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Reactivation of death receptor 4 (DR4) expression sensitizes medulloblastoma cell lines to TRAIL

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Abstract

Object—Apoptosis, a key cellular response to therapeutic agents is often inactivated in tumor cells. In this study, we evaluated the expression of the tumor necrosis family of death receptors, *DR4* and *DR5*, in medulloblastoma tumor samples and cell lines to determine if epigenetic modulation of gene expression could sensitize tumor cell lines to TRAIL-mediated apoptosis.

Methods—Human medulloblastoma samples and cell lines were analyzed for DR4 and DR5 expression by quantitative PCR and immunofluorescence assays. Cell lines with downregulated expression of one or both genes were treated with the histone deacetylase inhibitor, MS-275, and the expression of DR4 and DR5 measured by quantitative PCR, Western blotting, flow cytometry and chromatin immunoprecipitation assays. Induction of apoptosis in the presence of MS-275 was evaluated by TUNEL assay and its ability to augment TRAIL-mediated cytotoxicity was determined by MTT assays, Western blotting and flow cytometry.

Results—Compared to normal cerebellum, *DR4*, but not *DR5* expression was consistently downregulated in medulloblastoma tumor samples and in Daoy and D283 cell lines. Interestingly, MS-275 decreased cell growth and induced apoptosis in Daoy and D283 cells. In Daoy cells, this coincided with increased histone H3 and H4 acetylation at the *DR4* promoter and enhanced DR4 gene and protein expression as well as elevated Caspase-8 activity. The involvement of DR4 in the cellular response to MS-275 was further confirmed by the observation that knockdown of DR4 and FADD abrogated apoptosis. Further, addition of TRAIL to MS-275 treated cells resulted in an enhancement of apoptosis, suggesting that the upregulated death receptors were functional.

Conclusion—Our study provides an understanding of the role of DR4 in apoptosis of medulloblastoma cell lines and suggests a potential contribution of aberrant histone deacetylation to the resistance of medulloblastoma cells to therapeutic death.

Keywords

MS-275; Medulloblastoma; Apoptosis; DR4; DR5; TRAIL

Introduction

Medulloblastoma, the most common malignant pediatric brain tumor, originates in the cerebellum [1, 2]. Current therapeutic modalities have improved survival, however, the majority of long-term survivors suffer significant sequelae [3]. Thus, targeted therapies based on a better understanding of disease biology are necessary.

Apoptosis a process required for cellular homeostasis in normal tissues is often deregulated in tumors [4, 5]. In normal cells, apoptosis is induced in response to extracellular stimuli by activation of the death receptor or the extrinsic pathway and to intracellular perturbations through activation of the mitochondrial or intrinsic pathway. Activation of the extrinsic pathway is initiated by the binding of cognate ligands to the death receptors FAS, TNF receptor 1 (TNFR1), DR4 and DR5. The death effector domains of these receptors then interact with adaptor proteins, FAS-associated death domain (FADD) or TNF receptor-associated death domain (TRADD) to recruit the inactive effector pro-Caspase-8 [5]. Dimerization of pro-Caspase 8 results in its auto-proteolytic cleavage and activation of a downstream signaling cascade to initiate Caspase 3-mediated PARP cleavage and other morphologic changes linked to apoptosis.

Mutations or down-regulation of essential pro-apoptotic proteins, or expression of anti-apoptotic proteins exemplify some mechanisms by which tumor cells evade apoptosis. However, recent studies have implicated epigenetic deregulation of gene expression by aberrant histone deacetylation and DNA methylation in tumor development [6–8].

An involvement of epigenetic mechanisms in the silencing of the extrinsic or death receptor-mediated pathway in medulloblastoma cells has been suggested by the following studies. A report showed that a decrease in Caspase 8 protein expression is a negative prognostic indicator in childhood medulloblastomas [9]. This study also demonstrated that reactivation of *CASP8* gene expression using agents that inhibited DNA methylation restored apoptosis in medulloblastoma cell lines suggesting that *CASP 8* gene was silenced by DNA methylation. Loss of *CASP8* gene expression by aberrant DNA methylation has also been confirmed by a number of other studies [10–15]. In contrast, only a few studies have examined the expression of upstream death receptor expression in human brain tumor samples. For example, FAS/CD95 expression was assessed in patient samples by flow cytometry and immunohistochemistry and it was determined that although FAS/CD95 receptor and its cognate FASL ligand were expressed, tumor cells were resistant to treatment with etoposide [16]. A study by Zuzak et al. demonstrated by semi-quantitative RT-PCR experiments that *DR4* mRNA expression was downregulated in human samples of primitive neuroectodermal tumors (PNETS), however, the mechanism underlying its deregulated expression was not examined [10].

Because mis-regulation of the epigenome has been suggested to play a role in human medulloblastomas, we asked if the expression of *DR4* and *DR5* is deregulated in human tumors by such mechanisms and if so, can it be reversed by agents that target the epigenome? In the current study, we used immunohistochemical and real-time PCR analyses to show that *DR4* expression is downregulated in human medulloblastoma tumor samples and in Daoy and D283 medulloblastoma cell lines relative to that in the normal cerebellum. Importantly, the HDAC inhibitor, MS-275 is effective in decreasing the growth of Daoy and D283 cells in vitro through activation of the extrinsic apoptotic pathway. This occurs through MS-275-mediated increase in acetylation of histones H3 and H4 acetylation in the *DR4* promoter and reactivation of *DR4* expression in Daoy cells. A significant potentiation of apoptosis observed in the presence of both MS-275 and recombinant TRAIL suggests that the upregulated *DR4* receptors are cell-surface associated and functional. Our studies indicate a role for histone deacetylation in the aberrant silencing of *DR4* in medulloblastomas and suggest further pre-clinical and clinical investigation of MS-275 as a chemosensitizer in medulloblastoma therapy.

Materials and methods

Human patient samples

After Internal Board Review approval, a total of 47 human medulloblastoma tumor specimens, including 27 samples in a tissue microarray (TMA) obtained from the MD Anderson Cancer Center Brain Tumor Tissue Bank and The University Hospital at Munster were reviewed by Drs. Fuller, Paulus and Hasselblatt and provided to us for molecular analyses [1, 2]. Formalin-fixed paraffin embedded (FFPE) samples were obtained from primary tumor tissue of patients ranging in age from 1 to 59 years.

Cell culture

Human medulloblastoma cell lines (Daoy and D283) were obtained from the ATCC (Manassas, VA) and maintained in minimum essential medium (Eagle) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (MediaTech, Herndon, VA), 1%

penicillin and streptomycin (GIBCO, Carlsbad, CA) at 5% CO₂ tension and 37°C. Cell lines were used between 6 and 10 passages and routinely tested for mycoplasma.

Chemicals

N-(2-aminophenyl)-4-[(N-pyridine-3-ylmethoxycarbonyl) aminomethyl] Benzamide (MS-275) was purchased from Axxora (San Diego, CA). A 100 mM stock solution of MS-275 was prepared in methanol and stored at -20°C. Further dilutions were carried out in media to the appropriate concentrations.

