

Histone Deacetylase (HDAC) Inhibition Induces Growth Arrest and Apoptosis in Human Medulloblastoma Cells

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Abstract

Medulloblastoma is the most common brain tumor of childhood. Inhibitors of histone deacetylase (HDAC) are a promising new class of antineoplastic agents with the ability to induce apoptosis and growth arrest of cancer cells. This study was designed to investigate the anticancer efficacy of MI-192, a novel selective HDAC inhibitor, on the two human medulloblastoma cell lines, DAOY and SHS-Y5Y. Acute exposure to MI-192 was largely effective in suppressing cell expansion, proliferation, and subsequent induction of apoptosis in both DAOY and SHS-Y5Y cells in a time- and dose-dependent manner. SHS-Y5Y cells are more responsive to this compound than DAOY cells, which may be dependent on their original state of differentiation. At 24-hours of exposure, cell proliferation, but not cell expansion was significantly inhibited in both DAOY and SHS-Y5Y medulloblastoma cells. Washout experiments showed that MI-192 was most efficient with 50% of cell expansion and proliferation inhibition values after 48- and 72-hours ranging from $0.58 \pm 0.09 \mu\text{M}$ in SHS-Y5Y cells to $0.83 \pm 0.18 \mu\text{M}$ in DAOY cells. Our findings suggest that HDAC selective inhibitor MI-192 may provide a novel avenue to treat medulloblastoma as monotherapy.

Key words: Medulloblastomas; Epigenetics; Histone Deacetylases; Cell Proliferation; Apoptosis

Introduction

Neoplasms of the central nervous system (CNS) rank second only to leukemia in incidence among children (Sonnenmann *et al.*, 2010). Medulloblastoma is the most common embryonal tumors of the CNS of childhood, especially among those < 15 years of age (Packer *et al.* 1999). It is a highly malignant neoplasia. Despite aggressive multimodality treatment with surgery, ionizing radiation, and chemotherapy, more than a third of children with medulloblastoma dies from the disease within 5 years of diagnosis (Packer *et al.* 1999). Survivors of medulloblastoma cells commonly have severe treatment-induced neurocognitive sequelae (Mulhern *et al.* 2004). Therefore, more effective treatment strategies aimed to improve the chance of survival and reduce therapy-related long-term side-effects are urgently needed for this disease.

Several studies reported that manipulation of the epigenetic events may offer novel cure options. The two most crucial mechanisms of epigenetic modulation are DNA methylation and histone deacetylation, which are closely associated. Histone acetyltransferases (HATs) add acetyl groups and histone deacetylases (HDACs) remove acetyl groups from lysine residues in proteins. These enzymes play pivotal roles in epigenetic regulation of gene transcription by remodelling chromatin structure (Kornberg and Lorch, 1999). Histone acetylation causes a weaker association between DNA and histones, promoting a more open, more accessible chromatin structure. However, histone deacetylation causes a tighter association between the DNA and histones, promoting a more compact, less accessible chromatin conformation for transcriptional

machinery to initiate transcription (Kornberg and Lorch, 1999). There are four classes of HDACs: class I HDACs (1, 2, 3 and 8) are found within the cell nucleus where they can deacetylate histones; class II HDACs (4, 7, 9 and 10) shuttle between the nucleus and the cytoplasm as well as histones, they also deacetylate cytoplasmic proteins such as the microtubules; class III HDACs, also known as the sirtuins, couple deacetylation to NAD⁺ hydrolysis, and the single member of class IV HDACs, HDAC11, has features in common with both class I and II HDACs (Gregoret *et al.*, 2004).

Dysregulation of epigenetic processes such as acetylation have been shown to be crucial in the progression and development of tumorigenesis. Histones but also non-histone proteins like p53 and c-Myc can be acetylated. HDACs are shown to be highly expressed in various cancers and high expression of HDACs is associated with reduced patient survival. In clinical studies many classes of HDAC inhibitors demonstrated potent anticancer activities with remarkable tumor specificity, such as neuroblastoma (Glick *et al.*, 1999), breast cancer (Schmidt *et al.* 1999), prostate cancer (Butler *et al.* 2000), and renal cancer (Park *et al.* 2003).

Subsequently, they have been shown to prevent proliferation, activate differentiation, and/ or induce apoptosis of tumor cells. Inhibitors of HDACs function by inhibiting histone deacetylases, resulting in the accumulation of acetylated histones, which in turn leads to an increase in transcriptionally active chromatin (Johnstone, 2002). As a consequence, they reactivate



the gene expression of dormant tumor suppressor genes, such as CDKN1A (p21) (Blagosklonny *et al.* 2002). However, the molecular basis underlying their anticancer action is not fully understood.

