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Over-expression of Human Endosulfatase-1 Exacerbates Cadmium-induced Injury to Transformed Human Lung Cells *In Vitro*

Huiying Zhang[a,b], **Donna R. Newman**[a], **James C. Bonner**[b], and **Philip L. Sannes**[a,*]

[a]Department of Molecular Biomedical Sciences, Center for Comparative Molecular Translational Research, College of Veterinary Medicine, **NC State University, Raleigh, NC 27607**

[b]Department of Environmental and Molecular Toxicology, College of Agriculture and Life Sciences, NC State University, Raleigh, NC **27695**

Abstract

Environmental exposure to cadmium is known to cause damage to alveolar epithelial cells of the lung, impair their capacity to repair, and result in permanent structural alterations. Cell surface heparan sulfate proteoglycans (HSPGs) can modulate cell responses to injury through their interactions with soluble effector molecules. These interactions are often sulfate specific, and the removal of sulfate groups from HS side chains could be expected to influence cellular injury, such as that caused by exposure to cadmium. The goal of this study was to define the role 6-O-sulfate plays in cellular responses to cadmium exposure in **two** pulmonary epithelial cancer cell **lines** (H292 **and A549**) and in normal human primary alveolar type II (hAT2) cells. Sulfate levels were modified by transduced transient over-expression of 6-O-endosulfatase (HSulf-1), a membrane-bound enzyme which specifically removes 6-O-sulfate groups from HSPG side chains. Results showed that cadmium decreased cell viability and activated apoptosis pathways at low concentrations in hAT2 cells but not in the cancer cells. HSulf-1 over-expression, on the contrary, decreased cell viability and activated apoptosis pathways in H292 and A549 cells but not in hAT2 cells. When combined with cadmium, HSulf-1 over-expression **further decreased cell viability and** exacerbated the activation of apoptosis pathways in **the transformed** cells but **did** not **add to the toxicity** in hAT2 cells. The finding that HSulf-1 sensitizes **these cancer** cells and intensifies the injury induced by cadmium suggests that 6-O-sulfate groups on HSPGs may play important roles in protection against certain environmental toxicants, **such as heavy metals.**

Keywords

cadmium; HSulf-1; lung epithelial injury; 6-O-sulfate

*Corresponding author: Philip L. Sannes, Ph.D. Department of Molecular Biomedical Sciences College of Veterinary Medicine 1060 William Moore Dr. North Carolina State University Raleigh, NC 27607 (philip_sannes@ncsu.edu).

INTRODUCTION

Cadmium (Cd^{2+}) is a ubiquitous environmental pollutant and a contaminant of tobacco smoke (Chambers *et al.*, 1998). It is highly toxic and may cause multiple health problems. Its main route of exposure is through the respiratory system. Inhalation of cadmium results in pneumonitis, pulmonary edema, and emphysema, and may even promote development of certain lung cancers (Ress *et al.*, 2003). Although the detailed mechanisms of cadmium-induced diseases are still unclear, cadmium has been shown to induce apoptosis in a rat lung epithelial cell line and to increase cytokine production, including IL-8, in alveolar type II (AT2) epithelial cells of the lung (Hart *et al.*, 1999; Lag *et al.*, 2002; Lag *et al.*, 2010).

Lungs have the highest risk of all organs of being exposed to airborne toxicants (Valacchi *et al.*, 2004), and alveolar epithelial cells, forming the primary barrier in air exchange, are particularly vulnerable to damage with the subsequent loss of barrier integrity. Following injury, healthy alveolar epithelial cells must rapidly proliferate and differentiate to avoid irreversible lung damage. The extracellular matrix environment surrounding these cells, which is rich in sulfated components (Sannes, 1984), is believed to play an important role in repair following injury (Kainulainen *et al.*, 1998). Structural studies have shown that the cuboidal AT2 cells reside on a basement membrane (BM) that is under-sulfated and symmetrically layered, whereas the large squamous alveolar type I (AT1) cells are associated with more heavily and asymmetrically sulfated microdomains (Sannes, 1984). It has been suggested that these differences in sulfation of the BM, predominantly in the form of heparan sulfate proteoglycans (HSPGs), help maintain/stabilize phenotype, potentiate the AT2 cell's capacity to respond to growth factors and divide, and promote the differentiation process (Newman *et al.*, 2004). The production of HSPGs by some lung cells was found to decrease by up to 40% and their sulfation pattern was altered after exposure to cadmium (Chambers *et al.*, 1998; Fears and Woods, 2006). Kainulainen, et al., discovered that after non-specific lung injury, syndecan-1 and -4 ectodomains were shed into acute wound fluids where they bound to elastase and balanced the proteolytic environment near wounds (Kainulainen *et al.*, 1998). Thus, alterations in sulfation pattern, quantity, and bond type on relevant molecules could potentially jeopardize normal cellular protective mechanisms and repair processes following cadmium injury.

Human 6-O-endosulfatase 1 (HSulf-1) is an enzyme that cleaves the 6-O-sulfate group from HSPGs, modifies their molecular structure, and alters their capacity to modulate signaling pathways (Bret *et al.*, 2011). Due to its capacity to decrease or otherwise alter the sulfation of HSPGs, we hypothesized that over-expression of HSulf-1 would exacerbate cellular injury caused by cadmium. In this study, **the** pulmonary epithelial cancer cell **lines, H292 and A549,** and normal human primary AT2 (hAT2) cells were treated with cadmium after adenoviral transduction for HSulf-1 over-expression, and cell viability and apoptosis were quantitated by MTT and TUNEL assays, respectively. A PCR array was performed to assess the activation of the apoptosis pathway. The resulting data indicate that HSulf-1 over-expression sensitizes H292 **and A549** cells to injury by cadmium, while having no synergistic effect in normally cadmium-sensitive hAT2 cells, and suggest that 6-O-sulfate groups on HSPGs may play important roles in cellular protection against environmental toxicants.

MATERIALS AND METHODS

Cell preparation and maintenance

H292 **and A549** cells were obtained from American Type Culture Collection (*ATCC*, Manassas, VA) and cultured in RPMI1640 **or F-12K** medium, **respectively,** with 10% FBS and antibiotics. Human tissue for isolation of AT2 cells was obtained from organ donor

lungs from University of North Carolina Cystic Fibrosis/Pulmonary Research and Treatment Center Tissue Procurement and Cell Culture Core (Chapel Hill, NC). Following isolation (see below), hAT2 cells were maintained in **low-glucose** DMEM medium supplemented with 10% FBS and Antibiotic-Antimycotic solution (Mediatech, Manassas, VA).

hAT2 cell isolation

Cells were isolated according to a scaled-up, modified version of the original Dobbs (Dobbs, 1990) procedure, fibroblast-depleted using an anti-Thy-1 antibody (AS02, EMD Chemicals, Gibbstown, NJ) and pan-mouse IgG Dynabeads (Invitrogen, Carlsbad, CA), and seeded in **low-glucose** DMEM/10% FBS on rat-tail collagen-coated tissue culture dishes. After a medium change the next day, cells were cultured for 48 hours before further treatment.

Preparation of HSulf-1 adenovirus

Over-expression of **HSulf-1** in epithelial cells was accomplished by adenoviral delivery of the human Sulf-1 gene (HSulf-1) driven by a CMV promoter. An Ultimate ORF clone in the pENTR221 vector (Invitrogen) was used to introduce the protein coding sequence of HSulf-1 into the pAd/CMV/V5-DEST vector (Invitrogen) by LR Clonase II transfer and ligation reaction. The recombinant pAd-HSulf-1 plasmid was transformed into TOP10 E. coli hosts and successful transformants were selected on Ampicillin plates. The HSulf-1 coding DNA was completely sequenced by primer walking to confirm 100% fidelity, a perfect clone was amplified, and the purified **plasmid** was used to transfect 293A cells to produce adenovirus. Amplified adenoviruses were titered by the Hexon antibody/DAB method and used to infect experimental hAT2, **H292**, and **A549** cells for transduced transient over-expression of HSulf-1.

Adenoviral transduction and cell treatments

Cells were initially transduced at 10 MOI for lacZ or HSulf-1 over-expression, incubated overnight, and then treated with or without 10 μM cadmium chloride for 48 hours. Photomicrographs were taken with an Olympus IMT-2 inverted microscope under phase contrast and samples were harvested for apoptosis assessment using a TUNEL assay kit (Invitrogen) and SuperArray **Human** Apoptosis PCR arrays (SABiosciences, Frederick, MD). In further experiments, **hAT2,** H292, and **A549** cells over-expressing HSulf-1 were seeded onto 24-well plates at 25,000 cells per well. Cells were then exposed to a range of cadmium chloride concentrations, depending upon their relative sensitivity established in preliminary experiments (5 – 60 μM for H292 **and A549** cells and 1–30 μM for hAT2 cells), for 24 or 48 hours and viability was assessed by MTT assay.

MTT assay

A measure of cell proliferation/viability was obtained by a colorimetric assay which **utilizes** the capacity of live cells to change 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) from yellow to a purple **formazan** precipitate. Twenty-four hours after transduction for HSulf-1 over-expression, **hAT2,** H292, and **A549** cells were exposed to various concentrations of cadmium chloride for another 24 or 48 hours. At the end of the exposure, culture medium was discarded and the MTT solution (Sigma, St. Louis, MO) was added to a final concentration of 1 mg/ml. After 3 hours at 37ºC, the solution was removed and the formazan precipitate was dissolved in DMSO. Optical densities were measured at 570 nm using a microplate ELISA reader. Data was expressed as a percentage of control cells and analyzed with ANOVA, followed by Student's t-test.

TUNEL assay

hAT2, H292, and **A549** cells were transduced for lacZ or HSulf-1 over-expression and then treated with or without 10 μM cadmium chloride for 48 hours, as above. This cadmium concentration was chosen because it did not kill the cells too quickly while still decreasing viability over the 48 hr time course, as demonstrated in **dose response experiments performed over a** broader concentration **range**. All cells from each treated dish were detached using Detachin (Genlantis, San Diego, CA). TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay (Invitrogen), which detects the DNA fragmentation produced in apoptotic cells, was performed on detached cells, which were then **spread on** glass slides for photomicrography. Cells undergoing apoptosis (BrdU-labeled) exhibit a bright yellow-green fluorescence **(Alexa Fluor 488)** at 519 nm upon excitation at 495 nm. Cell nuclei, stained with **propidium iodide (PI),** were detected (blue) at excitation/emission wavelengths of 358/461 nm. Photomicrographs were taken of random fields on a Meiji MX fluorescence microscope at 200× magnification with an Infinity 3 camera; **PI** and BrdU pictures were merged using Image J. A minimum of 3 fields from TUNEL assay photomicrographs were randomly selected and total cells were counted in each field to achieve a minimum total number of 150 H292 **or A549** cells and 60 **hAT2 cells.** Apoptotic ratios (apoptotic cells/total cells) are expressed as a percentage of each total with Mean ± SD from different fields.

