed to heat shock at 43°C for 1 h with 5 h recovery. Luciferase activity was determined with a GloMax ™ 96 microplate luminometer (Promega). 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 versusthe no heat control. *, *P*<0.05; **, *P*<0.01 versus heat control.***F***, The structures of fisetin, quercetin, naringenin, kaempferol and baicalein.

Fig. 2. Fisetin inhibits heat shock-induced transcription of *hsp genes* and protein expression of HSPs.

(A-D) The heat-induced transcription of *hsp70*,*hsp47*,*hsp27*,and *bag3*, was repressed by fisetin in a dose-dependent manner. HCT-116 cancer cells were pretreated with fisetin at different concentrations (0, 10, 20, or 50 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*,*hsp27*, and *bag3*was performed using quantitative 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 ± SD. Statistical significance (*P*-value) was determined with an unpaired *t*-test. #, *p*<0.01 versus the no heat control. *, *P*<0.05; **, *P*<0.01 versus heat control.**(E)** Fisetin inhibited heat-induced HSP70,HSP27, and BAG3 expression in HCT-116 cancer cells. HCT-116 cancer cells were pretreated with the

indicated concentrations of fisetin for 30 min, exposed to heat shock at 43°C for 1 h, and then incubated at 37°C for 6 h. Whole cell lysates were analyzed by western blotting as described in "Experimental Procedures".

Fig. 3. Fisetin inhibits the expression of HSP70, BAG3, Mcl-1, Bcl-2 and Bcl-x_L proteins and induces apoptotic cell death.

(A) The western blot analysis of whole cell extracts of HCT-116 cancer cells treated with the indicated concentrations of fisetin or vehicle solvent (0.1% DMSO) for 48 h. **(B)**HCT-116 cancer cells were treated with 0.1% DMSO or fisetin (50 μM) for 3-48 h, and whole cell lysates were analyzed by western blotting as described under "Experimental Procedures."**(C**) The western blot analysis of whole cell extracts of HCT-116 cancer cells treated with the indicated concentrations of fisetin or vehicle solvent (0.1% DMSO) for 24 h.

Fig. 4. Fisetin inhibits the proliferation of HCT-116 cancer cells.

(A) HCT-116 cancer cells were treated with 0.1% DMSO or different concentrations of fisetin or quercetin. After incubation for 48 h, a cell proliferation assay was performed as described under "Experimental Procedures." Proliferation is expressed as the percentage of fisetin-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 fisetin 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-G_1 (<2N), G_2, M, and G_0/G_1 phases were determined by using the ModFit program (BD Biosciences).

Fig. 5. Fisetin inhibits heat shock-induced HSF1 binding to the *hsp70* promoter.

(A) The analysis of the subcellular localization of HSF1. HCT-116 cancer cells were treated with either no heat or heat at 43°C for 1 h in the presence of fisetin (50 μM). After heat shock, nuclear and

cytoplasmic protein extraction was performed as described under "Experimental Procedures."α-Tubulin and Histone H1 are markers for the purity of cytoplasmic and nuclear fractions, respectively. **(B and C)**ChIP analysis of HSF1 binding to the *hsp70* promoter. HCT-116 cancer cells were incubated with the indicated concentrations of fisetin, heat-shocked at 43°Cfor 1 h, and analyzed for the recruitment of HSF1 on the *hsp70* promoter by the ChIP assay as described under "Experimental Procedures." ChIP-enriched DNAs was prepared using preimmune IgG or anti-HSF1 antibodies. The quantification of the DNA fragment of the *hsp70* gene (-216 to -24) was performed using quantitative real-time PCR (**B**) and PCR (**C**). The ChIP assay was performed three times, and similar results were obtained. Relative promoter occupancy is expressed as fold induction compared to the control prepared from samples that were not heat-treated. Error bars indicate S.D. Statistical significance (p value) was determined with an unpaired t test. #, $p<0.01$ versus the no heat control. *, $P<0.05$; **, $P<0.01$ versus the heat control.**(D)**HCT-116 cancer cells were incubated with the indicated concentrations of fisetin, heat-shocked at 43°C for 1 h with no recovery. HCT-116 lysates were analyzed by western blotting using anti-HSF1 or GAPDH antibody as described under "Experimental Procedures."