Studies on mice xenograft models of human neoplasia showed anticancer effect of HDAC inhibitors on colorectal, lung, pancreatic and ovarian cancer (Saito *et al.*, 1999) as well as prostate cancer (Butler *et al.* 2000). Most notably, they have been demonstrated both *in vitro* and *in vivo* to affect cancer cells while leaving normal cells comparatively unscathed (Butler *et al.*, 2000). The clinical potential of these agents has been reported by various Phase I trials of different inhibitors of HDACs in patients with solid tumors or leukemias (Kelly *et al.* 2005; Sandor *et al.* 2002).

Many HDAC inhibitors (e.g. MS-275, phenylbutyrate, phenylacetate) have been assessed in brain tumors in many studies (Appelskog *et al.* 2004; Camphausen *et al.* 2005; Jaboin *et al.* 2002; Li *et al.* 2004; Sonnemann *et al.* 2010). It has been reported that HDAC inhibitors can synergize with other drugs in the up-regulation of pro-survival and anti-apoptotic genes, including heat shock protein-70 (HSP70) (Marinova *et al.* 2009) and Bcl-2 (Leng *et al.* 2010). The distinct effects of inhibitors of HDACs on medulloblastoma and neuroblastoma cells in culture accelerate the investigation into the effect of these substances might have on other malignances of the CNS in childhood.

Many studies have shown that the compound that selectively inhibits HDAC2 and 3, but not HDAC1 could be therapeutically better for treating brain tumors than the currently available non-selective general class I and II HDAC inhibitors. These studies reveal that there could be therapeutic potential from selectively inhibiting HDAC2 and 3. The University of Leeds developed the HDAC selective inhibitor MI-192 (Gillespie *et al.* 2011). This study was undertaken to evaluate *in vitro* the anti-tumour efficacy of MI-192 on human medulloblastoma cell lines using several assays. Our results showed that this compound significantly suppressed cell expansion in DAOY and SHS-Y5Y medulloblastoma cell lines. Even more importantly, we demonstrate that this selective HDAC inhibitor MI-192 is also capable of reducing the cell proliferation activity in these two human medulloblastoma cell lines.

Materials and methods

HDAC Inhibitor MI-192

MI-192 was dissolved in 1% dimethyl sulfoxide (DMSO, Sigma) to make a 10 μ M stock solution. Final concentrations ranging from 0.1 to 3 μ M were prepared by serial dilutions in the culture media.

Cell Line Cultures

DAOY and SHS-Y5Y medulloblastoma cells were a gift from Dr. Ian C. Wood (Leeds, UK). The cell suspension (2000 cells/ 100 μ l/ well) were seeded into 96-well plates. The cells were maintained in normal culture medium and allowed overnight at 37 °C in a humidified atmosphere with 5% CO₂ to reach exponential growth before 100 μ l of culture medium

containing the drug at different concentrations were added. Each drug concentration was tested in 4 replicate wells. The cell line cultures were routinely maintained in DMEM (Dulbecco's Modified Eagle Medium, Invitrogen) supplemented with 10% fetal bovine serum (Sigma) and 2% glutamine (Invitrogen). The cell cultures were routinely passaged (sub-cultured) approximately every 3 days when the cells were 70% confluent. Cells were regularly inspected to be free of mycoplasma. At 24, 48 and 72 hours of exposure, cell expansion was characterized by cell viability using trypan blue exclusion test.

Cell Proliferation Assay

Proliferative activity of DAOY and SHS-Y5Y medulloblastoma cell lines *in vitro* was characterized by using Colorimetric Immunoassay Kit (Roche) for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis. In brief, medulloblastoma cell lines in log-phase of growth were harvested by trypsinization, resuspended at a concentration of 2000 viable cells/180 μ l, and replated into 96-well plates. After 24 hours, the medium was replaced with media containing BrdU labelling solution (1: 10) and the drug at different concentrations were then added. The cells were centrifuged before replacing medium to also retain the population of cells in suspension. Each drug concentrations: 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 μ M MI-192 was tested in 4 replicate wells. After 24 hours, cell proliferation was measured.