SuperArray analysis of apoptosis signaling pathway activation

hAT2, **H292**, and **A549** cells were transduced for HSulf-1 or lacZ (control) over-expression and treated with or without **10 μM** cadmium for 48 hours, as above. Cells were harvested and total mRNA was isolated and purified using the RNeasy kit from QIAGEN (Valencia, CA). Concentrations were measured spectrophotometrically at 260 nm and 1 μg of total mRNA was used as template for cDNA synthesis utilizing the High Capacity Archive Kit (Applied Biosystems, Foster City, CA). Produced cDNA was added to SybrGreen PCR master mix (SABiosciences) and aliquoted into each well of the ready-to-use Human Apoptosis **RT^2 Profiler** PCR array PAHS-012 (SABiosciences). Duplicate samples were transduced, treated, reverse-transcribed, and analyzed in parallel for statistical analysis. The 84 apoptosis-related genes analyzed included members of the TNF ligand and receptor family, Bcl2 families, Caspases, Inhibitors of Apoptosis (IAP), caspase recruitment domain family, death domain, death effector domain, and the p53 family.

Western analysis

H292 and A549 cells, cultured and treated in 60 mm dishes, were rinsed with PBS. RIPA buffer (150 μl), containing PhosStop and Complete EDTA-free Protease Inhibitors (Roche, Indianapolis, IN), was added to dishes. Cells were scraped and collected in microcentrifuge tubes and sonicated three times. Samples were then shaken on ice for at least 30 minutes and centrifuged at 14,000 rpm for 30 minutes at 4°C. Supernatants were transferred to fresh microcentrifuge tubes and total proteins in each sample were quantitated by Pierce 660 nm Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Equal amounts of proteins were subjected to electrophoretic separation on NuPage 4–12% Bis-Tris gels using the Novex X-Cell II system (Invitrogen) and MOPS running buffer at 200V for 60 minutes. Proteins were transferred to nitrocellulose membranes and blocked with Tris Buffered Saline/0.1% Tween20/5% milk (TBS/T/milk) for 1 hour. Blocked membranes were incubated in TBS/T containing 5% BSA (TBS/T/BSA) with primary antibodies to Caspase-3, BAX, and GAPDH (Cell Signaling Technology, Danvers, MA) overnight at 4°C with agitation. After washing in TBS/T, blots were incubated in TBS/T/milk containing secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) for 2 hours with

agitation. Bands on the membrane were detected by chemiluminescence using SuperSignal West Pico or Dura substrate (Pierce) and visualized by autoradiography.

Data analysis and statistics

PCR array data were analyzed with the delta-delta Ct method for gene regulation and visualized by scatter-plot, with online data analysis tools supplied by SABiosciences, Inc. ANOVA was utilized to analyze the MTT assay data, followed by Student's *t*-test. Two-tailed Student's *t*-test was performed on the quantitative PCR data and factorial ANOVA was performed for the association test.

RESULTS

HSulf-1 and low-dose cadmium change cell morphology and reduce cell numbers

Preliminary data using various concentrations of sodium chlorate (5 to 200 mM) as a global inhibitor of sulfation revealed that chlorate itself induces cytotoxicity in H292 cells, and it was found that this cytotoxicity could be reduced by heparin (500 **μg**/ml), indicating that there is a lower limit to the amount of sulfation crucial for cell growth and proliferation (Supplementary Data 1). Matrix HSPGs are decorated with a variety of sulfate moieties, so to dissect the contribution of a single type of sulfation to cell viability we specifically targeted the 6-O-sulfate moiety with a HSulf-1 over-expression adenovirus.

Previously, we reported that HSulf-1 over-expression reduced H292 cell numbers in a dose-dependent fashion but had no effect upon hAT2 cells, and that the mechanism behind this decrease was apoptosis, likely due to the reduction in growth factor signaling caused by selective removal of 6-O-sulfates from HSPGs by HSulf-1 (Zhang *et al.*, 2012). In hAT2, H292, and A549 cells over-expressing HSulf-1, the sulfated glycosaminoglycan (GAG) content was indeed reduced, which confirmed the selective modification of extracellular matrix by HSulf-1 (Supplementary Data 2).

Preliminary data **also** indicated that exposure to even a low level of cadmium reduces viability in hAT2 cells, while H292 **and A549** cells are much more resistant to cadmium's effects. Because many cancer cells exhibit increased resistance to heavy metals, such as cadmium, we wanted to determine whether HSulf-1 over-expression might sensitize H292 cells to cadmium and/or intensify the effects of cadmium on H292 cells.

H292, **A549,** and hAT2 cells (two to three days after isolation) were infected with adenoviruses at 10 MOI (multiplicity of infection) for transient transduced over-expression of lacZ (control) or HSulf-1, incubated for 24 hours to allow expression of the gene, and then treated with 10 μM cadmium for 48 hours. The hAT2 cells infected with lacZ adenovirus only were essentially similar to untreated cells (data not shown) and, by the fifth or sixth day after isolation, had assumed a phenotype similar to hAT1 (type I) cells, which are typically large, round, and squamous in appearance with a centrally located nucleus (Figure 1A). H292 cells infected with lacZ adenovirus only (Figure 1E) appeared as small, polygonal cells with a centrally positioned nucleus, essentially similar to untreated cells (data not shown). A549 cells transduced with lacZ appeared similar to untreated cells as well (data not shown). With cadmium treatment, even at this relatively low concentration (10 μM), there was a visible decrease in hAT2 cell density and size along with assumption of more stellate or fusiform shapes (Figure 1B), while H292 **and A549** cells were relatively unaffected by comparison (Figure 1F **and 1J**). As expected, a low MOI of HSulf-1 adenovirus did not appear to reduce the density of hAT2 cells (Figure 1C), but did induce some reduction in size and cell density (apparent cell numbers) of H292 cells (Figure 1G).

In A549 cells, HSulf-1 induced increased cell refractility, but not much cell density reduction (Figure 1K).

Interestingly, treatment with cadmium in the presence of HSulf-1 over-expression **markedly** reduced the cell densities of both hAT2 (Figure 1D) and H292 (Figure 1H) cells and increased the refractility as well as detachment of A549 cells (Figure 1L). In addition to **these reductions** in **cell density, increases in the relative numbers of** stellate or fusiform cell shapes and in the **numbers** of floating cells and **amounts of** surface blebbing, as suggested by increased refractility of cell borders, were seen in **all three** cell types. Western blot confirmed the high basal level of HSulf-1 protein expression in hAT2 cells, the lack of HSulf-1 protein expression in non-transduced H292 **and A549** cells, and its over-expression in HSulf-1-transduced cells of **all three** types **(Supplementary Data 3).**

HSulf-1 significantly reduces H292 and A549 cell viability with cadmium treatment

Since HSulf-1 over-expression worsened the effects of low concentrations of cadmium on hAT2, **H292**, and **A549** cells, further experiments using a commercial MTT assay were performed to determine the optimal dose of cadmium to use on each cell type in order to trigger some adverse or signaling effects without inducing **significant and rapid** cell death from toxicity. The survival rates of lacZ over-expressing hAT2 cells treated with cadmium concentrations ranging from 1 to 30 μM decreased from 100 to 27% of untreated controls after 24 hours (Figure 2A). Similarly, the transduction of HSulf-1 into hAT2 cells subsequently treated with the same range of cadmium concentrations did not significantly change the survival rates at 24 hours, which decreased from 100% to 25% of untreated, nontransduced control cells (Figure 2A). At 48 hours, in lacZ over-expressing hAT2 cells, the survival rates ranged from 100% of untreated controls at 1 μM to 14% at 30 μM with a steep decline at 20 μM, suggesting toxicity at that concentration. Similarly, HSulf-1 over-expression along with increasing levels of cadmium further decreased hAT2 survival percentage from 100% down to 8%, a decrease that was not significantly different from that of lacZ-transduced cells over most of the dose range and with the same steep decline at 20 μM (Figure 2B).

In H292 cells, by comparison, the survival rates of lacZ over-expressing cells at 24 hours gradually decreased from 98% down to 55% of untreated controls over cadmium concentrations ranging from 5 to 60 μM (Figure 2C), with only a slightly greater decrease over most of the range at 48 hours, from 92% to 37% of untreated controls (Figure 2D), confirming some resistance to cadmium's effects in these cancer cells. However, H292 cells over-expressing HSulf-1 and treated in culture with cadmium chloride at the same range of concentrations had viability rates that gradually decreased from 98% to 33% of untreated controls after 24 hours, significantly less than control at the higher doses (Figure 2C), and from 95% down to 18% of untreated controls at 48 hours, with significance over most of the dose range (Figure 2D).

Similarly, in A549 cells, the survival rates of lacZ over-expressing cells at 24 hours gradually decreased from 96% down to 60% of untreated controls over cadmium concentrations ranging from 5 to 60 μM (Figure 2E), with only a slightly greater decrease over most of the range at 48 hours, from 99% to 41% of untreated controls (Figure 2F). However, A549 cells over-expressing HSulf-1 and treated in culture with cadmium chloride at the same range of concentrations had viability rates that gradually decreased from 96% to 42% of untreated controls after 24 hours, significantly less than control at the higher doses (Figure 2E), and from 99% down to 33% of untreated controls at 48 hours, with significance over most of the dose range (Figure 2F).

These results confirm that H292 **and A549** cells are more resistant to cadmium than are hAT2 cells but indicate that **in these transformed cell lines**, HSulf-1 over-expression can specifically exacerbate injury from high concentrations of cadmium chloride with short exposure times and from a range of concentrations after long exposures.

Because **all three** cell types exhibited some **phenotypic response with lowest** toxicity **to** 10 μM **cadmium**, this dose was used for further experiments to explore the mechanism behind the observed reduction in cell numbers in cadmium-treated HSulf-1-expressing cells.