Fig. 6. Fisetin inhibits the growth of HCT-116 cells in a nude mouse xenograft regression model. **(A)**For the evaluation of the *in vivo* anti-tumor activity of fisetin, HCT-116 cells were implanted subcutaneously into to the right flank of nude mice on day 0. Vehicle control and fisetin (30 mg/kg, intraperitoneal injection)were administered three times per week for 24 days. Tumor volumes were estimated by the formula length (mm) X width (mm) X height (mm)/2.**(B)** Body weight was measured on each indicated day. **(C)** Tumor weights were measured on day 25.**(D)**Proposed mechanism for pro-apoptotic activity of fisetin. The expression of HSP70 and BAG3 is inhibited by fisetin by blocking HSF1 binding to the promoters of these genes. The down-regulation of the HSP70/BAG3 chaperone complex decreases the stability of anti-apoptotic Bcl-2, Bcl-x_L, and Mcl-1 proteins, enhancing their

degradation and apoptotic cell death. ABT-737 inhibits Bcl-2 and Bcl-x_L but not Mcl-1, which allows Mcl-1-overexpressing cancer cells to overcome this treatment.

Figure. 1

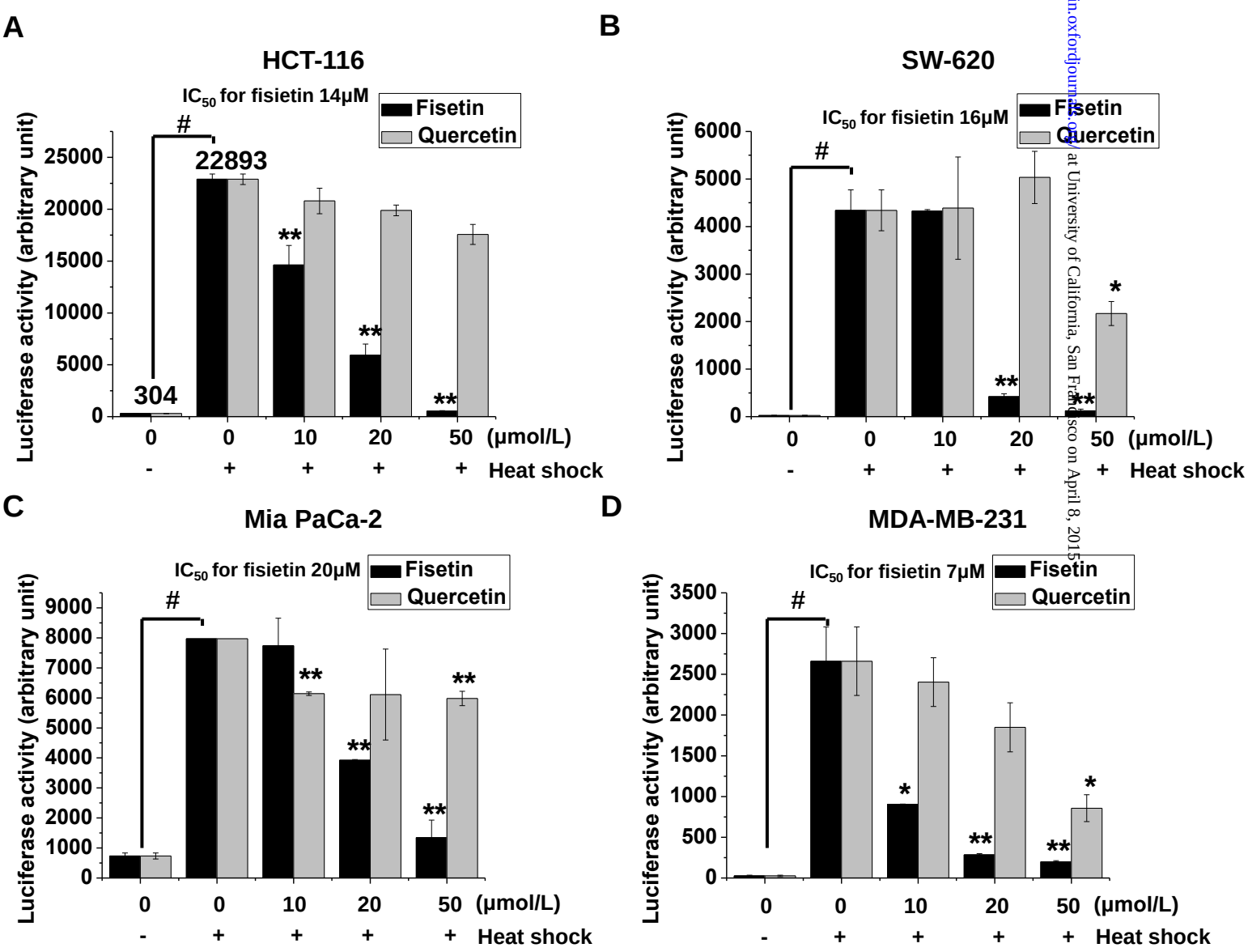
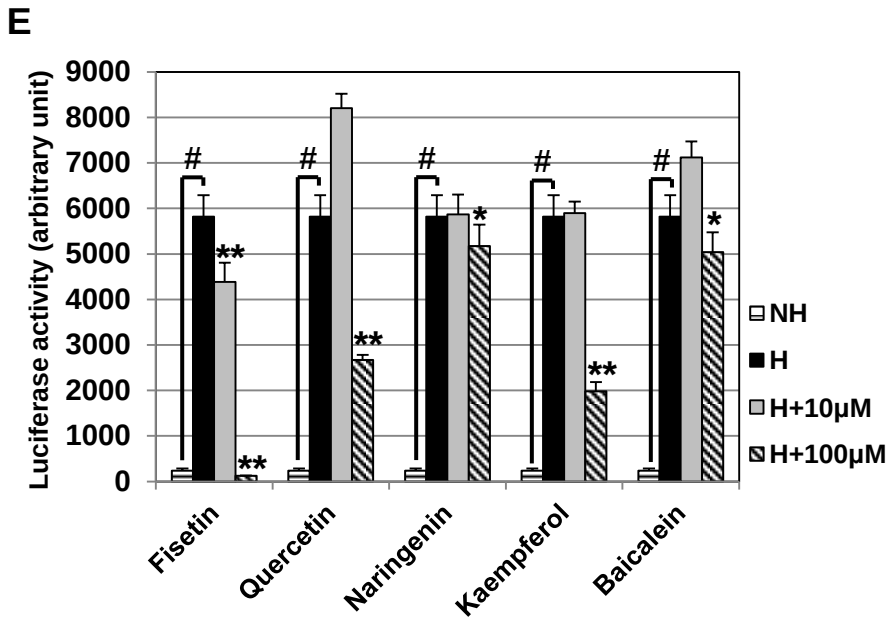