To study the reversibility of the antiproliferation effects of MI-192, washout experiments were done at various time points (48 and 72 hours). Media containing MI-192 were removed, cells washed and the media replaced with MI-192-free DMEM on the first 24 hours, and cells maintained in drug-free medium for the remaining duration of the experiment. The cell proliferation was determined by measuring the absorbance of the specimen in an ELISA plate reader at 450 nm using FLUOstar Omega (MBG LABTECH). The measurements were carried out within 5 minutes after adding stop solution.

Apoptosis Measurement

The cell lines were seeded into 96-well plates (2000 cells/ 190 μ l/ well). The cells were allowed overnight at 37°C in normal culture medium to reach exponential growth before 10 μ l of culture medium containing the annexin-v (3 μ g/ ml) (1: 50) and the drug at different concentrations were added. Each drug concentration was tested in 4 replicate wells. One 96-well plate was used as control. The cells were then incubated at room temperature (10 minutes), fluorescence intensity of the specimens in an ELISA plate reader was measured using FLUOstar Omega (MBG LABTECH). Apoptosis was measured at various time points (24, 48 and 72 hours).

Statistics

Statistical significance was determined using analysis of variance (ANOVA) (SPSS, version 20). The



impact of HDAC inhibitor MI-192 on cell expansion, cell proliferation, and apoptosis was analyzed with one-way ANOVA with Tukey post hoc analysis for multiple comparisons. 2 tailed Student's *t* test was used when only two independent groups were compared. Results were considered to be statistically significant at $P < 0.05$. Data are presented as means $\pm$ SEM.

Results

Effect of MI-192 on the Cell Morphology

Here we evaluated *in vitro* the impact of HDAC inhibitor MI-192 on the morphology (outgrowth, cell density) of two human medulloblastoma cell lines, DAOY and SHS-Y5Y. It should be noted that there was no pronounce effect of DMSO (vehicle) on the morphology of the DAOY and SHS-Y5Y cell lines at 24 (Figure 1A-D), 48, and 72 hours (data not shown). The results of this study showed that concentrations as low as 0.001, 0.003, 0.01 or 0.03 of MI-192 had no effect on the cell morphology of both cell lines as seen in Figure 1E-F. Concentrations of 1 μ M and above were toxic to both cell lines and were therefore not used in subsequent experiments as shown in Figure 1G-H. There was no significant effect of HDAC inhibitor MI-192 on cell morphology at 24, 48, and 72 hours in DAOY and SHS-Y5Y cells (Figure 1I-N).

MI-192 Reduces Cell Expansion

The possibility of cytotoxic effect of MI-192 on DAOY and SHS-Y5Y medulloblastoma cells *in vitro*, cell expansion was characterized by counting cell numbers at serial time points. DAOY and SHS-Y5Y cell expansion was not significantly suppressed following 24 hours of MI-192 exposure; however, the cell number continued to increase, albeit at a slower rate than untreated cells (Figure 2A). It should be noted that there was not pronounced effect of DMSO (vehicle) on cell expansion of DAOY and SHS-Y5Y cells at 24, 48, and 72 hours as shown in Figure 2. In addition, the results of washout experiments revealed that following 24 hours of exposure to MI-192 significantly suppressed expansion of medulloblastoma cells (Figure 2B-C). For example, at the end of 48 hours, 27% of the DAOY cells and 18% of the SHS-Y5Y cells were viable (Figure 2B). With continuous exposure, however, the cell counts decreased significantly, such that at 72 hours, only 18% of the DAOY cells and 5% of the SHS-Y5Y cells were viable (Figure 2C). It should be pointed out that the growth curves of the untreated cells in both cell lines reached plateau at 72 hours mainly due to the limited growth areas that were available in 96-well plates that were used in this assay.

MI-192 Suppresses Proliferative Activity

Next we evaluated *in vitro* the anti-proliferative efficacy of MI-192 on DAOY and SHS-Y5Y medulloblastoma cells using DNA mitotic marker BrdU. The results of this study demonstrated that HDAC inhibitor MI-192 had significant anti-proliferative effects in a dose- and time-dependent manner (Figure 3). MI-192 was a potent compound at concentration less than 1 μ M. It should be noted that

cells treated with lower MI-192 concentrations ($< 0.1 \mu$ M) began to proliferate at similar rates as the untreated cells, whereas higher MI-192 concentrations ($> 1 \mu$ M) were too toxic to the both cell lines (Figure 3A and B). Our study demonstrated that both cell lines were sensitive to MI-192. This compound was more effective in SHS-Y5Y cells (proliferation inhibition; PI50 mean: 0.58 μ M after 24 hrs) than in DAOY cells (PI50 mean: 0.83 μ M after 24 hrs). In DAOY cells, treatment with PI50 MI-192 for 24 hours resulted in a $\sim 55\%$ suppression of proliferation (Figure 3C), whereas treatment of SHS-Y5Y cells with PI50 MI-192 for 24 hours resulted in a $\sim 65\%$ suppression of cell proliferation (Figure 3C).