Cell growth

Daoy and D283 cells were incubated with various concentrations of MS-275 (0.625–20 µM) for 24–144 h. Metabolic activity was measured spectrophotometrically at 570 nm following addition of 3-(4, 5 dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) reagent and 20% SDS. Data was analyzed with Softmax software and results expressed as percentages relative to untreated cells.

Western blotting

Extracts were prepared from Daoy cells treated with 2.5 µM MS-275 for 4–72 h and subjected to SDS polyacrylamide gel electrophoresis and Western blotting using anti-histone H3 (1:1000) (Abcam, Cambridge, MA), histone H4, acetylated histones H3 and H4 (1:1000) (Upstate, Waltham, MA), cleaved PARP (1:1000), pan-Actin (1:1000), Caspase 8, (1:500) (Cell Signaling Technology, Beverly, MA), and DR4 (1:500), DR5 (1:500) and FADD (1:500) (Millipore, Cambridge, MA).

Quantitative PCR analyses

RNA was extracted from FFPE tumor tissue using the pin-point Slide RNA Isolation system II kit (Zymo Research, Orange, CA) [17, 18]. Since RNA from paired normal cerebellar samples were substantially degraded, normal cerebellar RNA was purchased from Ambion (Austin, TX). cDNA was synthesized using the Sensiscript RT kit (Qiagen, Valencia CA) following addition of random nonamers (US Biologicals, Swampscott, MA) and oligo dT (Promega, Madison, WI). The primer/probe for Taqman gene expression assays were as follows: *DR4* (Hs00269492_m1), *DR5* (Hs00366272_m1) and *TRAIL* (Hs00234356_m1) (Applied Biosystems, Foster City, CA). These 3 sets of primers and probe were chosen because their amplicon sizes were less than 100 bp long, making them suitable for work with RNA from archival materials [15, 16]. Quantitative PCR (QPCR) was performed using an iCycler (Bio-Rad Laboratories, Hercules, CA), with reactions in triplicate and using the following parameters: 50°C for 2 min and 95°C for 10 min, followed by 60 cycles at 95°C for 15 s and 60°C for 1 min. Data were analyzed using iCycler software (Bio-Rad Laboratories, Valencia, CA). *18S* ribosomal RNA (rRNA) and *GAPDH* were used for normalization. Standard deviations were calculated based on mean threshold cycle (Ct) values of triplicates (below 1.0) of each run.

Immunofluorescence of paraffin embedded tissues

FFPE tumor and normal cerebellar TMAs were stained with hematoxylin–eosin (H&E) and visualized by light microscopy. For immunostaining, sections were deparaffinized, subjected to antigen retrieval and blocked with TBST containing 20% normal goat serum. This was followed by incubation with antibody against human DR4 and DR5 (1: 500) (Millipore, Cambridge MA) in TBST containing 2% normal goat serum. After removal of unbound antibody and incubation with Alexa-488 conjugated antirabbit secondary antibody (1:2000), cells were washed and nuclei were stained with Draq-5 (1:1000) (Axxora, San Diego, CA). The stained sections were covered with Slowfade-Antifade (Invitrogen, Carlsbad, CA),

sealed with a cover slip and examined using a Nikon Fluorescence microscope. Image analysis was done using Metamorph software (Molecular Devices, Downington, PA).

Flow cytometric analysis

For cell cycle analyses, Daoy and or D283 cells were incubated with 2.5 μ M MS-275 for 24–96 h, harvested and re-suspended in PBS containing 1 mg/ml of sodium citrate, 0.1% of Triton X-100 and 0.05 mg/ml propidium iodide (Sigma Aldrich, St Louis, MO). Flow cytometry was performed on a Becton Dickinson FACScan Instrument (San Jose, CA) and the data analyzed using Cell Quest 3.2 software (Becton Dickinson, Franklin Lakes, NJ).

Flow cytometric analyses to measure cell surface expression of DR4 and DR5 was performed with Daoy cells treated with 2.5 μ M MS-275 for 24 h. Cells were harvested, washed and re-suspended in 100 μ L of fluorescence-activated cell-sorter buffer (FACS) (PBS 1% BSA, 0.02% sodium azide) containing either Phytoerythrin (PE)-labeled mouse anti-human DR4 and DR5 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), or mouse antihuman IgG control antibody (BD Pharmingen, San Jose, CA) plus PE-labeled goat anti-mouse antibody.

For flow cytometric measurement of apoptosis at 48 h was done with MS-275 at a concentration of 0.312 or 0.625 μ M MS-275 and TRAIL at a concentration of 2.5 ng (12.5 ng/ml).

Caspase activity

Caspase 8 enzymatic activity was measured using the fluorogenic caspase-like enzyme substrate (N-Acetyl-IETD-AMC) (BioMol, Plymouth Meeting, PA). Daoy cells were either untreated or treated with 2.5 μ M MS-275 for 24–72 h. Cell lysates prepared by multiple freeze-thaw cycles were diluted in Caspase 8 buffer (100 mM HEPES, 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 5 mM DTT). Extracts from equal number of cells were mixed with N-Acetyl-IETD-AMC to a final concentration of 50 μ M and fluorescence emitted was measured with SpectraMAX GeminiEM (Molecular Devices, Downington, PA) at 460 nm.

Immunofluorescence assays

Daoy cells were seeded in CC2 chamber slides and treated with 2.5 μ M MS-275 for 24 h. Cells were fixed with 2% paraformaldehyde, blocked and then incubated with antihuman DR4 and DR5 (1:1000) (BD Pharmingen, Franklin Lakes, NJ) for 90 min. After washing, the cells were incubated with Cy3-conjugated goat anti-mouse antibody (Amersham, Piscataway, NJ), covered with Slowfade Gold[®] antifade (Invitrogen, Eugene, OR) containing 1 μ g/ml DAPI. Images were visualized using a Nikon Fluorescence microscope and analyzed using Metamorph software.

Transfection with SiRNA

Daoy cells were transfected with the On target plus Smart pool FADD and DR4 SiRNA (includes four SiRNAs directed to FADD and DR4) or control non-targeting scrambled SiRNA (Dharmacon, Lafayette CO) using DharmaFect transfection reagent. Briefly, Daoy cells were seeded and allowed to grow for 24 h in serum-containing media. The media was replaced with media lacking serum and antibiotics, but containing 100 nM of FADD or DR4 SiRNA or control non-target SiRNA and incubated for 24 h. Transfection efficiency was monitored by inclusion of siGLO and was determined to be around 80%.

TUNEL assay

Daoy cells were cultured in CC2 chamber slides and transfected with 100 nM of FADD siRNA or control scrambled siRNA. Twenty-four hours post-transfection, the cells were treated with 2.5 μ M MS-275 for an additional 24 h and TUNEL assay performed with a DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) as follows. Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100, followed by incubation with rTdT buffer (rTdT Enzyme, nucleotide mix and equilibration buffer) for 1 h. The nuclei were stained with 1 μ g/ml DAPI contained in Slowfade Gold[®] antifade reagent (Invitrogen, Eugene, OR) and immediately visualized using a Nikon fluorescence microscope. Images were analyzed using Metamorph software. Over one thousand cells were counted for each sample.