Figure. 1



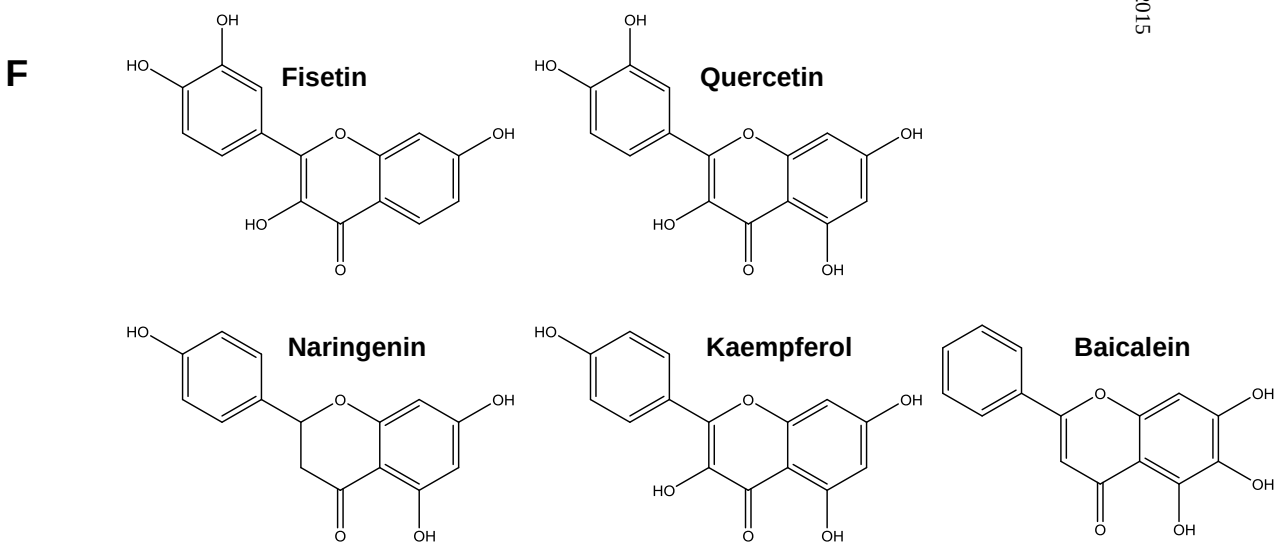


Figure. 2