To evaluate whether human medulloblastoma cells regained proliferative capacity following cessation of the HDAC inhibitor exposure, MI-192-containing medium was replaced at the end of 24 hours of treatment with MI-192-free medium. With washout experiments, the antiproliferative effect of 24 hours of exposure to MI-192 on DAOY and SHS-Y5Y cells was found to be irreversible even at the end of 48 or 72 hours (Figure 3D-E). At 48 hours, only 40% of the SHS-Y5Y cells were proliferative, whereas 50% of the DAOY cells were proliferative (Figure 3D). With continuous measurement, however, the cell proliferation was further decreased significantly ($P < 0.05$). At the end of 72 hours, only 10% of the SHS-Y5Y cells were proliferative, whereas 20% of the DAOY cells were proliferative at the end of 72 hours (Figure 3E).

MI-192 Induces Apoptosis

To investigate the proapoptotic effect of MI-192 on DAOY and SHS-Y5Y medulloblastoma cell lines, annexin-V staining was performed. There was no significant difference in the rate of apoptosis in MI-192-treated versus vehicle-treated DAOY or SHS-Y5Y cell line cultures at serial time points (24, 48 and 72 hours) (Table 1). Moreover, the rate of apoptosis was not statistically different in MI-192-treated versus non-treated DAOY cell cultures at 48 and 72 hours after 24 hours of exposure and a similar tendency was found in the SHS-Y5Y cell line cultures as shown in Table 1.

Discussion

Several studies support the use of histone deacetylase inhibitors as potent anticancer agents through remodeling of chromatin structure and dynamic changes in nucleosomal packaging of DNA. This study was undertaken to assess the efficacy of HDAC selective inhibitor MI-192 on DAOY and SHS-Y5Y human medulloblastoma cell lines, which might aid the search for potential therapeutic interventions. This compound is predicted to have better therapeutic potential, due to its lack of efficacy to inhibit HDAC1 and other HDACs, which can contribute to the production of side effects associated with currently available general non-selective class I and II HDAC inhibitors. In so doing, a number of important insights were gained into the contribution of this compound in modulating cell expansion and proliferation of these two human medulloblastoma cell lines.

The author concludes that potent anti-medulloblastoma activities of HDAC inhibitor MI-192 in medulloblastoma cells are included with reduction of cell expansion as well as cell proliferation. Some of these responses in these cell lines may be related to their original state of differentiation and genetic backgrounds, including pathways that were not evaluated in the current study. With minimal apoptosis, the DAOY cell line seems less sensitive to MI-192 than SHS-Y5Y cell line.

There have been ongoing attempts to identify effective antitumor agents for the treatment of human

brain cancers, such as medulloblastoma; however, there is still a lack of HDAC inhibitor that is ready for clinical trials. This study shows the possibility of replacing non-specific HDAC inhibitors such as valproic acid and SAHA with more selective compounds as potential therapies for some cancers. As discussed earlier and reviewed by (Li et al., 2005; Li et al., 2004), general non-selective HDAC inhibitors have been well reported as efficacious protective agents against tumorigenesis.