Chromatin immunoprecipitation (ChIP) assay

Untreated or MS-275 (2.5 μ M)- or Trichostatin A (TSA) (300 nM)-treated Daoy cells were cross linked with formaldehyde for 10 min at room temperature in cross-linking buffer (50 mM HEPES pH 8.0, 140 mM NaCl, 1 mM EDTA, 0.4% Igepal CA-630, 0.2% Triton X-100 and a cocktail of protease inhibitors). The cross-linking was then neutralized by addition of glycine and nuclei harvested by centrifugation. Nuclei were sonicated and centrifuged to remove cellular debris. The lysate was precleared with Protein-A and then incubated with either control non-immune serum (IgG) or anti-histone H3 antibody (Millipore, Danvers, MA) or anti-acetyl histone H4 antibody (Millipore, Danvers, MA) overnight at 4°C in buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and protease inhibitors. The beads were washed several times to remove non-specifically bound protein and then eluted using a buffer containing 2% SDS and 0.2 M sodium bicarbonate. After reversal of cross-linking, the bound DNA was purified and eluted using the QiaQuick PCR purification kit (Qiagen, Valencia, CA). Quantitative PCR amplification was done using the following primers:

DR4-1.2 Kb forward: 5'-GAGACGGAGTTTCCCTCTT GTGGG-3',

DR4-1.2 Kb reverse: 5'-GACATGGAGAAACGCAGC CTCTAC-3

DR4-0.7 Kb forward: 5'-TCTCCCGTGGTTTAAGGAC TTTCA-3

DR4-0.7 Kb reverse: 5'-CTGCTTTTGGTTTCAGGTC TCCAT-3

DR4-TS forward: 5'-CTGGGACCAAGTGGCAAAAC GAC-3

DR4-TS reverse: 5'-CTTCGCATTTCGGAGTTCAGG GC-3'.

Normalization and analyses were carried out as described previously.

Results

Analysis of DR4 and DR5 protein expression in human medulloblastoma samples

To investigate if death receptors were aberrantly expressed in medulloblastoma samples, we first carried out H&E staining of 27 medulloblastoma tumor samples in a TMA along with normal cerebella obtained from the M.D. Anderson Cancer Center Brain Tumor Tissue Bank. The tumor samples were determined to be morphologically different from the normal cerebella. H&E staining revealed the presence of granular cell neurons with uniform round nuclei in normal cerebella, whereas medulloblastoma tumor samples had densely packed tumor cells with irregular and hyperchromatic nuclei (Fig. 1a).

These samples were analyzed for changes in the expression of the TNF-family of death receptors, DR4 and DR5, by immunostaining and fluorescence microscopy. As seen in Fig.

1a, DR4 expression could not be detected in 95% of the tumor samples examined, whereas demonstrable staining (green) was observed in normal cerebellar samples. However, a majority of the samples (75–80%) showed DR5 staining that was comparable to normal cerebellum, while a few lacked DR5 staining. Mouse xenografts of human bladder cancers served as our positive control for both proteins. Staining observed in medulloblastoma tumor, normal cerebellum and bladder cancer is shown in Fig. 1a. Similar experiments carried out with Daoy and D283 cells showed DR4 expression to be completely absent in Daoy and D283 cells, whereas DR5 expression was observed in both cell lines (Fig. 1b). These results were also confirmed by Western blotting and flow cytometry in Daoy cells (Fig. 3b, c respectively). Thus, DR4 protein expression was absent in most human tumor samples and medulloblastoma cell lines examined, whereas, DR5 protein was present in 75–80% of tumor samples examined and in Daoy and D283 cells.

Analysis of *DR4*, *DR5* and *TRAIL* gene expression in human medulloblastomas

To further confirm our findings described above and to determine if the absence of death receptor expression was due to aberrant silencing of gene expression, we carried out QPCR experiments using RNA extracted from paraffin embedded tumor tissue and normal cerebellar tissue. We asked if the expression of gene encoding *DR4* was down regulated in tumor cells relative to normal cerebellar tissue. The expression of *DR5* and the ligand, *TRAIL*, was also determined in these samples. The levels of the house keeping gene, *GAPDH*, and that of *18S* ribosomal RNA was measured as controls for normalization of *DR4*, *DR5* and *TRAIL* gene expression.

The expression of *DR4* in tumor samples shown in Fig. 1c, is plotted relative to that in normal cerebellum which was set to 1. Interestingly, *DR4* expression was not detected in any of the 20 samples examined by QPCR. *DR5* expression could be detected in 1/20 (5%) of samples at levels that were the same or up to 3–4-fold elevated compared to normal human cerebellum. The results for *DR4* correlated well with our results in Fig. 1a. *TRAIL* expression relative to normal human cerebellum was up regulated and ranged from 3–400 fold in 8/20 (40%) of tumor samples.

We also carried out similar measurements in the human medulloblastoma cell lines, Daoy and D283. While *DR4* expression (normalized to the mean of *18S* RNA and *GAPDH*) was not detected in either cell line, *DR5* expression could be measured in Daoy and D283 medulloblastoma cell lines and was 10–20 fold elevated compared to normal human cerebellum (Fig. 1d). *TRAIL* gene was expressed in Daoy cells but not in D283 cells. These results were similar to that observed in human tumors where *DR4* expression was down regulated in all the tumor samples, whereas *DR5* and *TRAIL* displayed a more variable expression pattern. Thus, our results suggest that down regulated expression of *DR4* and *DR5* genes is a common feature of human medulloblastoma tumors and cell lines studied.

Effect of MS-275 on the growth of Daoy and D283 cell lines in vitro

To examine if aberrant histone deacetylation contributed to the silencing of *DR4* and *DR5* genes in Daoy and D283 cell lines, we studied the effects of the histone deacetylase inhibitor, MS-275, on the growth of Daoy and D283 cells. Both cells were treated with different concentrations of the drug (0.625–20 μ M) for various incubation periods (24–144 h). As shown in Fig. 2a, cell growth measured by MTT assays decreased as a function of concentration and time of exposure to the drug. Approximately 70% of Daoy cells were viable upon treatment with 0.625 μ M MS-275 for 48 h, whereas cell growth decreased to 25% with 2.5 μ M MS-275. Increasing the time of exposure to MS-275 for any given concentration caused a further decrease in cell growth. In general, D283 cells were more resistant to the effects of MS-275 compared to Daoy cells. D283 cells exposed to 0.625 μ M

MS-275 for 48 h did not exhibit a decline in cell growth and required up to 144 h of drug treatment or higher concentrations of MS-275 to observe a decrease in cell growth (Fig. 2a).