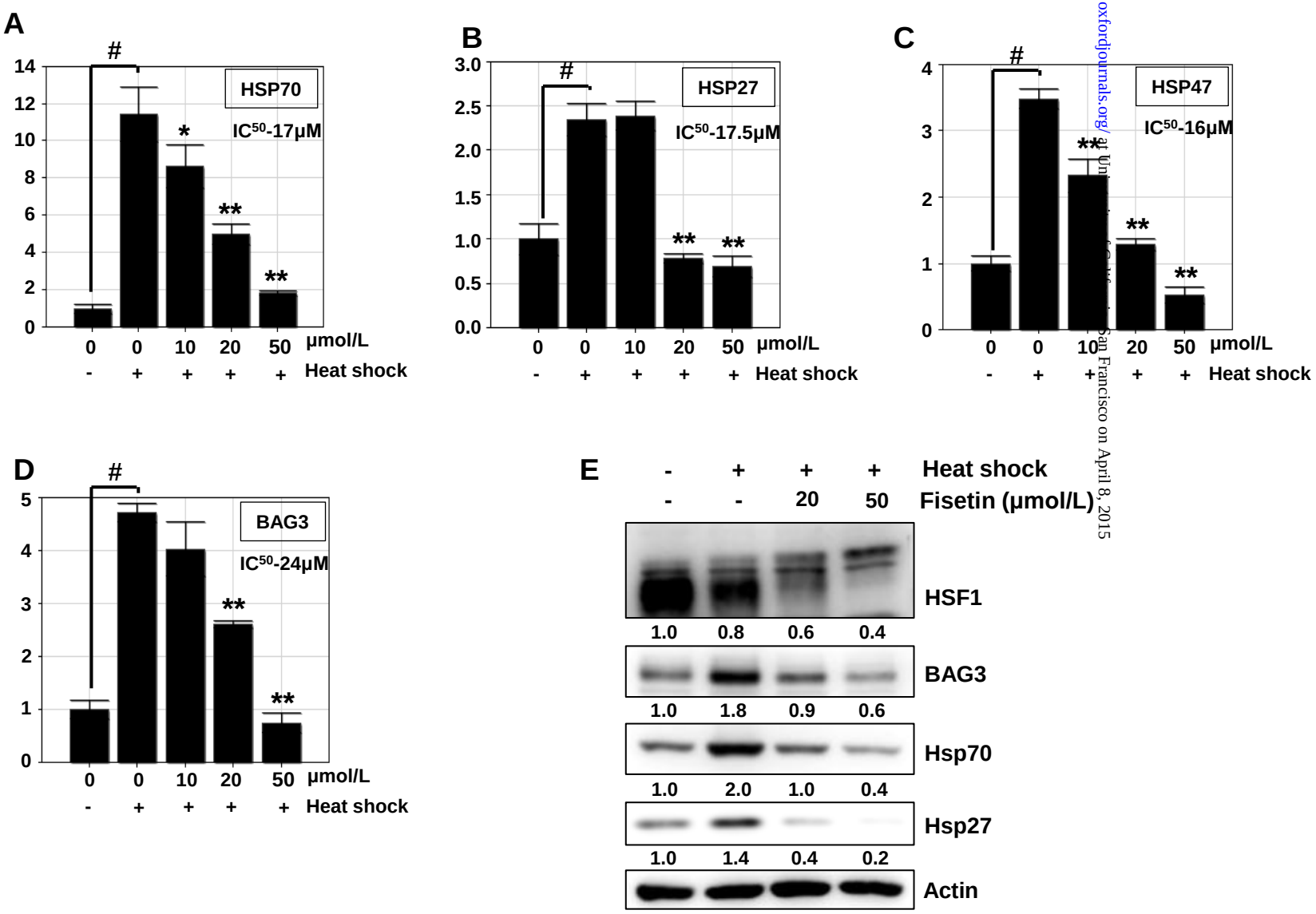


Figure. 3