Apoptosis is exacerbated by HSulf-1 over-expression in cadmium-treated H292 and A549 cells

To determine whether the observed reduced viability of HSulf-1-expressing cells treated with low-dose cadmium is caused by apoptosis, hAT2, **H292**, and **A549** cells were transduced at 10 MOI with lacZ or HSulf-1 over-expression adenoviruses, incubated overnight, and then treated with 10 μM cadmium chloride as above. After 48 hours of cadmium treatment, cells were detached; TUNEL assay was performed and analyzed by light microscopy. Results, confirmed in representative photographed fields (Figure 3, A–D), indicated that HSulf-1 transduction at 10 MOI did not induce apoptotic cell death in hAT2 cells, with only rare or no co-localization of yellow-green BrdU-labeled foci (DNA fragments) with blue (PI) nuclei (Figure 3C), but did induce apoptotic cell death in both H292 and A549 cells (Figures 4C and 5C). However, cadmium at 10 μM did induce an increased number of FITC-labeled foci indicative of apoptosis in hAT2 cells (Figure 3B) but not in H292 or A549 cells (Figures 4B and 5B). The combination of HSulf-1 and cadmium induced apoptosis in all three cell types (Figures 3D, 4D, and 5D).

Apoptotic cell numbers were normalized to total cell numbers to obtain apoptotic ratios. In hAT2 cells, over-expression of HSulf-1 alone induced apoptosis in only 4% of cells, which was not significantly different from the lacZ over-expressing control. However, cadmium, in the presence of lacZ or HSulf-1 over-expression, induced apoptosis in 35% **or** 32% of cells, respectively, at a significantly higher level than apoptosis in untreated cells but not significantly different due to the transduced gene (Figure **3E)**.

In contrast to hAT2 cells, cadmium treatment of H292 **or A549** cells alone was not a significant factor in induction of apoptosis, while HSulf-1 over-expression alone induced apoptosis in 15% **or 12%** of transduced cells**, respectively (Figures 4E and 5E).** Further, the combination of HSulf-1 and cadmium induced apoptosis in up to 28% **or 20%** of total H292 **or A549** cells, **respectively,** which was significantly greater than for HSulf-1 over-expression alone (Figures 4E and 5E). Thus, it appears that the reductions in H292 **and A549** cell numbers caused both by HSulf-1 alone and by the combination of cadmium and HSulf-1 over-expression are due primarily to an increase in apoptosis.

Cadmium induces activation of apoptosis pathway genes in hAT2 cells but not in H292 or A549 cells

Apoptosis array data demonstrated that in hAT2 cells over-expressing lacZ, **expression of** fifteen pro-apoptotic genes (APAF1, CARD8, CASP10, DAPK1, FAS, NOD1, TNFRSF21, TNFSF10, BCL2L11, BIK, BNIP3, CIDEA, HRK, LTA, and *TNFRSF9*), three anti-apoptotic genes (*BAG3, BIRC3*, and *BNIP1*), and two genes with functions unrelated to apoptosis (*CD27, TNFSF8*) **was** altered by cadmium treatment (Table 1). Of the fifteen proapoptotic genes, eight (*APAF1, NOD1, CARD8, CASP10, DAPK1, FAS, TNFRSF21*, and *TNFSF10*) were down-regulated and seven (*BCL2L11, BIK, BNIP3, CIDEA, HRK, LTA*, and *TNFRSF9*) were up-regulated **by cadmium**, while all of the three anti-apoptotic genes were up-regulated. However, three of the genes **(*HRK, LTA*, and *BCL2L11*),** all pro-

apoptotic, were up-regulated with much higher fold changes than any of the other genes (Table 1). **These results are** further illustrated by scatter plot analysis (Figure **6A**), in which the distance outside the 2-fold difference from identity with no-cadmium **control** (lacZ only) for these three genes (labeled) is clearly evident and which, on balance, indicates apoptosis pathway activation.

In contrast, in H292 cells over-expressing lacZ, only three apoptotic genes were altered by cadmium alone. Of them, two pro-apoptotic genes (*BIK* and *CIDEA*) were slightly down-regulated and one pro-apoptotic gene (*DFFA*) was up-regulated to a small extent, which suggests a lack of significant pathway activation in H292 cells by cadmium (Table 2). This **is illustrated** by scatter plot analysis, which **shows** a clustering of relevant genes at identity with the no-cadmium **control** (lacZ only), and only a small fold change for those outside the non-significant range (Figure **6B).**

Similarly, in A549 cells, cadmium treatment of lacZ over-expressing cells only activated seven genes, including four anti-apoptotic genes and one apoptotic gene. Of the four anti-apoptotic genes, two were down-regulated (*BCL2A1* and *NFkB1*) and two were up-regulated (*BCL2* and *NOL3*). The only pro-apoptotic gene, *TP53*, was down-regulated (Table 3). This relative lack of apoptosis pathway activation by cadmium is illustrated by scatter plot analysis (Figure 6C).

HSulf-1 over-expression with cadmium treatment activates apoptosis pathway genes in H292 and A549 cells

Initially, normalizing the “HSulf-1 + cadmium” array data to the “lacZ only” data gave scatter plots (Figure **7)** and gene tables (Tables 1, 2, and 3) that confirmed that apoptosis was triggered by the combination of HSulf-1 and cadmium in **all three** cell types. However, this analysis obscured the separate contributions of the HSulf-1 over-expression and cadmium to apoptosis caused by the combination in the cells. Normalizing the “HSulf-1 + cadmium” data to that of “lacZ + cadmium” revealed that in hAT2 cells, **expression of** only two pro-apoptotic genes (*TNFRSF9* and *HRK*), one anti-apoptotic gene (*BAG3*), and a pro-proliferation gene (*CD70*) **was** specifically **altered** by the HSulf-1 contribution to cadmium's effects (Table **4**). One of the pro-apoptotic genes was up-regulated and the other was down-regulated, the anti-apoptotic gene was down-regulated, and the pro-proliferation gene was up-regulated. This **relative** lack of significant pathway activation is **illustrated** by scatter plot analysis, which shows a clustering of relevant genes with no significant change and small fold change for those outside the range (Figure **8A**).

The contribution of HSulf-1 to cadmium-induced injury was much more striking in the H292 **and A549** cells. **In H292 cells, eight** pro-apoptosis genes (*BAX, BIK, BNIP3, CASP7, CIDEA, FAS, TNF*, and *TNFRSF9*) were significantly up-regulated, several by nearly 10-fold or better. Two pro-apoptosis genes (*CASP9* and *TNFSF10*) were moderately down-regulated and two anti-apoptosis genes (*AKT1* and *BCL2A1*) were moderately up-regulated. Two pro-proliferation genes (*CD70* and *TNFSF8*) and a gene thought to be involved with apoptosis but with an unidentified function (*CD27*) were up-regulated (Table 5). Furthermore, in A549 cells, fourteen of the array genes showing activation due to HSulf-1's contribution were pro-apoptotic. Twelve pro-apoptosis genes (BAK1, BAX, CASP3, CASP6, CD40LG, LTA, MCL1, NOD1, RIPK2, TNFRSF9, TNFRSF25, and *TNFSF10*) were up-regulated while only two pro-apoptosis genes were down-regulated (*BCL2L11* and *LTBR*). Two anti-apoptosis genes (*CFLAR* and *TNFRSF1B*) were up-regulated and one anti-apoptosis gene (NFkB1) was down-regulated (Table 6). Scatter plot analysis illustrates these results (Figures 8B and 8C).

Importantly, whereas cadmium itself strongly induced activation of several pro-apoptotic genes in hAT2 cells, almost no apoptotic pathway activation in H292 **or A549** cells was triggered by cadmium alone. **In contrast,** the HSulf-1 contribution to **cadmium's** effects **up-regulated** many more pro-apoptosis genes in **both** H292 **and A549** cells than **it did** in hAT2 cells. **Data** points representing those genes in H292 **and A549** cells (Figures 8B and 8C) showed increased **and significant** activation compared to genes in hAT2 cells (Figure **8A**). These results indicate that HSulf-1 **exacerbates** apoptosis **pathways activated** by cadmium in H292 **and A549** cells but not in hAT2 cells.

Interactions between HSulf-1 over-expression and cadmium treatment

In hAT2 cells, synergistic/antagonistic effects of cadmium and HSulf-1 were seen by the Association Test on three genes (*HRK, TNFRSF9,* and *CD70*), which accounted for only 13% of total activated genes (Figure **9**). In the interaction graph for each of these genes, the X-axis indicates the lacZ control cells at the left with the HSulf-1 over-expressing cells at the right, while the Y-axis represents the regulatory fold-changes revealed in the PCR array. The dashed line **(NA)** represents "No Cadmium treatment" and the solid line **(Cd)** represents cadmium chloride (10 μM) treatment. Horizontal lines indicate no change, positively-sloped lines indicate up-regulation, and negatively-sloped lines indicate down-regulation. Thus, **for example**, the solid line in each graph indicates the change in expression of the examined gene from that seen in the lacZ over-expression control to that seen with HSulf-1 over-expression, both when in the presence of cadmium. Solid and dashed lines in parallel indicate **that there are** no synergistic or antagonistic effects. A solid line with a more positive slope than the dashed line indicates a synergistic effect between HSulf-1 and cadmium on that gene, while a solid line with a more negative slope than the dashed line indicates there is an antagonistic effect. Accordingly, in hAT2 cells, the HSulf-1 and cadmium combination showed synergistic effects on *TNFRSF9* (pro-apoptotic) and *CD70* (pro-proliferation) (Figures 9A and 9B). Interestingly, the combination exhibited significant and large antagonistic effects on *HRK* (pro-apoptotic) in hAT2 cells (Figure **9C**), suggesting that HSulf-1 may actually counteract the up-regulation of *HRK* by cadmium in these **normal** cells.

In H292 cells, of the 25 genes whose expression was either up- or down-regulated by cadmium after over-expression of HSulf-1, compared to the "No Cadmium" lacZ control, synergistic/antagonistic effects were seen on eleven genes (BAX, BIK, BNIP3, CASP4, CASP7, FAS, GADD45A, TNFSF10, TP53, TNF, and *TNFRSF9*), which accounted for 44% of the activated genes (**Figure 10**, A – K). Based on the slopes of the lines, the HSulf-1 and cadmium combination showed significant synergistic effects on six pro-apoptotic genes (*BAX, BIK, BNIP3, CASP4, CASP7,* and *FAS*) (Figure **10**, A – F) and **on** an anti-proliferation gene (*GADD45A*) (Figure **10G**). Small but significant antagonistic effects were seen on two additional pro-apoptotic genes (*TNFSF10* and *TP53*) (Figure **10**, H **and** I). Although the cadmium and HSulf-1 combination did not show any significant synergistic effects on two pro-apoptotic genes (*TNF* and *TNFRSF9*) (Figure **10**, J **and** K), HSulf-1 does up-regulate the expression of these two genes compared to cadmium treatment alone.