Effect of MS-275 on cell cycle distribution of Daoy cells

To assess the effects of MS-275 on the cell cycle distribution of medulloblastoma cells, we carried out flow cytometric analyses after treatment of Daoy and D283 cells with 2.5 μ M MS-275 for various time points (24–96 h). Treatment with MS-275 for 24 h caused an accumulation of cells with a sub-G1 DNA content (40%), whereas 8% untreated controls had a sub-diploid DNA content at the same time period. Longer exposure to the drug (48–96 h) resulted in a substantial elevation in the percentage of cells with a sub-diploid DNA content (65–90%). While 30% of untreated D283 cells had a sub-G1 DNA content, exposure to MS-275 resulted in a substantial accumulation of cells with degraded DNA, confirming that both Daoy and D283 cells were sensitive to the drug (Fig. 2b). Since MS-275 caused a decline in cell growth and induced DNA degradation in both Daoy and D283 cell lines, further analyses were carried out with Daoy cells that are more amenable to experimental analyses.

Induction of apoptosis in MS-275-treated Daoy cells

To further confirm activation of apoptosis in MS-275-treated cells, we measured PARP cleavage by Western blotting. As shown in Fig. 2c, cleaved PARP could be detected as early as 4 h and continued to increase at longer periods of incubation with the drug. Actin served as the loading control and remained unaffected upon MS-275 treatment. Drug activity was monitored by measuring the levels of acetylated histones H3 and H4 in drug-treated cells compared to untreated control cells using pan-acetyl histone antibodies. As seen in Fig. 2d, an increase in the levels of acetylated H3 could be discerned at about 8 h and remained elevated up to 24 h. While acetylation of histone H4 could not be detected in untreated cells, treatment with MS-275 promoted a significant increase in levels of acetylated histone H4. These results suggest that MS-275 inhibited HDAC activity and induced the acetylation of histones H3 and H4.

Evaluation of *DR4*, *DR5* and *TRAIL* gene and protein expression upon MS-275 treatment

Since *DR4* gene and protein expression was down regulated in human tumor samples and in Daoy cells and because MS-275 treatment promoted apoptosis in these cells, we asked if this occurred through drug-mediated remodeling of chromatin and reactivation of *DR4* gene expression in these cells. To this end, cDNA was prepared using RNA extracted from Daoy cells that were either untreated or treated with 2.5 μ M MS-275 and subjected to QPCR using commercially available Taqman primers for human *DR4*, *GAPDH* and *18S* rRNA were included as controls and for normalization. We also included *DR5*, and *TRAIL* in our analyses to evaluate if MS-275 had an effect on the expression of these genes. As shown in Fig. 3a, *DR4* and *DR5* gene expression were determined to be 17- to 63-fold, and 25- to 114-fold higher at 12 and 20 h, respectively, in drug-treated compared to untreated cells. Interestingly, MS-275 promoted a 4–6 fold increase in *TRAIL* expression in Daoy cells in the same period of drug treatment. These results suggest a potential regulation of *DR4*, *DR5* and *TRAIL* gene expression through histone acetylation-deacetylation in Daoy cells.

Western blotting, flow cytometry and immunofluorescence analyses were also used to measure the levels and subcellular localization of DR4 and DR5 proteins. Western blotting revealed that DR4 protein levels increased as early as 4 h and continued to be elevated up to 24 h in cells exposed to 2.5 μ M MS-275 (Fig. 3b). However, a similar increase in DR5 protein was not detected. Flow cytometric analyses using antibodies specific to human DR4 and DR5, demonstrated a substantial increase in cell surface staining of DR4 but a very small increase in DR5 staining upon MS-275 treatment compared to that with isotype

matched control antibodies or in untreated cells (Fig. 3c). Elevated expression of DR4 was also demonstrated by immunofluorescence assays carried out with Daoy cells treated in a similar manner (Fig. 3d).

Since, DR5 protein expression was only slightly increased in the presence of MS-275, we focused on understanding the mechanism by which MS-275 altered DR4 expression. To ask if the increase in DR4 expression occurred directly through changes in histone acetylation in the *DR4* gene, we carried out chromatin immunoprecipitation assays with extracts prepared from drug-treated cells or untreated controls and using antibodies against acetylated histones H3 and H4 or control IgG and primers designed to amplify the *DR4* promoter region between the transcription start site and 1.2 kb upstream. The amplification obtained with anti-acetyl H3 and H4 antibodies was expressed relative to that obtained with IgG controls. As seen in Fig. 3e, relative to untreated controls, MS-275 promoted a substantial increase in the acetylation of histone H3 at the transcription start site and 0.7 kb upstream. A similar pattern of increase in acetylated histone H3 was observed with another HDAC inhibitor, trichostatin-A (TSA) confirming that this change in histone H3 acetylation was due to inhibition of HDAC activity. Likewise, histone H4 acetylation was also significantly elevated in the *DR4* promoter in MS-275 and TSA-treated cells. Thus, our studies demonstrate that MS-275 reactivates *DR4* gene expression through acetylation of histones H3 and H4 at the *DR4* promoter.

TRAIL enhances MS-275-mediated apoptosis

To verify the activity of the upregulated death receptors, we monitored changes in the metabolic activity of Daoy cells treated with either 2.5 μ M MS-275 alone or coexposed to various concentrations of commercially available soluble TRAIL ligand for different time periods. Cells exposed to TRAIL alone were also included as controls. As shown in Fig. 4a, while treatment with MS-275 and TRAIL alone promoted a 10% and 2–5% decrease in metabolic activity respectively at 24 h, only 40% of tumor cells were metabolically active upon addition of 2.5, 5 or 10 ng of TRAIL and 0.625 μ M MS-275. A similar pattern of decline in metabolic activity was observed at 48 and 72 h of treatment. Interestingly, TRAIL alone caused a modest decline in cell growth of 25% at the highest concentration studied.

We also carried out Western blotting analyses with Daoy cells treated with either 0.312 μ M MS-275 or 2.5 ng TRAIL alone or with both agents together for 48 h. As shown in Fig. 4b, a substantial increase in PARP cleavage was observed in cells treated with both agents compared to either agent alone. These results were also confirmed by flow cytometry where treatment with 0.312 μ M or 0.625 μ M MS-275 and 2.5 ng TRAIL resulted in a sub-G1 DNA content in approximately 70% of Daoy cells at 48 h (Fig. 4c). In contrast, treatment with 0.312 μ M/0.625 μ M MS-275 or 2.5 ng TRAIL alone resulted in a sub-G1 DNA content in 10–15% and 7% of Daoy cells respectively. Thus, our results demonstrate enhancement of apoptosis in Daoy cells in the presence of both MS-275 and TRAIL.

Activation of Caspase-8 in MS-275 treated medulloblastoma cell lines

To confirm the activation of the extrinsic pathway in Daoy cells in the presence of MS-275, we determined the activation of Caspase-8 in drug-treated and untreated cells using a fluorogenic assay. Extracts prepared from Daoy cells treated with 2.5 μ M MS-275 for 24, 48 or 72 h and comparable untreated cells were incubated with the fluorogenic substrate, N-Acetyl-IETD-AMC. As shown in Fig. 5a, Caspase-8 activity showed a 2-fold increase as early as 24 h and continued to increase up to 6-fold relative to that in untreated cells at 72 h.