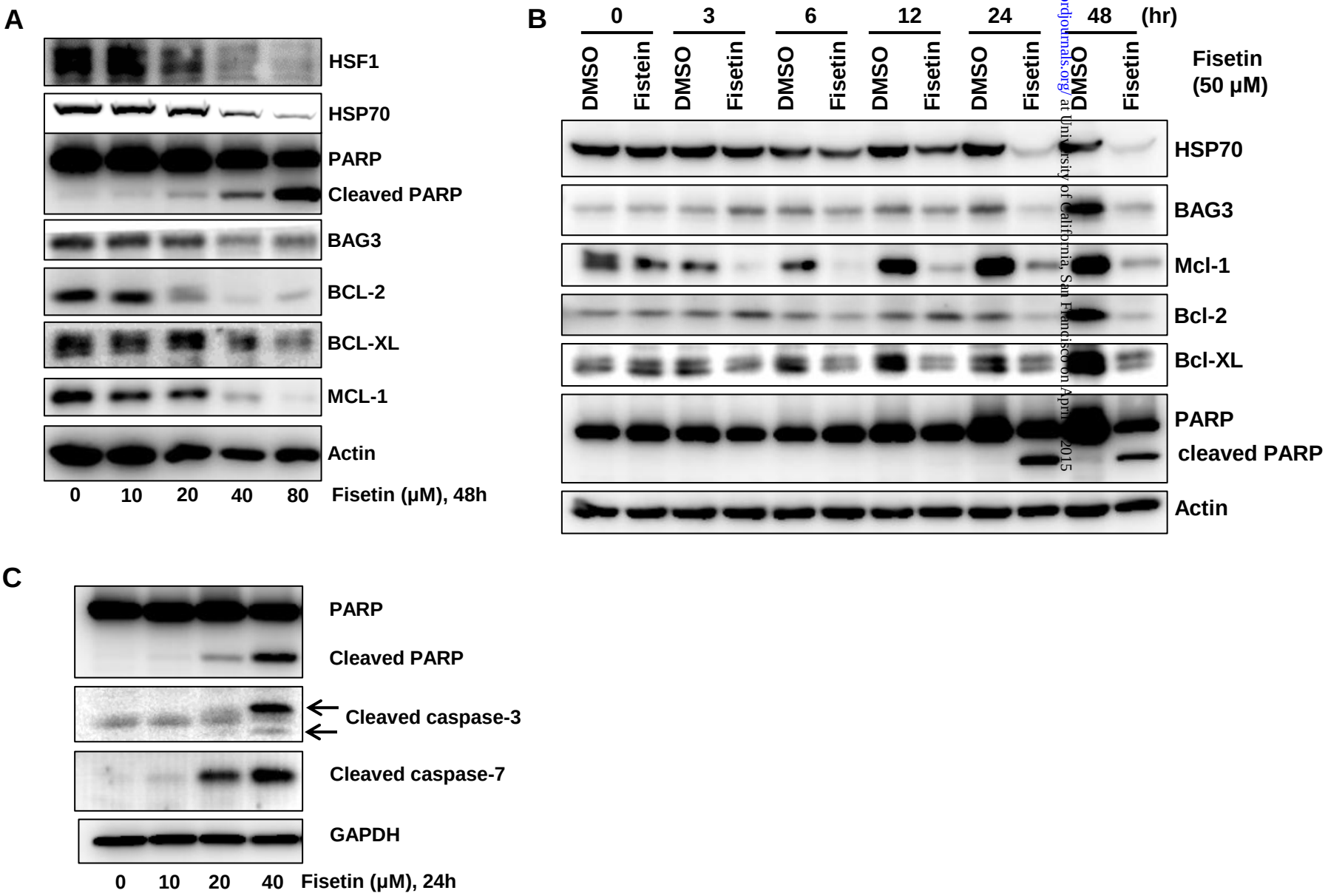
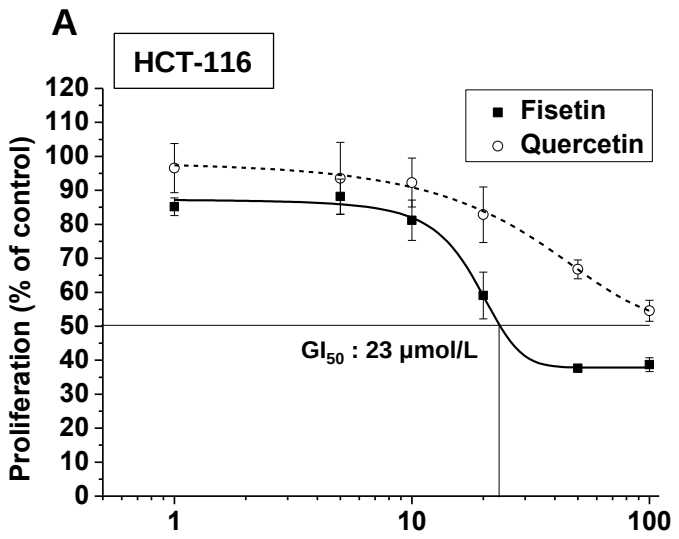