Of the 21 genes in A549 cells that were activated by the combination of HSulf-1 and cadmium, synergistic/antagonistic effects or associations were seen on 6 genes (*BCL2L11, LTA, TNFRSF9, NOL3, CD40LG,* and *TP73*), which accounted for 29% of activated genes (Figure 11, A – F). Based on the slopes of the lines, antagonistic effects were seen on one pro-apoptotic gene (*BCL2L11*) (Figure 11A) while synergistic effects were seen on three pro-apoptotic genes (*LTA, TNFRSF9,* and *CD40LG*) (Figure 11, B, C, and E). In the anti-apoptotic gene *NOL3* (Figure 11D), there is an association effect between the HSulf-1 over-expression and cadmium treatment, but there is no synergistic/antagonistic effect. In *TP73*,

although no significant synergistic effects were seen, HSulf-1 does up-regulate its expression compared to cadmium treatment alone (Figure 11F).

The results of the Association Test **indicate** that **pathway activation** in **both** H292 **and A549** cells treated with cadmium and over-expressing HSulf-1 exacerbated apoptosis in these transformed lung cells, while the combination, although lethal, did not add to cadmium's effects **on** normal human alveolar epithelial cells.

Western analysis confirmation of selected PCR array results

Western analysis was performed on H292 and A549 cells which were treated with cadmium after transduction of HSulf-1. In H292 cells, *BAX* was up-regulated by HSulf-1 alone and in combination with cadmium, and the increase in BAX protein was confirmed by Western analysis. In A549 cells, *CASP3,* which was up-regulated by HSulf-1 alone and in combination with cadmium, showed increased protein expression in Western analysis as well (Figure 12).

DISCUSSION

HSPGs are important components of extracellular matrix and have been shown to play key roles in tissue structure, cell signaling, responses to injury/disease, wound healing, and, importantly, in protecting the tissues and cells from extracellular injury (Barash *et al.*, 2010; Dreyfuss *et al.*, 2009; Elenius *et al.*, 2004; Fears and Woods, 2006; Iozzo, 2001). Depending upon their location and cell of origin, the functional characteristics of HSPGs can be largely attributed to their degree/level of sulfation and their specific sulfate bonds **(Dreyfuss *et al.*, 2009; Gupta *et al.*, 1998)**. It has been suggested that the heavy sulfation of some HSPGs may function as an effective barrier between cells and their soluble and insoluble environments (Bode *et al.*, 2008; Levy-Adam *et al.*, 2010; Tuve *et al.*, 2008). This function would be of particular importance in the lung, where exposure to potentially toxic agents is virtually constant and protection is crucial for survival. Pulmonary exposure to heavy metals remains a very real occupational and environmental hazard (Jarup, 2003). Of interest here is cadmium, a ubiquitous toxicant with industrial emissions and cigarette smoke as major sources (Jarup, 2003). Acute exposures to cadmium can cause bronchial and pulmonary irritation in the lung and chronic exposures can induce bronchiolitis and emphysema. In addition, studies in rats have shown that long term exposure to cadmium induces lung cancers, and occupational studies have indicated a positive relation between lung cancer and cadmium inhalation (Agency for Toxic Substances and Disease Registry (ATSDR), 2008). The main mechanisms leading to acute toxicity are apoptosis at low concentrations (0 – 10 μM) and necrosis at high concentrations (> 50 μM) (Kwon *et al.*, 2006; Lee and Thevenod, 2008).

HSPGs and polysaccharides have been suggested to play a protective role against cellular and tissue injury. For example, heparan sulfate has been found to protect cells from injuries caused by calcium oxalate and herpes simplex virus (Iida *et al.*, 2003; Witvrouw and De Clercq, 1997). Glycosaminoglycans (GAGs), components of proteoglycans, were also found to limit the *in vitro* oxidative injury to skin fibroblasts caused by iron (Campo *et al.*, 2005). More importantly, metals which utilize reduced sulfur as a ligand, such as cadmium and mercury, were found to be more toxic than others to cells, and they inhibit the incorporation of sulfate into HSPGs (Templeton and Chaitu, 1990). Based on these collective observations, we hypothesized that HSPGs, especially because of their high degree of sulfation, function as a barrier capable of protecting cells from metals such as cadmium and that modification of the sulfation patterns of HSPGs has the potential to change their biological activities and influence protection/injury.

The goal of this study was to determine whether alterations in the expression of HSulf-1, and thus the 6-O-sulfate composition of HSPGs, induce changes in cellular response to cadmium exposure in **two** transformed human lung epithelial cell **lines** and/or in normal human primary alveolar type II (hAT2) cells. Elsewhere, HSulf-1 over-expression was shown to have inhibitory effects on VEGF-mediated signaling in breast carcinoma cells; chlorate treatment, used as a mimic of HSulf-1, confirmed that the reduced sulfate content of HSPGs, rather than any other attribute of HSulf-1 or artifact of its over-expression, was responsible (Narita *et al.*, 2006). Here, we investigated the role of HSulf-1 over-expression in altering the response of **two** transformed lung cell **lines** to cadmium, and not whether this sulfate-dependent response occurs because of decreases in the total amount of sulfation or from specific removal of the 6-O-sufates.

Results initially showed that neither cadmium at low concentrations nor HSulf-1 transduction at a low adenoviral MOI reduced visible cell density in H292 **or A549 cells**. However, the combination of HSulf-1 over-expression with cadmium treatment clearly reduced cell density in **all three** cell types. Cadmium alone appeared to reduce cell density via apoptosis in primary hAT2 cells, which confirms previous work in which rat AT2 cells were **found** to be sensitive to cadmium-**induced apoptosis (Lag *et al*., 2002).**

TUNEL assay indicated that H292 and A549 cells respond with apoptosis to HSulf-1 over-expression (while hAT2 cells do not). Previous findings that liver epithelial cancer cells may acquire resistance to apoptosis after cadmium treatment suggest that some cancer cell lines may have a low level of natural resistance to cadmium that can be selected for (Qu *et al.*, 2006). **Our data** indicate that the exposure to cadmium combined with over-expression of HSulf-1 results in an apparently synergistic exacerbation of apoptosis in H292 **and A549** cells, which suggests that cancer cells which have acquired cadmium resistance could be re-sensitized to it by targeted de-6-O-sulfation of relevant biological reactive sites at cell surfaces or in the surrounding matrix, altering the barrier function of HSPGs.

Previously, we found that in H292 cells, HSulf-1 over-expression activated apoptotic pathways and inhibited ERK signaling **(Zhang *et al*., 2012)**. However, in hAT2 cells, HSulf-1 over-expression inhibited ERK signaling but did not cause apoptosis. A perhaps relevant difference between H292 and hAT2 cells lies in the basal expression level of HSulf-1, which is much lower in H292 **and A549** cells than in hAT2 cells **(Supplementary Data 3 and (Zhang *et al*., 2012)).** Forced over-expression of HSulf-1 would thus be expected to result in a relatively greater change in HSPG sulfation levels in **these transformed** cells. And H292 **and A549** cells, which have high growth factor requirements for maintenance, are likely to be more sensitive to reduced sulfate in their surroundings, **as this** would compromise the optimal level of sulfates maintained as co-factors for growth factor signaling. Indeed, the inhibition of growth factor signaling is known to trigger apoptosis in cancer cells (Lu *et al.*, 2010), and we find that 6-O-sulfate removal activates specific key regulators of apoptosis, such as *BAX, CIDEA, TNF*, and *FAS*, in H292 cells.

It is likely that hAT2 cells are better adapted to or tolerant of the lower sulfated environments by virtue of their high basal expression of HSulf-1. Due to their facultative cell nature, their growth factor requirements for homeostasis would be low as well, and their under-sulfated BMs *in vivo* reflect this (Sannes, 1984). Thus, hAT2 cells, which already express HSulf-1 at higher levels, are neither compromised by HSulf-1 nor further sensitized to the toxic effects of cadmium by the additional decrease in 6-O-sulfation. The matrix surrounding the hAT2 cell may be already too low in sulfate to protect them from cadmium toxicity. Interestingly, low-dose cadmium triggered apoptosis, rather than outright necrosis, in isolated hAT2 cells. By the time they were treated with cadmium (three or four days after isolation), the freshly-isolated hAT2 cells had already begun to differentiate into the hAT1

phenotype, which produces high amounts of HSulf-1 as well as a highly-sulfated matrix *in vivo*. Thus, it might be expected that the increased matrix production would counteract HSulf-1 forced over-expression and physically block some of the cadmium, decreasing the effective concentration of cadmium to a level that triggers apoptosis rather than toxicity.

Extracellular matrix HSPGs have been recognized for their functional involvement in cell/tissue shape, mechanisms of cell recognition, and facilitation of cell signaling (Dreyfuss *et al.*, 2009). However, the potential protective roles of HSPGs against metal toxicity and the possible mechanisms involved are less well understood. Studies have demonstrated that injuries caused by metals such as zinc, barium, and mercury were related to the sulfation status of HSPGs and that sulfation of proteoglycans was decreased in response to treatment with these metals (Purdey, 2004; Templeton and Chaitu, 1990). In the current study, it was proposed that the alteration of sulfate moieties would affect cellular injury induced by cadmium, and HSulf-1, an enzyme that hydrolyzes 6-O-sulfate groups, was used to modify the sulfation levels and characteristics of relevant HSPGs. We report that HSulf-1 over-expression (resulting in lower levels of 6-O-sulfation) reduced the tolerance of **both** H292 **and A549** cells to cadmium and synergistically induced more cell death and apoptosis. Since HSulf-1 itself drives apoptosis in H292 cells through growth factor signaling inhibition, there must be a separate feature of HSulf-1 activity that allows the toxic effects of cadmium to reach the cell's surface. We propose that the sulfate moieties on HSPGs can act by charge-charge interactions to stabilize cadmium in the matrix and away from the cell surface until the structure and charge, and thus the barrier function, of the matrix are disrupted by enzymatic sulfate depletion.