These results were also confirmed by Western blotting (Fig. 5b), where cleavage of pro-Caspase-8 to its active form was observed between 8 and 24 h. Interestingly, the levels of

pro-Caspase-8 protein itself was elevated upon MS-275 treatment. To determine if this elevation was due to an effect on the cognate gene expression, we carried out real-time PCR analyses with RNA prepared from untreated and MS-275 treated Daoy cells using Taqman primers specific for *CASP-8* gene. As seen in Fig. 5c, MS-275 treatment resulted in a 20- to 100-fold increase in *CASP-8* transcript in Daoy cells between 12 and 20 h of drug exposure.

Involvement of FAS associated death domain (FADD) associated death receptors in MS-275-induced apoptosis

To evaluate the contribution of upregulated *DR4* expression to MS-275-mediated apoptosis, we first asked if knocking down expression of FADD, a component of the death-inducing signaling complex (DISC), would perturb cell death in the presence of the drug. Daoy cells were transfected with a pool of commercially available siRNAs against *FADD* or control scrambled siRNA and the decrease in FADD protein expression studied by Western blotting using extracts prepared from cells treated with 2.5 μ M MS-275 for various time periods. As shown in Fig. 6a, FADD protein could be detected at all time points in scrambled siRNA treated cells. In contrast, FADD siRNA knocked down FADD protein as early as 24 h.

Once FADD knock down was established, we performed TUNEL assays to measure apoptosis in Daoy cells treated as above. Shown in Fig. 6b, is a representative field of cells captured with 10X (top panel) and 40X (bottom panel) objectives respectively. We observed that in control cells, approximately 74% of cells were TUNEL positive upon MS-275 treatment. In contrast, only 8.5% of MS-275 treated cells were TUNEL positive in cells where FADD was down regulated, suggesting that FADD-dependent death receptors such as DR4 were important for mediating apoptosis in response to MS-275 treatment.

To further confirm the relevance of elevated *DR4* expression to MS-275-mediated apoptosis, we transfected Daoy cells with scrambled siRNA or *DR4*-specific siRNA and first measured the efficiency of knockdown by Western blotting analyses. As shown in Fig. 6c, a substantial decrease in protein expression was observed in cells transfected with *DR4*-specific siRNA, but not in control siRNA transfected cells. Next, the effect of *DR4* knockdown on PARP cleavage in MS-275 treated cells was evaluated by Western blotting analyses. As demonstrated in Fig. 6d, DR4 knockdown caused a large decline in PARP cleavage, whereas measurable cleaved PARP was observed in control scrambled siRNA treated cells.

Taken together, these results suggest that the upregulation of *DR4* in the presence of MS-275 is important for drug-induced apoptosis. Our studies also suggest a role for aberrant histone deacetylation in the silencing of death receptors in medulloblastoma cell lines.

Discussion

Apoptosis or programmed cell death is critical for the maintenance of cellular homeostasis. This pathway is often perturbed in tumors and results in evasion of apoptosis in response to chemotherapy and radiation treatment, two mainstays of cancer therapy. In medulloblastoma, there is growing evidence that inactivation of the extrinsic pathway contributes to poor therapeutic response [9–15]. For example, recent studies demonstrated down regulation of *CASP-8* gene expression in approximately 62–75% of medulloblastoma tumor samples examined [9, 11–14]. This absence or decrease in *CASP-8* mRNA expression was strongly associated with deletion or methylation mediated silencing of the gene [11–14]. Furthermore, loss of *CASP-8* expression correlated with an unfavorable survival outcome in

childhood medulloblastomas [9]. These findings suggest an important role for Caspase-8 and the extrinsic pathway in inducing apoptosis in medulloblastoma tumor cells.

However, other molecular aberrations in the extrinsic pathway have not been carefully examined in medulloblastoma samples. A study by Zuzak et al. [10], showed that the expression of TRAIL-dependent death receptor, *DR4* gene, was down regulated in a number of human primitive neuroectodermal tumor samples and could be detected in only 8% of tumor tissues. The observation that *DR4* gene and protein expression is absent or severely impaired in all of the samples we analyzed and in both Daoy and D283 cell lines in the current study is consistent with the above observations of Zuzak et al. [10]. In our experiments, *DR5* gene expression was more variable and was absent or down regulated in comparison to normal brain cerebellar tissue, by quantitative RT-PCR in approximately 95% of the samples we examined. However, immunohistochemical analyses showed expression of *DR5* in 80–90% of the tumor samples analyzed. This difference in experimental outcome could be explained by technical issues arising from optimizing amplification conditions for RNA extracted from archival formalin fixed paraffin-embedded brain tumor samples [15, 16]. Alternatively, it is possible that *DR5* gene expression in tumor samples could be affected through a decrease in mRNA stability. The dependence of mRNA stability and degradation on AUUUA-rich elements present within their 3 untranslated regions is well established [19]. Our finding that the MS-275-associated increase in *DR5* gene expression did not result in a corresponding increase in protein levels suggest that *DR5* could also be regulated by post-transcriptional mechanisms such as protein stability or degradation.

We are currently determining the implications of downregulated *DR4* and *DR5* gene expression for survival or therapeutic outcome for patients with medulloblastoma. For example, the expression of death receptors, *DR4* and *DR5* in other brain tumors such as glioblastoma are independent prognostic factors and are positively correlated with patient survival [20].

Interestingly, the mechanism by which *DR4* expression was silenced was not determined in the afore-mentioned study by Zuzak et al. [10]. The fact that the HDAC inhibitor, MS-275, could reactivate and upregulate the expression of both *DR4* and *DR5* in Daoy cells suggests that aberrant histone deacetylase activity and chromatin remodeling may contribute to the silencing of both genes in these cells and potentially in tumor samples. This is in contrast to other cancers like neuroblastoma, melanoma, ovarian, prostate and bladder cancer, where *DR4* expression is silenced by DNA methylation [21–25]. However, it has been shown that histone deacetylation can occur prior to DNA methylation during silencing of gene expression, and a similar mechanism may operate for *DR4* and *DR5* silencing in medulloblastoma tumors [26].

In our experiments, we observed a 60- and 114-fold upregulation of *DR4* and *DR5* expression respectively after 20 h of treatment with MS-275, whereas a 4-fold increase in TRAIL expression was obtained. Our results suggest that either the small increase in TRAIL levels was sufficient to engage the elevated *DR4* and *DR5* receptors or that ligand-independent clustering could account for all or part of the apoptotic response in MS-275 exposure. Similar ligand-independent effects have been shown in several human cell lines upon exposure to cytotoxic drugs [27]. A previous study has also shown a synergistic increase in TRAIL-associated cytotoxicity in the presence of HDAC inhibitors, however the mechanism of synergy was not examined [28]. Our studies demonstrate that this synergy may result from an increase in *DR4* levels. The role of *DR5* is less clear since the increase in *DR5* gene expression in the presence of MS-275 did not result in a corresponding increase in protein levels. Also, the addition of TRAIL to Daoy cells caused only a modest increase in

apoptosis (5–10%) in the absence of MS-275, suggesting that the receptors were non-functional.