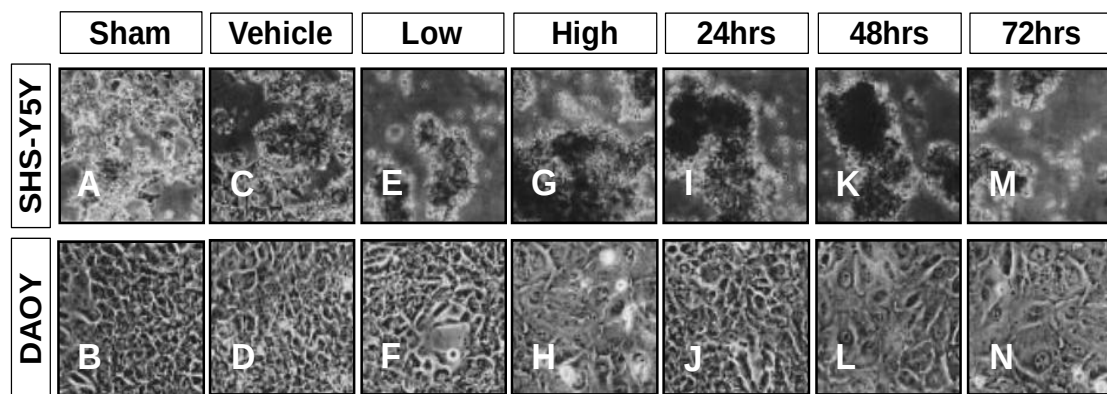


Figure 1. The effect of HDAC selective inhibitor MI-192 treatment on the morphology of DAOY and SHS-Y5Y medulloblastoma cell lines at serial time points (24, 48 and 72 hours). (A and B) Non-treated and (C and D) treated DAOY and SHS-Y5Y cells with DMSO alone. Treated DAOY and SHS-Y5Y cells with (E and F) low and (G and H) high MI-192 concentrations. Treated DAOY and SHS-Y5Y cells with IC50 MI-192 at (I & J) 24, (K and L), 48 and (M and N) 72 hours, phase contrast microscopy 100X magnification. Scale bar: 20µm.

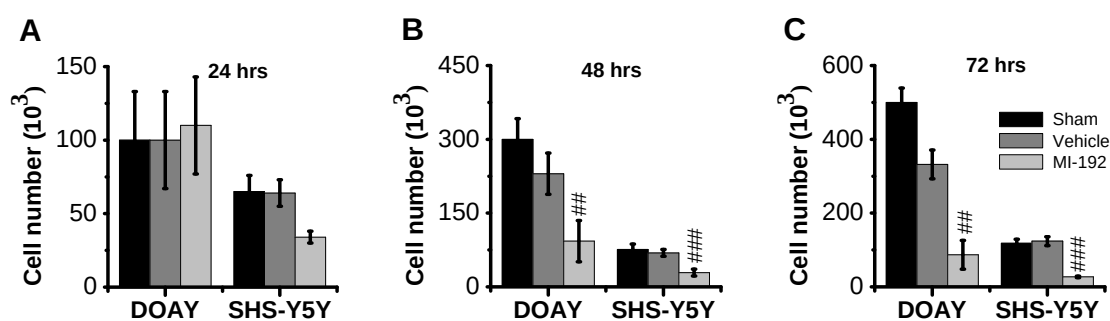


Figure 2. Quantitative analysis of DAOY and SHS-Y5Y medulloblastoma cell expansion at serial time points following treatment with HDAC selective inhibitor MI-192. Minimum required doses for suppressing cell expansion in DAOY and SHS-Y5Y cell lines at (A) 24 hours of exposure were $0.58 \pm 0.23 \mu\text{M}$ and $0.83 \pm 0.18 \mu\text{M}$ MI-192, respectively. (B-C) Washout experiments. Data are represented as mean $\pm$ SEM for at least 4 replicates for each concentration. # vs. vehicle. ##P ≤ 0.01 , ###P ≤ 0.001 . One-way ANOVA followed by Post-Tukey test.