Although it is plausible to suggest that it is HSulf-1's alteration of the structure and charge of matrix that is primarily responsible for the re-sensitization of H292 cells to cadmium, further study is needed to determine whether it is the specific 6-O-sulfation or the total level of sulfation in the cellular environment that is critical for resistance to cadmium. Challenging chlorate-cultured cells with cadmium and/or adding differently-sulfated heparins back to chlorate-treated cells prior to cadmium challenge should help answer this question.

Acknowledgments

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Highlights

Human lung alveolar type 2 (hAT2) cells and transformed H292 and A549 cells were used.

Cadmium induced apoptosis in hAT2 cells but not in H292 or A549 cells.

HSulf-1, which hydrolyzes the 6-O sulfate groups from extracellular heparan sulfate proteoglycans, exacerbates the apoptosis induced by cadmium in H292 and A549 cells but not in hAT2 cells

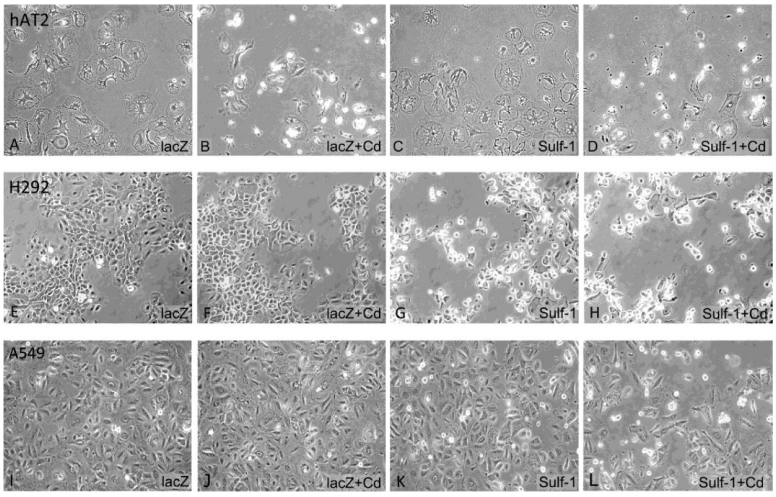

Figure 1.

Representative photomicrographs of hAT2, **H292**, and **A549** cells over-expressing HSulf-1 and/or treated with cadmium. hAT2 cells (A – D), H292 cells (E – H)**, and A549 cells (I – L)** were exposed to 10 μM cadmium for 48 hours after adenoviral transduction for lacZ or HSulf-1 over-expression. Photomicrographs were taken under a phase contrast microscope (200× magnification). (A), (**E**), and (**I**), lacZ transduction; (B), (**F**), and (**J**), lacZ plus cadmium; (C), (**G**), and (**K**), HSulf-1 transduction; (D), (**H**), and (**L**), HSulf-1 plus cadmium.

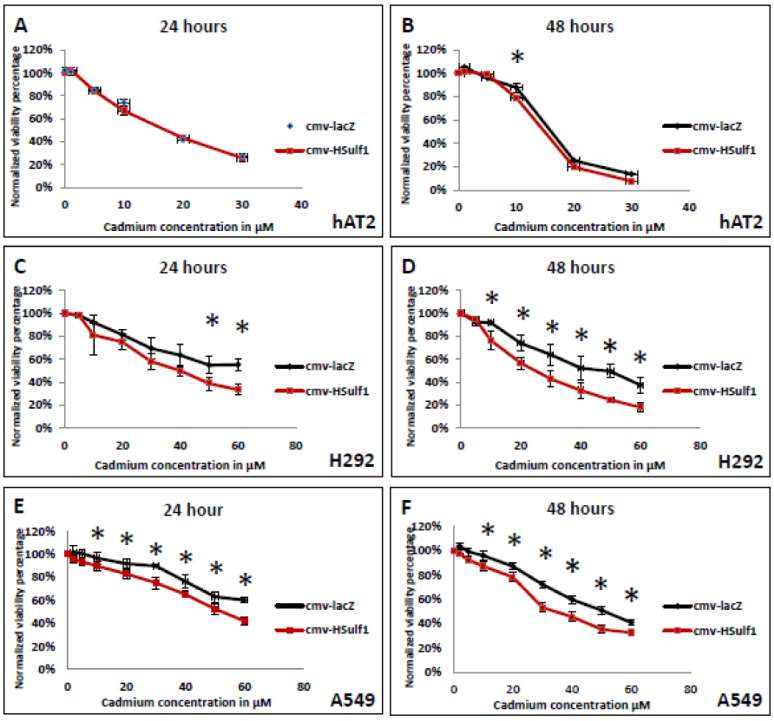

Figure 2.

Dose response to cadmium of hAT2, **H292,** and **A549** cells over-expressing lacZ or HSulf-1. Cells transduced at 10 MOI for lacZ or HSulf-1 over-expression were exposed to increasing concentrations of cadmium for 24 or 48 hours. Cells were cultured in 1 mg/ml MTT solution and resultant formazan metabolic products were read at 570 nm **on** an ELISA reader. Data were expressed as a percentage of the value of untreated control (lacZ over-expressing) cells as **Mean** ± SD from duplicate experiments. * Significant differences at $p < 0.05$ as determined by ANOVA, followed by Student's t-test.

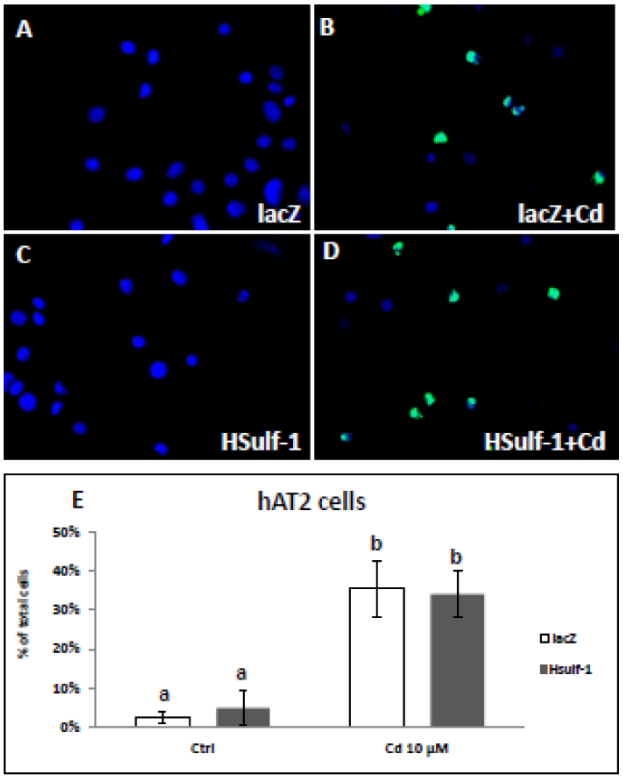

Figure 3.

TUNEL assay in hAT2 cells treated with HSulf-1 and/or cadmium. hAT2 cells were adenovirally-transduced (10 MOI) for lacZ or HSulf-1 over-expression and treated with or without 10 μM cadmium for 48 hours. TUNEL assay was performed on **harvested** cells. **(A – D) Random** fields were selected **for photomicrography** and total cells were counted in each field to achieve a minimum number of 60 total cells. (A) control adenovirus; (B) Cadmium only; (C) HSulf-1 adenovirus only; (D) HSulf-1 + cadmium. PI (blue), nuclei; Alexa Fluor 488 (green), apoptotic cells. (E) Apoptotic ratios as apoptotic cells/total cells are expressed as Mean ± SD from different fields. Significant differences between groups at $p < 0.05$ as determined by Student's t-test are indicated by **the letters above each bar, with** different letters **indicating significant differences between groups.**

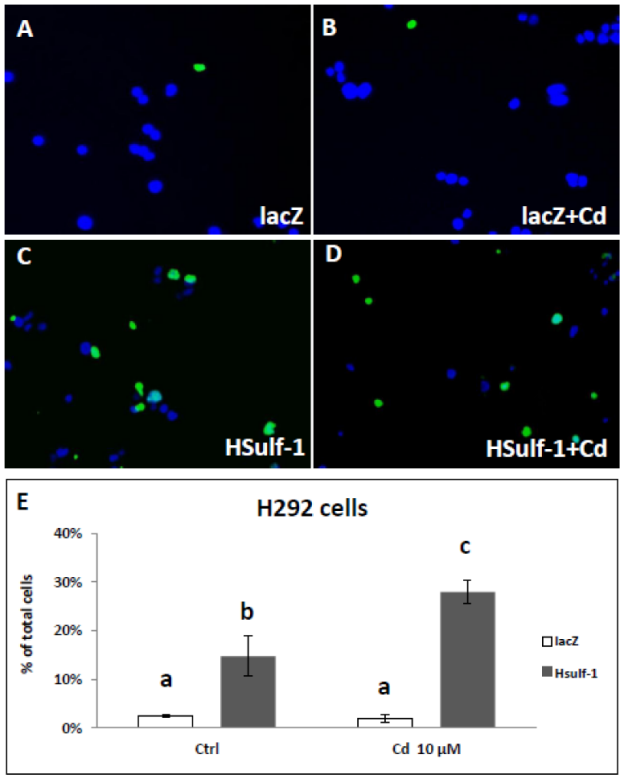

Figure 4.

TUNEL assay in H292 cells treated with HSulf-1 and/or cadmium. H292 cells were adenovirally-transduced (10 MOI) for lacZ or HSulf-1 over-expression and treated with or without 10 μM cadmium for 48 hours. TUNEL assay was performed on harvested cells. (A – D) Random fields were selected for photomicrography and total cells were counted in each field to achieve a minimum number of 150 total cells. (A) control adenovirus; (B) Cadmium only; (C) HSulf-1 adenovirus only; (D) HSulf-1 + cadmium. PI (blue), nuclei; Alexa Fluor 488 (green), apoptotic cells. (E) Apoptotic ratios as apoptotic cells/total cells are expressed as Mean ± SD from different fields. Significant differences between groups at $p < 0.05$ as determined by Student's t-test are indicated by the letters above each bar, with different letters indicating significant differences between groups.