Although *CASP-8* gene expression was also increased in the presence of MS-275, the decline in TUNEL staining upon FADD knock down suggests that molecular players upstream of FADD such as DR4, DR5 and FAS were important for the induction of apoptosis in the presence of MS-275. *FAS*, could also be potentially reactivated in our experiments, however, our observation that TRAIL, the agonist for DR4 and DR5, promoted a significant decrease in metabolic activity in Daoy cells indicates that the engagement of these receptors may be more important for MS-275-mediated apoptosis in these cells. This conclusion is also supported by our findings that knockdown of *DR4* attenuated PARP cleavage in drug-treated Daoy cells. Also, a previous study showed that the FAS receptor and ligand were expressed in several medulloblastoma samples studied, yet a significant resistance to the topoisomerase II inhibitor, etoposide, was observed, suggesting that non-FAS dependent mechanisms played a more important role in inducing apoptosis in medulloblastoma cells [16].

Finally, the observation that both MS-275 and TSA changed the acetylation status of histones H3 and H4 in the DR4 promoter region suggest that these changes were due to inhibition of HDAC activity in these cells. The DR4 promoter houses binding sites for NF-kappa B, AP1 and p53, whether these transcription factors play a role in the MS-275 and TSA-associated reactivation of *DR4* expression remains to be determined [29–31].

The apparent reversibility of epigenetic silencing of gene expression is of clinical relevance since targeting enzymes involved in the reactivation of the epigenome have emerged as powerful and novel approaches to cancer therapy. Several studies have demonstrated a potential for the use of HDAC inhibitors in pediatric brain tumors [32–41]. The HDAC inhibitors, Valproic acid (VPA), suberoylanilide hydroxamic acid (SAHA) and MS-275 are of particular interest since these agents have been shown to cross the blood-brain barrier and have acceptable toxicity profiles [34, 35, 37–39]. An in vitro study comparing the growth inhibitory properties of various HDACIs on several embryonal tumors of the nervous system revealed SAHA and MS-275 to be more potent compared to VPA in inducing apoptosis [36]. These HDAC inhibitors have also demonstrated ability to potentiate the effects of radiation and therapeutic agents such as gamma interferon [28, 38–41]. Early clinical trials with SAHA and MS-275 have demonstrated acceptable toxicity and similar trials for pediatric patients are currently underway (www.clinicaltrials.gov).

Since TRAIL targets tumor cells, leaving normal cells largely unaffected, the use of TRAIL for cancer therapy, especially brain tumors is of growing clinical interest [42]. However, many tumors including medulloblastoma, as shown in our study, down regulate the death receptors that bind TRAIL, and these cancers will offer resistance to TRAIL treatment. Therefore, agents such as HDAC inhibitors that reactivate death receptor expression have the potential to sensitize cancer cells to TRAIL and offer a novel approach to medulloblastoma therapy.

Although our experiments have focused on FADD-dependent extrinsic pathways in the induction of apoptosis by MS-275, we cannot exclude a potential role for the intrinsic pathway in this process. The role of post-transcriptional mechanisms such as acetylation of non-histone proteins in MS-275-induced cytotoxicity also remains to be evaluated.

In summary, our study has shown that human medulloblastoma samples and cell lines have down regulated expression of the death receptor, *DR4*. Interestingly, our finding that the expression of *DR4* and *DR5* could be upregulated by the HDAC inhibitor, MS-275, suggests that aberrant HDAC activity in tumors may promote evasion of apoptosis by silencing the

expression of these pro-apoptotic molecules. Also, our observation that soluble TRAIL potentiates the cytotoxic effects of MS-275 indicates that further studies in animal models to test the in vivo efficacy of these agents and for potential clinical application in children with medulloblastoma are warranted.

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Abbreviations

FADD	FAS-associated death domain
MTT	3-(4, 5 dimethylthiazole-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-Tetrazolium
TRAIL	TNF-related apoptosis-inducing ligand
MS-275	N-(2-aminophenyl)-4-[(N-pyridine-3-ylmethoxycarbonyl) aminomethyl] Benzamide

References

1. Louis DN, Ohgaki H, Wiestler OD, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* 2007; 114:97–109. [PubMed: 17618441]
2. Eberhart CG, Burger PC. Anaplasia and grading in medulloblastomas. *Brain Pathol.* 2003; 13:376–385. [PubMed: 12946027]
3. Packer RJ, Goldwein J, Nicholson HS, et al. Treatment of children with medulloblastomas with reduced-dose craniospinal radiation therapy and adjuvant chemotherapy: a Children's Cancer Group Study. *J Clin Oncol.* 1999; 17:2127–2136. [PubMed: 10561268]
4. Askenazi A, Dikshit VM. Death receptors: signaling and modulation. *Science.* 1998; 281:1305–1308. [PubMed: 9721089]
5. Ozoren N, El-Deiry WS. Cell surface death receptor signaling in normal and cancer cells. *Semin Cancer Biol.* 2003; 13:135–147. [PubMed: 12654257]
6. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov.* 2006; 5:769–784. [PubMed: 16955068]
7. Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. Histone deacetylases and cancer: causes and therapies. *Nat Rev Cancer.* 2001; 1:194–202. [PubMed: 11902574]
8. Esteller M. Epigenetics in cancer. *N Engl J Med.* 2008; 358:1148–1159. [PubMed: 18337604]
9. Pingoud-Meier C, Lang D, Janss AJ, et al. Loss of caspase-8 protein expression correlates with unfavorable survival outcome in childhood medulloblastoma. *Clin Cancer Res.* 2003; 9:6401–6409. [PubMed: 14695141]
10. Zuzak TJ, Steinhoff DF, Sutton LN, Phillips PC, Eggert A, Grotzer MA. Loss of caspase-8 mRNA expression is common in childhood primitive neuroectodermal brain tumour/ medulloblastoma. *Eur J Cancer.* 2002; 38:83–91. [PubMed: 11750844]
11. Ebinger M, Senf L, Wachowski O, Scheurlen W. Promoter methylation pattern of caspase-8, P16INK4A, MGMT, TIMP-3, and E-cadherin in medulloblastoma. *Pathol Oncol Res.* 2004; 10:17–21. [PubMed: 15029256]
12. Gonzalez-Gomez P, Bello MJ, Inda MM, et al. Deletion and aberrant CpG island methylation of caspase 8 gene in medulloblastoma. *Oncol Rep.* 2004; 12:663–666. [PubMed: 15289853]