Figure. 4



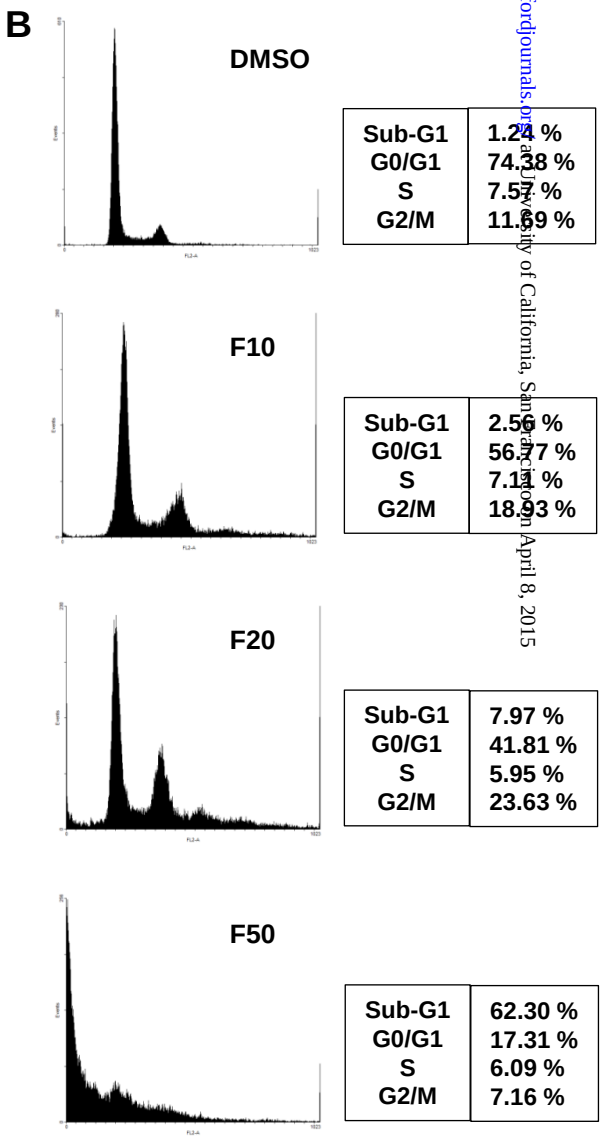


Figure. 5

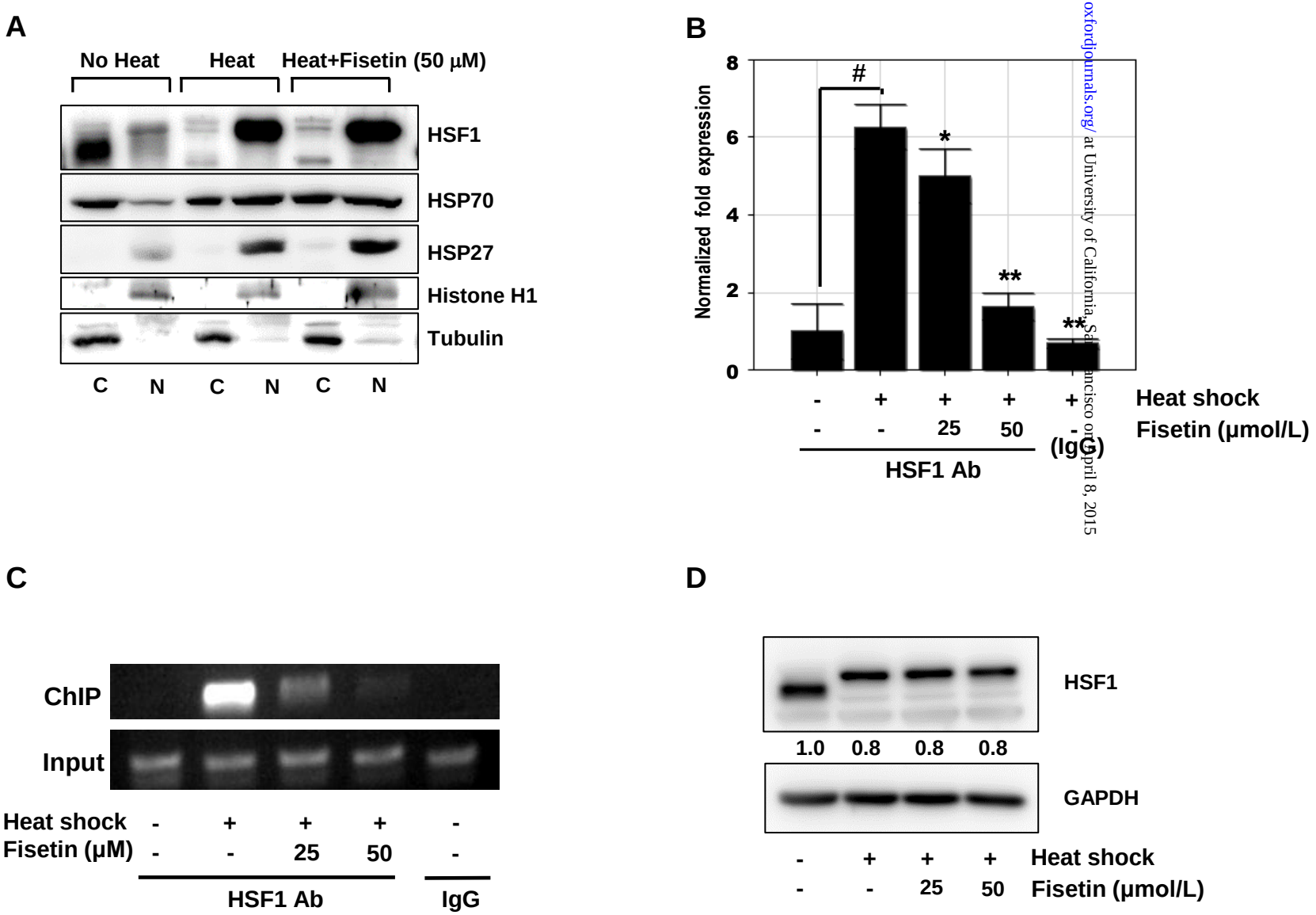