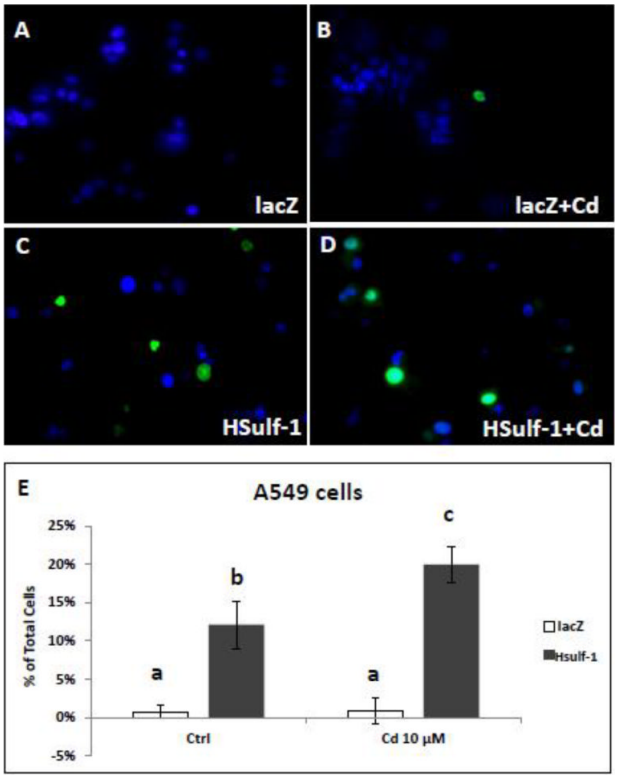

Figure 5.

TUNEL assay in A549 cells treated with HSulf-1 and/or cadmium. A549 cells were adenovirally-transduced (10 MOI) for lacZ or HSulf-1 over-expression and treated with or without 10 μM cadmium for 48 hours. TUNEL assay was performed on harvested cells. (A – D) Random fields were selected for photomicrography and total cells were counted in each field to achieve a minimum number of 150 total cells. (A) control adenovirus; (B) Cadmium only; (C) HSulf-1 adenovirus only; (D) HSulf-1 + cadmium. PI (blue), nuclei; Alexa Fluor 488 (green), apoptotic cells. (E) Apoptotic ratios as apoptotic cells/total cells are expressed as Mean ± SD from different fields. Significant differences between groups at $p < 0.05$ as determined by Student's t-test are indicated by the letters above each bar, with different letters indicating significant differences between groups.

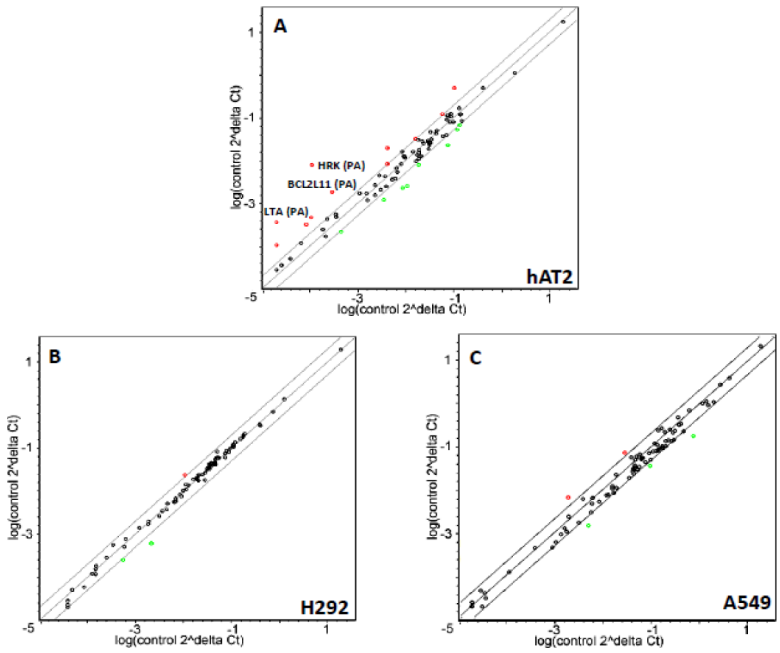

Figure 6.

Scatter plot analysis of Apoptosis PCR array results for (A) hAT2, (B) H292, and (C) A549 cells exposed to cadmium. hAT2, H292, and A549 cells over-expressing lacZ were exposed to cadmium for 48 hours. Total RNA was isolated and reverse-transcribed, and an Apoptosis PCR array was performed on each cDNA. Genes up- or down-regulated more than 2-fold compared to the lacZ-transduced control cells are labeled red or green, respectively. Several significant genes are labeled: (PA) pro-apoptosis.

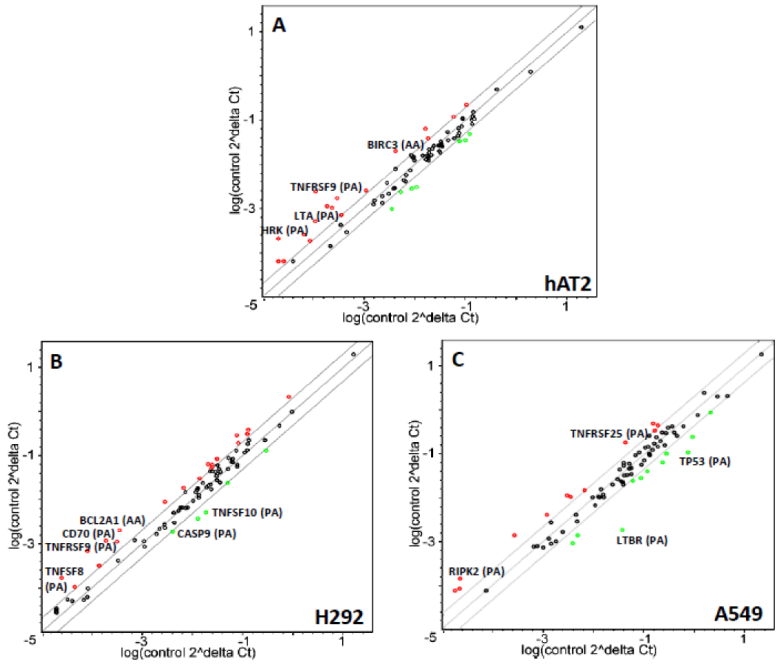

Figure 7.

Scatter plot analysis of Apoptosis PCR array results for **(A)** hAT2, **(B) H292**, and **(C) A549** cells exposed to the combination of HSulf-1 over-expression and cadmium treatment. hAT2, **H292,** and **A549** cells were exposed to cadmium for 48 hours after adenovirally-transduced over-expression of lacZ or HSulf-1. Genes up- or down-regulated more than 2-fold compared to the lacZ-transduced control cells are labeled red or green, respectively. Several significant genes are labeled: (PA) pro-apoptosis; (AA) anti-apoptosis.

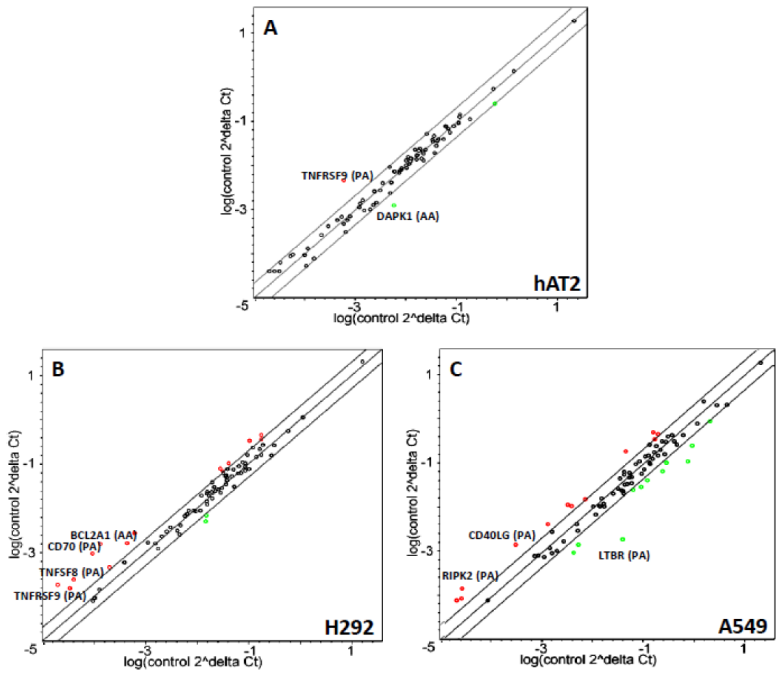

Figure 8.

Scatter plot analysis of the contribution of HSulf-1 to **the** apoptotic effects of cadmium. **(A)** hAT2, **(B) H292**, and **(C) A549** cells were exposed to cadmium for 48 hours after adenovirally-transduced over-expression of lacZ or HSulf-1. Genes significantly up- or down-regulated (more than 2-fold) by cadmium in HSulf-1-expressing cells compared to those regulated by cadmium in lacZ-expressing cells are shown in red or green, respectively, and the most significant genes are labeled: (PA) pro-apoptosis; (AA) anti-apoptosis.

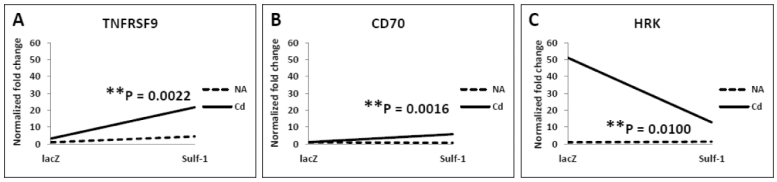

Figure 9.

Gene expression interaction analysis in hAT2 cells. Genes up- or down-regulated in hAT2 cells by HSulf-1/Cadmium were tested for synergistic or antagonistic interactions by the Association Test. Dashed line, No Cadmium; Solid line, Cadmium treatment at 10 μM. See text for details. Data was analyzed by Factorial ANOVA**. * p< 0.05; ** p<0.01.**

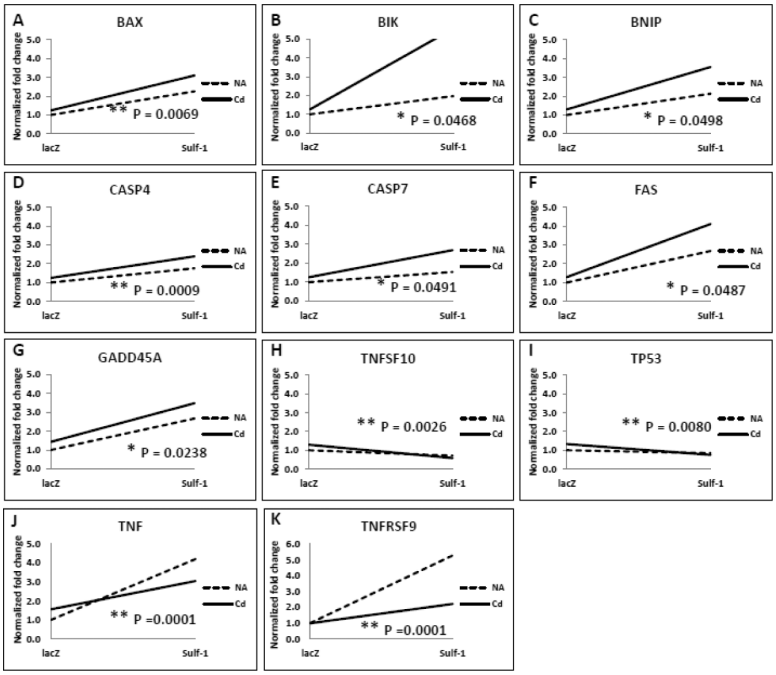

Figure 10.