13. Lindsey JC, Lusher ME, Anderton JA, et al. Identification of tumour-specific epigenetic events in medulloblastoma development by hypermethylation profiling. *Carcinogenesis*. 2004; 25:661–668. [PubMed: 14688019]
14. Fulda S, Debatin KM. 5-Aza-2 -deoxycytidine and IFN-gamma cooperate to sensitize for TRAIL-induced apoptosis by upregulating caspase-8. *Oncogene*. 2006; 25:5125–5133. [PubMed: 16607283]
15. Muhlich J, Bajanowski T, Rickert CH, et al. Frequent but borderline methylation of p16 (INK4a) and TIMP3 in medulloblastoma and sPNET revealed by quantitative analyses. *J Neurooncol*. 2007; 83:17–29. [PubMed: 17206475]
16. Riffkin CD, Gray AZ, Hawkins CJ, Chow CW, Ashley DM. Ex vivo pediatric brain tumors express Fas (CD95) and FasL (CD95L) and are resistant to apoptosis induction. *Neurooncol*. 2001; 3:229–240.
17. Antonov J, Goldstein DR, Oberli A, et al. Reliable gene expression measurements from degraded RNA by quantitative real-time PCR depend on short amplicons and a proper normalization. *Lab Invest*. 2005; 85:1040–1050. [PubMed: 15951835]
18. Sinnapah-Kang ND, Mrak RE, Paulsen DB, Marchetti D. Heparanase expression and TrkC/p75NTR ratios in human medulloblastoma. *Clin Exp Metastasis*. 2006; 23:55–63. [PubMed: 16826429]
19. Chen CY, Shyu AB. AU Rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci*. 1995; 20:465–470. [PubMed: 8578590]
20. Kuijlen JM, Mooij JJ, Platteel I, et al. TRAIL-receptor expression is an independent prognostic factor for survival in patients with a primary glioblastoma multiforme. *J Neurooncol*. 2006; 78:161–171. [PubMed: 16544055]
21. Horak P, Pils D, Haller G, et al. Contribution of epigenetic silencing of tumor necrosis factor-related apoptosis inducing ligand receptor 1 (DR4) to TRAIL resistance and ovarian cancer. *Mol Cancer Res*. 2005; 3:335–343. [PubMed: 15972852]
22. van Noesel MM, van Bezouw S, Voute PA, Herman JG, Pieters R, Versteeg R. Clustering of hypermethylated genes in neuroblastoma. *Genes Chromosomes Cancer*. 2003; 38:226–233. [PubMed: 14506696]
23. van Geelen CM, deVries EG, Le TK, van Weeghel RP, deJong S. Differential modulation of the TRAIL receptors and CD95 in colon carcinoma. *Br J Cancer*. 2003; 89:363–373. [PubMed: 12865931]
24. Suzuki M, Shigematsu H, Shivapurkar N, et al. Methylation of apoptosis related genes in the pathogenesis and prognosis of prostate cancer. *Cancer Lett*. 2006; 242:222–230. [PubMed: 16458425]
25. Bae SI, Cheriya V, Jacobs BS, Reu FJ, Borden EC. Reversal of methylation silencing of Apo2L/TRAIL receptor 1 (DR4) expression overcomes resistance of SK-MEL-3 and SK-MEL-28 melanoma cells to interferons (IFNs) or Apo2L/TRAIL. *Oncogene*. 2008; 27:490–498. [PubMed: 17653094]
26. Bachman KE, Park BH, Rhee I, et al. Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell*. 2003; 3:89–95. [PubMed: 12559178]
27. Boldin MP, Mett IL, Varfolomeev EE, et al. Self-association of the “death domains” of the p55 tumor necrosis factor (TNF) receptor and Fas/APO1 prompts signaling for TNF and Fas/APO1 effects. *J Biol Chem*. 1995; 270:387–391. [PubMed: 7529234]
28. Sonneman J, Kumar KS, Heesch S, et al. Histone deacetylase inhibitors induce cell death and enhance susceptibility to ionizing radiation, etoposide and TRAIL. *Int J Oncol*. 2006; 28:755–766. [PubMed: 16465382]
29. Ravi R, Bedi GC, Engstrom LW, Zeng Q, Mookerjee B, Gelinas C, Fuchs EJ, Bedi A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-kappaB. *Nat Cell Biol*. 2001; 3:409–416. [PubMed: 11283615]
30. Guan B, Yue P, Lotan R, Sun SY. Evidence that human death receptor protein 4 is regulated by activator protein 1. *Oncogene*. 2002; 21:3121–3129. [PubMed: 12082627]
31. Liu X, Yue P, Khuri FR, Sun SY. p53 Upregulates death receptor 4 expression through an intronic p53 binding site. *Cancer Res*. 2004; 64:5078–5083. [PubMed: 15289308]

32. Li XN, Shu Q, Su JM, Perlaky L, Blaney SM, Lau CC. Valproic acid induces growth arrest, apoptosis, and senescence in medulloblastomas by increasing histone hyperacetylation and regulating expression of p21Cip1, CDK4, and CMYC. *Mol Cancer Ther.* 2005; 4:1912–1922. [PubMed: 16373706]
33. Jaboin J, Wild J, Hamidi H, et al. MS-27-275, an inhibitor of histone deacetylase, has marked in vitro and in vivo antitumor activity against pediatric solid tumors. *Cancer Res.* 2002; 62:6108–6115. [PubMed: 12414635]
34. Shu Q, Antalffy B, Su JM, et al. Valproic acid prolongs survival time of severe combined immunodeficient mice bearing intracerebellar orthotopic medulloblastoma xenografts. *Clin Cancer Res.* 2006; 12:4687–4694. [PubMed: 16899619]
35. Graham C, Tucker C, Creech J, et al. Evaluation of the antitumor efficacy, pharmacokinetics, and pharmacodynamics of the histone deacetylase inhibitor depsipeptide in childhood cancer models in vivo. *Clin Cancer Res.* 2006; 12:223–234. [PubMed: 16397046]
36. Kumar KS, Sonnemann J, Beck JF. Histone deacetylase inhibitors induce cell death in supratentorial primitive neuroectodermal tumor cells. *Oncol Rep.* 2006; 16:1047–1052. [PubMed: 17016591]
37. Spiller SE, Ravanpay AC, Hahn AW, Olson JM. Suberoylanilide hydroxamic acid is effective in preclinical studies of medulloblastoma. *J Neurooncol.* 2006; 79:259–270. [PubMed: 16645722]
38. Camphausen K, Cerna D, Scott T, et al. Enhancement of in vitro and in vivo tumor cell radiosensitivity by valproic acid. *Int J Cancer.* 2005; 114:380–386. [PubMed: 15578701]
39. Camphausen K, Scott T, Sproull M, Tofilon PJ. Enhancement of xenograft tumor radiosensitivity by the histone deacetylase inhibitor MS-275 and correlation with histone hyperacetylation. *Clin Cancer Res.* 2004; 10:6066–6071. [PubMed: 15447991]
40. Das CM, Aguilera D, Vasquez H, et al. Valproic acid induces p21 and topoisomerase-II (alpha/beta) expression and synergistically enhances etoposide cytotoxicity in human glioblastoma cell lines. *J Neurooncol.* 2007; 85:159–170. [PubMed: 17534580]
41. Banuelos CA, Banath JP, MacPhail SH, et al. Radiosensitization by the histone deacetylase inhibitor PCI-24781. *Clin Cancer Res.* 2007; 13:6816–6826. [PubMed: 18006784]
42. Nagane M, Huang HJ, Cavenee WK. The potential of TRAIL for cancer chemotherapy. *Apoptosis.* 2001; 6:191–197. [PubMed: 11388668]

Fig. 1.