Figure. 6

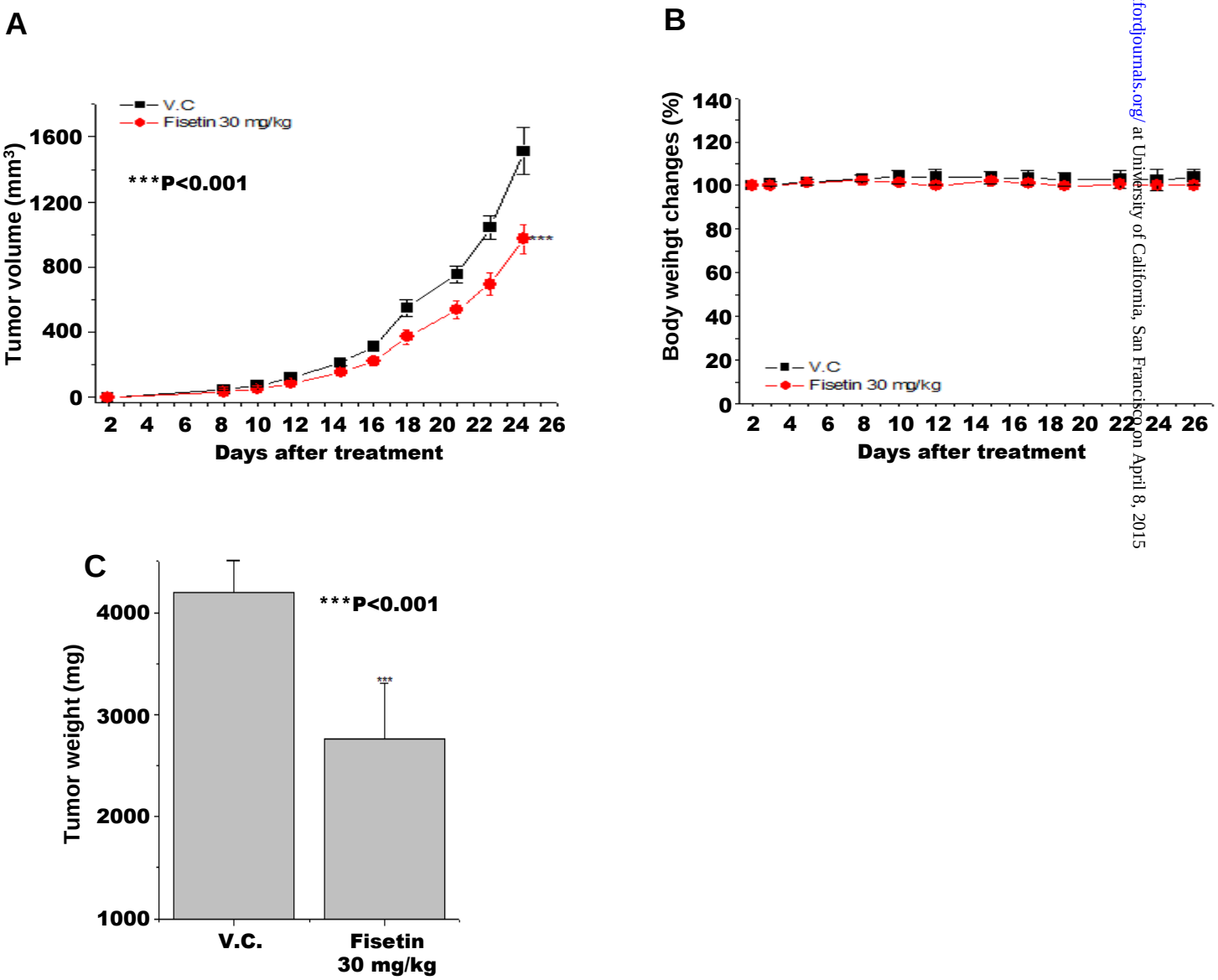


Figure. 6

D