Gene expression interaction analysis in H292 cells. Genes up- or down-regulated in H292 cells by HSulf-1/Cadmium were tested for synergistic or antagonistic interactions by the Association Test. Dashed line, No Cadmium; Solid line, Cadmium treatment at 10 μM. See text for details. Data was analyzed by Factorial ANOVA**. * p< 0.05; ** p<0.01.**

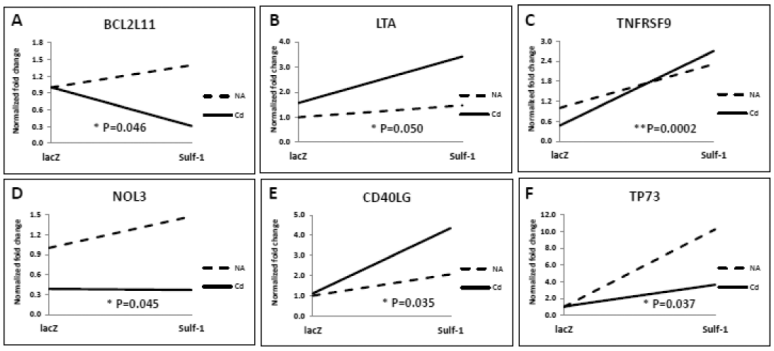

Figure 11.

Gene expression interaction analysis in A549 cells. Genes up- or down-regulated in A549 cells by HSulf-1/Cadmium were tested for synergistic or antagonistic interactions by the Association Test. Dashed line, No Cadmium; Solid line, Cadmium treatment at 10 μM. See text for details. Data was analyzed by Factorial ANOVA. * $p<0.05$; ** $p<0.01$.

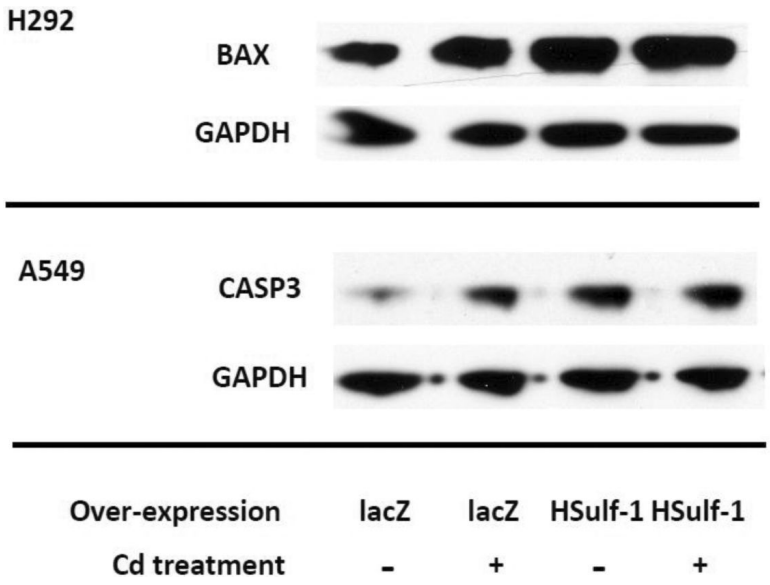

Figure 12.
Western blot confirmation of selected PCR array results. Apoptosis array analysis found that *BAX* in H292 cells and *CASP3* in A549 cells were up-regulated by HSulf-1 alone and in combination with cadmium. Western analysis confirms that the up-regulation of these pro-apoptotic genes led to their increased protein expression.

Table 1

Genes that were up- or down-regulated in hAT2 cells by HSulf-1 or cadmium

hAT2 cells		lacZ		HSulf-1		
Gene	**Description**	**Ctrl**	**Cd**	**Ctrl**	**Cd**	**Function**
AKT1	v-akt murine thymoma viral oncogene homolog 1	1	**−1.8**	**−1**	**−2.3**	Anti-apoptosis
BAG3	Bcl-2-associated athanogene 3	1	**4.5**	**−1.1**	**2.2**	Anti-apoptosis
BCL2A1	BCL2-related protein A1	1	**2**	**−2**	**2.8**	Anti-apoptosis
BIRC3	Baculoviral IAP repeat containing 3	1	**5**	**1.2**	**5.4**	Anti-apoptosis
BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1	1	**2**	**1.2**	**1.7**	Anti-apoptosis
IGF1R	Insulin-like growth factor 1 receptor	1	**−1.7**	**−2.3**	**−2**	Anti-apoptosis
APAF1	Apoptotic peptidase activating factor 1	1	**−2.7**	**−1.8**	**−1.5**	Pro-apoptosis
BCL2L11	BCL2-like 11 (apoptosis facilitator)	1	**5.7**	**2.1**	**4.9**	Pro-apoptosis
BIK	BCL2-interacting killer	1	**3.3**	**−1.3**	**3.8**	Pro-apoptosis
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	1	**2.2**	**1.3**	**2**	Pro-apoptosis
NOD1	Nucleotide-binding oligomerization domain 1	1	**−4**	**1.1**	**−3.4**	Pro-apoptosis
CARD8	Caspase recruitment domain family, member 8	1	**−2.5**	**−1.2**	**−1.6**	Pro-apoptosis
CASP10	Caspase 10	1	**−2.9**	**−1.3**	**−5.5**	Pro-apoptosis
CASP7	Caspase 7	1	**1.2**	**2.1**	**2.4**	Pro-apoptosis
CIDEA	Cell death-inducing DFFA-like effector a	1	**2.3**	**−1.4**	**2.8**	Pro-apoptosis
DAPK1	Death-associated protein kinase 1	1	**−3.6**	**−4.2**	**−3.8**	Pro-apoptosis
FAS	TNF receptor superfamily member 6	1	**−2.2**	**1.3**	**−1.7**	Pro-apoptosis
HRK	Activator of apoptosis harakiri	1	**56.7**	**1.6**	**14.3**	Pro-apoptosis
LTA	Lymphotoxin alpha	1	**16.9**	**0.83**	**9.2**	Pro-apoptosis
TNFRSF21	TNF receptor superfamily member 21	1	**−2.6**	**−1.1**	**−2.7**	Pro-apoptosis
TNFRSF9	TNF receptor superfamily member 9	1	**3.3**	**4.5**	**21.8**	Pro-apoptosis
TNFSF10	TNF superfamily member 10	1	**−3.5**	**1.2**	**−2.3**	Pro-apoptosis
CD40	TNF receptor superfamily member 5	1	**−2**	**−1.2**	**−2.2**	Pro-proliferation
CD70	TNF superfamily member 7	1	**1.1**	**−1.7**	**5.8**	Pro-proliferation
TNFSF8	TNF superfamily member 8	1	**2**	**1.7**	**3.4**	Pro-proliferation
BRAF	v-raf murine sarcoma viral oncogene homolog B1	1	**−1.7**	**1**	**−2.7**	Unknown
CD27	TNF receptor superfamily member 7	1	**5.3**	**1.4**	**5.1**	Unknown

hAT2 cells		lacZ		HSulf-1		
Gene	**Description**	**Ctrl**	**Cd**	**Ctrl**	**Cd**	**Function**
TP73	Tumor protein P73	1	**1.2**	**−1.3**	**2.2**	Pr/An-apoptosis

hAT2 cells were transduced for lacZ or HSulf-1 over-expression and treated with or without cadmium. Total mRNA **of duplicate samples** was isolated, Apoptosis PCR **arrays were** performed, **and results were combined** to analyze gene expression. Genes that were up- or down-regulated more than 2-fold over “lacZ control” levels are indicated. Red, up-regulation; Blue, down-regulation; Black, non-significant change.

Table 2

Genes that were up- or down-regulated in H292 cells by HSulf-1 or cadmium

H292 cells		lacZ		HSulf-1		
Gene	**Description**	**Ctrl**	**Cd**	**Ctrl**	**Cd**	**Function**
AKT1	v-akt murine thymoma viral oncogene homolog 1	**1**	**1.2**	**2**	**2.4**	Anti-apoptosis
BAG1	Bcl-2-associated athanogene 1	**1**	**−1.2**	**−2.3**	**−2**	Anti-apoptosis
BAG4	Bcl-2-associated athanogene 4	**1**	**−1**	**−2**	**−1.3**	Anti-apoptosis
BCL2A1	BCL2-related protein A1	**1**	**1.2**	**5.3**	**5.4**	Anti-apoptosis
IGF1R	Insulin-like growth factor 1 receptor	**1**	**−1.2**	**−2.2**	**−1.4**	Anti-apoptosis
APAF1	Apoptotic peptidase activating factor 1	**1**	**1.1**	**1.5**	**2.1**	Pro-apoptosis
BAX	BCL2-associated X protein	**1**	**1.2**	**2.1**	**2.9**	Pro-apoptosis
BIK	BCL2-interacting killer	**1**	**−2**	**1.1**	**2.2**	Pro-apoptosis
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	**1**	**1.1**	**1.7**	**3**	Pro-apoptosis
CASP4	Caspase 4	**1**	**1.2**	**1.7**	**2.3**	Pro-apoptosis
CASP6	Caspase 6	**1**	**−1.1**	**−4.5**	**−1.1**	Pro-apoptosis
CASP7	Caspase 7	**1**	**1**	**1.2**	**2.2**	Pro-apoptosis
CASP8	Caspase 8	**1**	**−1.1**	**−2.1**	**1.1**	Pro-apoptosis
CASP9	Caspase 9	**1**	**−1.1**	**−1.7**	**−3.2**	Pro-apoptosis
CIDEA	Cell death-inducing DFFA-like effector a	**1**	**−3.4**	**3.1**	**3.4**	Pro-apoptosis
DFFA	DNA fragmentation factor	**1**	**2.2**	**2.2**	**2.7**	Pro-apoptosis
FAS	TNF receptor superfamily member 6	**1**	**1.1**	**2.3**	**3.6**	Pro-apoptosis
HRK	Activator of apoptosis harakiri	**1**	**−1.2**	**−1.9**	**−2.1**	Pro-apoptosis
TNFRSF10A	TNF receptor superfamily member 10A	**1**	**1.2**	**1.7**	**2.4**	Pro-apoptosis
TNFRSF9	TNF receptor superfamily member 9	**1**	**−1.3**	**7.3**	**7.7**	Pro-apoptosis
TNFSF10	TNF superfamily member 10	**1**	**−1.6**	**−2.8**	**−3.5**	Pro-apoptosis
TP53	Tumor protein 53	**1**	**−1.2**	**−1.9**	**−2.2**	Pro-apoptosis
GADD45A	Growth arrest and DNA-damage-inducible, alpha	**1**	**1.1**	**2**	**2.6**	Anti-proliferation
CD70	TNF superfamily member 7	**1**	**1.6**	**6.6**	**5.8**	Pro-proliferation
TNFSF8	TNF superfamily member 8	**1**	**1**	**5.3**	**6.2**	Pro-proliferation
CD27	TNF receptor superfamily member 7	**1**	**−1**	**1.7**	**2.3**	Unknown
TRAF3	TNF receptor associated factor 3	**1**	**1.1**	**1.7**	**2.1**	Unknown