Expression of *DR4*, *DR5* and *TRAIL* in human medulloblastomas. Immunostaining to measure expression of *DR4*, *DR5* and *TRAIL* in (a) human medulloblastoma tissue microarray and (b) in DAOY and D283 cells. Tumor samples and normal cerebella were stained with H&E. Additional sections were stained with polyclonal rabbit antibodies against DR4, DR5 and TRAIL followed by Alexa 488-conjugated goat anti-rabbit secondary antibodies. Nuclei were stained with DRAQ-5 and sections were visualized using a Nikon Fluorescence microscope. Quantitative RT-PCR to measure gene expression in (c) medulloblastoma tumor samples and (d) DAOY and D283 medulloblastoma cell lines. Total RNA was extracted from paraffin embedded tumor sections or normal cerebella (nch) and DAOY/D283 cell lines and quantitated using a two-step real-time PCR method using Taqman primers specific for human *DR4*, *DR5*, *TRAIL*, *GAPDH* and *18s* ribosomal RNA (rRNA). The signal intensity in tumor samples was normalized to the gene mean of *GAPDH* and *18s*RNA.

Fig. 2. MS-275 Decrease the viability of medulloblastoma cell lines in vitro. (a) Daoy cells and (b) D283 cells were treated with various concentrations of MS-275 ranging from 0.625 to 20 μ M for increasing time periods of 24, 48, 72, 96 and 144 h. Metabolic activity (as a measure of cell growth) in drug treated cells was determined using MTT assays and normalized to that obtained with untreated controls for each time point. The data was analyzed using Softmax software and results expressed as percentages relative to untreated cells. All experiments were performed in quadruplets. (b) Cell cycle distribution of Daoy and D283 cells treated with 2.5 μ M of MS-275 for 24, 48, 72 and 96 h was determined by flow

cytometric analyses following staining with 0.05 mg/ml propidium iodide. One hundred thousand events were analyzed for each cell sample. Values represent the means $\pm$ SD for at least 3 different experiments. **(c)** Induction of PARP cleavage in Daoy cells upon exposure to MS-275 was demonstrated by Western blot analyses using antibodies that recognize the cleaved 89 kDa PARP protein. Pan-Actin (45 kDa) was used as a loading control. **(d)** Inhibition of HDAC activity was determined by measuring the levels of acetylated histones H3 and H4 relative to total H3 and H4 proteins using anti-AcH3, -AcH4, -H3 or -H4 antibodies

Fig. 3.

MS-275 Increases DR4, DR5 and TRAIL expression in Daoy cells. (a) Quantitative PCR assays to measure *DR4*, *DR5* and *TRAIL* and *18S* rRNA were carried out with RNA extracted from Daoy cells that were either untreated or treated with 2.5 μ M MS-275 for 12, 16, 20 and 24 h. Data were normalized to *18S* rRNA levels. Values represent mean $\pm$ SD based on mean Ct values of triplicates. (b) Western blotting and (c) Immunofluorescence assays to detect DR4 and DR5 expression upon treatment of Daoy cells with 2.5 μ M MS-275 for 4, 8 and 24 h. Daoy cells were treated with 2.5 μ M MS-275 for 24 h and stained with polyclonal anti-human DR4 and DR5 antibodies, followed by Cy3-conjugated goat anti-mouse secondary antibodies. Stained cells were covered with slow-fade anti-fade solution containing 1 μ g/ml DAPI and the images were visualized using a Nikon Fluorescence microscope. (d) Flow Cytometry to measure cell surface expression of FAS, DR4 and DR5 using PE-labeled antibodies. Cells stained with isotype matched anti-human IgG antibodies served as controls. (e) Chromatin Immunoprecipitation Assay to measure histone acetylation in the *DR4* promoter upon treatment with MS-275 and TSA. Daoy cells were treated with 2.5 μ M MS-275 or 300 nM TSA for 10 h and extracts subjected to immunoprecipitation and quantitative PCR analyses as described in “Materials and methods”. Control immunoprecipitation with extracts prepared from untreated cells were also carried out. Normalization was done by subtracting values obtained from reactions carried out with nonimmune serum

Fig. 4.

Activity of TRAIL-dependent receptors in Daoy cells exposed to MS-275. (a) Daoy cells were treated with either a sub-toxic concentration of MS-275 (0.625 μ M) alone or in conjunction with various concentrations of soluble TRAIL (2.5–10 ng) for 24, 48 and 72 h. Cells treated with TRAIL alone (2.5–10 ng) were as controls. Decrease in metabolic activity was determined using MTT assays and normalized using absorbance obtained using extracts from untreated cells. (b) Western blotting to measure PARP cleavage in untreated Daoy cells or cells treated with 0.312 μ M MS-275 alone, 2.5 ng TRAIL alone or both agents together was carried out using antibodies specific to cleaved PARP. (c) Flow cytometry to measure the DNA content of Daoy cells treated with 0.312 or 0.625 μ M MS-275 alone, 2.5

ng TRAIL alone or both agents together was carried out as described in ‘‘Materials and methods’’

Fig. 5. Increase in Caspase-8 activity in MS-275 treated Daoy Cells. **(a)** Activation of Caspase-8 was in Daoy cells treated with 2.5 μ M MS-275 was studied using a fluorophore-conjugated caspase-like enzyme substrate at a final concentration of 50 μ M. Fluorescence emitted upon cleavage of substrate was measured at an emission wavelength of 460 nm. **(b)** Cleavage of pro-Caspase-8 was demonstrated by Western blotting with extracts prepared from Daoy cells treated with 2.5 μ M MS-275 for various time periods. Antibodies specific to human Caspase-8 or the loading control, pan-Actin were used to probe protein expression. **(c)** Real-time Quantitative PCR assays to measure the expression *CASP-8* and *18S* rRNA were

carried out with RNA extracted from Daoy cells that were either untreated or treated with 2.5 μ M MS-275 for various time periods. Data were normalized to *18S* rRNA levels. Values represent mean $\pm$ SD based on mean Ct values of triplicates

Fig. 6. Knock-down of FADD and DR4 Attenuates MS-275 mediated Apoptosis in Daoy Cells. **(a)** Extracts prepared from Daoy cells transfected with Smartpool SiRNA against human FADD or scrambled SiRNA were subjected to Western blotting using anti-human FADD antibodies. **(b)** TUNEL Assays were performed with Daoy cells transfected with Smartpool SiRNA against human FADD or scrambled SiRNA. TUNEL staining was visualized using a Nikon Fluorescence microscope and analyzed using Metamorph Software (10 $\times$ magnification, *top panel*, 40 $\times$ magnification, *bottom panel*). **(c)** Extracts prepared from Daoy cells transfected with Smartpool SiRNA against human DR4 or scrambled SiRNA for 48 h were subjected to Western blotting using anti-human DR4 antibodies. **(d)** Apoptosis in MS-275 treated cells after transfection of DR4 SiRNA or scrambled SiRNA was determined by measuring PARP cleavage by Western blotting analyses using antibodies specific to cleaved PARP