H292 cells		lacZ		HSulf-1		
Gene	**Description**	**Ctrl**	**Cd**	**Ctrl**	**Cd**	**Function**
MCL1	Myeloid cell leukemia sequence 1	**1**	**1.3**	**2.1**	**2**	Pro/anti-apoptosis
TP73	Tumor protein 73	**1**	**1.1**	**2.5**	**3**	Pro/anti-apoptosis

H292 cells were transduced for lacZ or HSulf-1 over-expression and treated with or without cadmium. Total mRNA **of duplicate samples** was isolated, Apoptosis PCR **arrays were** performed, **and results were combined** to analyze gene expression. Genes that were up- or down-regulated more than 2-fold over “lacZ control” levels are indicated. Red, up-regulation; Blue, down-regulation; Black, non-significant change.

Table 3

Genes that were up- or down-regulated in A549 cells by HSulf-1 or cadmium

A549 cells		**lacZ**		**HSulf-1**		
Gene	**Description**	**Ctrl**	**Cd**	**Ctrl**	**Cd**	**Function**
ABL1	c-abl oncogene 1	1	**−1.2**	**−1.3**	**−2.2**	Anti-apoptosis
BCL2A1	BCL2-related protein A1	1	**3.2**	**2.5**	**1.8**	Anti-apoptosis
BCL2	B-cell lymphoma 2	1	**−3.2**	**1.3**	**−3.7**	Anti-apoptosis
BNIP2	BCL-2 and adenovirus E1B 19 kDa protein	1	**1.4**	**1.0**	**2.9**	Anti-apoptosis
BFAR	Bifunctional apoptosis regulator	1	**−2.0**	**−1.5**	**−3.1**	Anti-apoptosis
NAIP	NLR family, apoptosis inhibitory protein	1	**−1.8**	**−1.1**	**−2.1**	Anti-apoptosis
NFkB1	Nuclear factor k B1	1	**2.4**	**1.0**	**−1.1**	Anti-apoptosis
NOL3	Nucleolar protein 3	1	**−2.6**	**1.5**	**−2.7**	Anti-apoptosis
AIFM1	Apoptosis inducing factor, mitochondria associated 1	1	**−1.9**	**−1.1**	**−2.5**	Pro-apoptosis
BCL2L11	BCL2-like 11 (apoptosis facilitator)	1	**1.0**	**1.4**	**−3.1**	Pro-apoptosis
CASP3	Caspase 3	1	**−1.6**	**2.3**	**2.1**	Pro-apoptosis
CASP6	Caspase 6	1	**−1.3**	**2.2**	**3.0**	Pro-apoptosis
CD40LG	T cell antigen Gp39	1	**1.1**	**2.1**	**4.4**	Pro-apoptosis
LTA	Lymphotoxin alpha	1	**1.5**	**1.5**	**3.4**	Pro-apoptosis
LTBR	Lymphotoxin β receptor	1	**−1.4**	**−2.6**	**−15.2**	Pro-apoptosis
MCL1	Myeloid cell leukemia sequence 1	1	**1.5**	**1.6**	**3.7**	Pro-apoptosis
RIPK2	Receptor-interacting serine-threonine kinase 2	1	**−1.3**	**6.0**	**5.8**	Pro-apoptosis
TNFRSF9	TNF receptor superfamily member 9	1	**−2.0**	**2.3**	**2.7**	Pro-apoptosis
TNFRSF11B	TNF receptor superfamily member 11B	1	**−1.5**	**−1.2**	**−2.5**	Pro-apoptosis
TNFRSF25	TNF receptor superfamily member 25	1	**1.2**	**2.8**	**3.5**	Pro-apoptosis
TNFSF10	TNF superfamily member 10	1	**−1.5**	**3.0**	**2.3**	Pro-apoptosis
TP53	Tumor protein P53	1	**−3.9**	**−1.3**	**−5.5**	Pro-apoptosis
TP73	Tumor protein P73	1	**1.0**	**10.4**	**3.3**	Pr/An-apoptosis

A549 cells were transduced for lacZ or HSulf-1 over-expression and treated with or without cadmium. Total mRNA of duplicate samples was isolated, Apoptosis PCR arrays were performed, and results were combined to analyze gene expression. Genes that were up- or down-regulated more than 2-fold over “lacZ control” levels are indicated. Red, up-regulation; Blue, down-regulation; Black, non-significant change.

Table 4

Genes that were up- or down-regulated in hAT2 cells by the HSulf-1/cadmium combination, normalized to lacZ/cadmium results

Gene	Description	Fold change	Function
BAG3	Bcl-2-associated athanogene 3	**−2.0**	Anti-apoptosis
HRK	Activator of apoptosis harakiri	**−4.0**	Pro-apoptosis
TNFRSF9	TNF receptor superfamily member 9	**6.7**	Pro-apoptosis
CD70	TNF superfamily member 7	**5.3**	Pro-proliferation

hAT2 cells were transduced for lacZ or HSulf-1 over-expression and treated with or without cadmium. Total mRNA **of duplicate samples** was isolated, Apoptosis PCR **arrays were** performed, **and results were combined** to analyze gene expression. Genes that were up- or down-regulated more than 2-fold over “lacZ/cadmium” levels are indicated. Red, up-regulation; Blue, down-regulation.

Table 5

Genes that were up- or down-regulated by HSulf1/cadmium combination vs cadmium in H292 cells

Gene	Description	Fold change	Function
AKT1	v-akt murine thymoma viral oncogene homolog 1	**2.0**	Anti-apoptosis
BCL2A1	BCL2-related protein A1	**4.5**	Anti-apoptosis
BAX	BCL2-associated X protein	**2.5**	Pro-apoptosis
BIK	BCL2-interacting killer	**4.5**	Pro-apoptosis
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	**2.7**	Pro-apoptosis
CASP7	Caspase 7	**2.2**	Pro-apoptosis
CIDEA	Cell death-inducing DFFA-like effector a	**11.7**	Pro-apoptosis
FAS	TNF receptor superfamily member 6	**3.2**	Pro-apoptosis
TNF	Tumor necrosis factor	**9.4**	Pro-apoptosis
TNFRSF9	TNF receptor superfamily member 9	**10.1**	Pro-apoptosis
CASP9	Caspase 9	**−2.9**	Pro-apoptosis
TNFSF10	TNF superfamily member 10	**−2.2**	Pro-apoptosis
CD70	TNF superfamily member 7	**3.7**	Pro-proliferation
TNFSF8	TNF superfamily member 8	**6.2**	Pro-proliferation
CD27	TNF receptor superfamily member 7	**2.3**	Unknown

H292 cells were transduced for lacZ or HSulf-1 over-expression and treated with or without cadmium. Total mRNA **of duplicate samples** was isolated, Apoptosis PCR **arrays were** performed, **and results were combined** to analyze gene expression. Genes that were up- or down-regulated more than 2-fold over “lacZ/cadmium” levels are indicated. Red, up-regulation; Blue, down-regulation.

Table 6

Genes that were up- or down-regulated by HSulf1/cadmium combination vs cadmium in A549 cells

Gene	Description	Fold change	Function
CFLAR	Caspase 8 and FADD-like apoptosis regulator	**3.7**	Anti-apoptosis
TNFRSF1B	TNF superfamily member 1B	**2.3**	Anti-apoptosis
NFkB1	Nuclear factor B1	**−2.8**	Anti-apoptosis
BAK1	BCL2 antagonist/killer 1	**2.3**	Pro-apoptosis
BAX	BCL2-associated X protein	**2.5**	Pro-apoptosis
BCL2L11	BCL2-like 11	**−3.2**	Pro-apoptosis
CASP3	Caspase 3	**3.3**	Pro-apoptosis
CASP6	Caspase 6	**3.8**	Pro-apoptosis
CD40LG	CD40 ligand	**3.9**	Pro-apoptosis
LTA	Lymphotoxin alpha	**2.2**	Pro-apoptosis
LTBR	Lymphotoxin beta receptro	**−10.9**	Pro-apoptosis
MCL1	Myeloid cell leukemia sequence 1	**2.5**	Pro-apoptosis
NOD1	Nucleotide-binding oligomerization domain containing 1	**2.2**	Pro-apoptosis
RIPK2	Receptor-interacting serine threonine kinase 2	**6.3**	Pro-apoptosis
TNFRSF25	TNF receptor superfamily member 9	**3.2**	Pro-apoptosis
TNFSF10	TNF superfamily member 10	**3.3**	Pro-apoptosis
TNFRSF9	TNF superfamily member 9	**5.8**	Pro-apoptosis

A549 cells were transduced for lacZ or HSulf-1 over-expression and treated with or without cadmium. Total mRNA of duplicate samples was isolated, Apoptosis PCR arrays were performed, and results were combined to analyze gene expression. Genes that were up- or down-regulated more than 2-fold over “lacZ/cadmium” levels are indicated. Red, up-regulation; Blue, down-regulation.