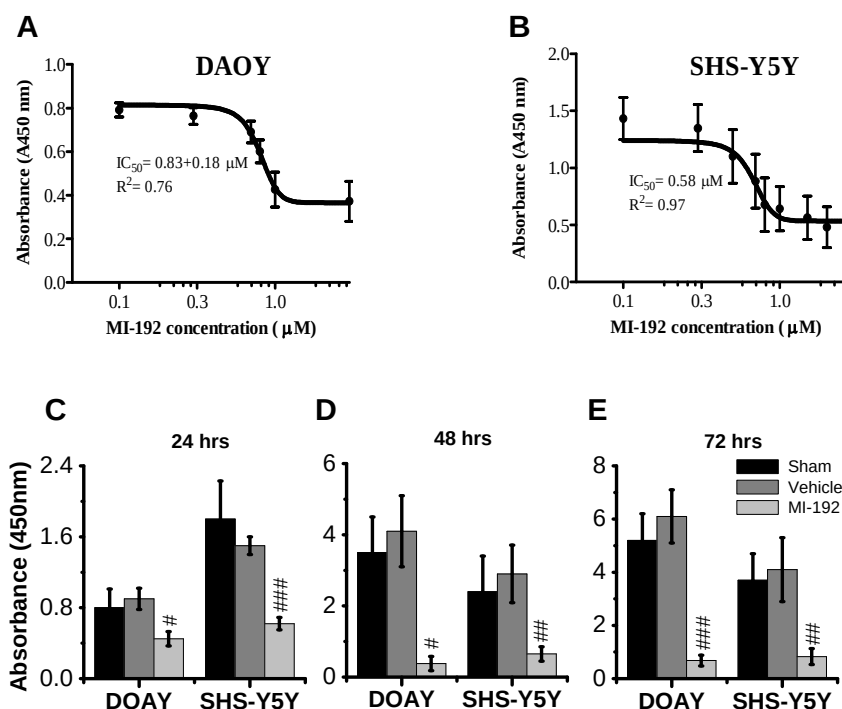


Figure 3. Proliferation inhibition (PI) of DAOY and SHS-Y5Y medulloblastoma cells following treatment with a novel HDAC selective inhibitor MI-192. (A and B) Measurement of 50% cell proliferation inhibition (PI₅₀). (C) Quantification of cell proliferation of DAOY cells (PI₅₀ mean 0.83 μM) and SHS-Y5Y cells (PI₅₀ mean 0.58 μM) at the end of 24 hours of exposure to the HDAC inhibitor MI-192. Quantification of proliferation of DAOY cells (PI₅₀ mean 0.83 μM) and SHS-Y5Y (PI₅₀ mean 0.58 μM) cell proliferation at the end of (D) 48 and (E) 72 hours of HDAC inhibitor exposure. Data are represented as mean ± SEM for at least 4 replicates for each concentration. # vs. vehicle. #*P* ≤ 0.05, ##*P* ≤ 0.01, ###*P* ≤ 0.001. One-way ANOVA followed by Post-Tukey test.

Table 1. Quantitative analysis of apoptosis in human medulloblastoma cells *in vitro* at serial time points following 24, 48 and 72 hours of exposure to HDAC selective inhibitor MI-192.

Cell line	Groups	24 hrs	48 hrs	72 hrs
DAOY	Vehicle	15.28 ± 6.23	29.33 ± 3.71	67.92 ± 6.42
	MI-192	23.41 ± 4.63	35.33 ± 7.83	71.78 ± 5.11
SHS-Y5Y	Vehicle	9.12 ± 2.94	15.33 ± 4.13	31.75 ± 6.23
	MI-192	13.41 ± 1.43	21.48 ± 3.26	39.83 ± 4.89

Annexin-v fluorescence intensity of the specimens in an ELISA plate reader was measured and apoptosis given as mean ± SEM.

Our novel selective HDAC2 and 3 inhibitors largely protected against tumour activities in our *in vitro* cell culture model; therefore, our study shows the potential of moving away from general inhibitors towards more selective ones, such as those against HDAC2 and 3 and there is no compromise in protective efficacy. The exact molecular mechanisms of protection by inhibiting HDAC2 and 3 are not yet known. However, it is likely that the mechanisms responsible involve the increased transcription of pro-survival and anti-apoptotic genes (Appelskog et al., 2004; Camphausen et al., 2005; Leng

et al., 2010), but may also involve the prevention of toxicity associated with increased HDAC2 and 3 activities.

Cell culture models have been employed to test HDAC inhibitors as protective agents against tumorigenesis (Appelskog et al. 2004; Camphausen et al. 2005; Jaboin et al. 2002; Li et al. 2004). In this study, we have used a culture model system to evaluate the anti-tumor efficacy of HDAC inhibitor MI-192 in two human medulloblastoma cell lines, DAOY and SHS-Y5Y using several *in vitro* assays.



Our study demonstrates that *in vitro* anti-medulloblastoma effects of MI-192 were time- and dose-dependent, and irreversible inhibition of cell expansion was also achieved. This finding is in an agreement with prior results with another inhibitor of HDACs such as phenylbutyrate (Li *et al.* 2004). More importantly, we further showed that HDAC inhibitor MI-192 possesses strong inhibitory activities on cell proliferation of medulloblastoma cells, similar to those described in many other human malignant tumors (Li *et al.* 2004; Sonnemann *et al.* 2010). Our results also showed that following continuous exposure to MI-192 for at least 24 hours the suppression of cell proliferation became irreversible in the medulloblastoma cells as seen at 48 and 72 hours.

Treatment with IC₅₀ (5.8 μ M and 0.83 μ M, respectively) of MI-192 for 24 hours resulted in a significant decrease of cell expansion in both DOAY and SHS-Y5Y cells and significantly reduced proliferation (down to 50%) which had no effect on the amount of cell apoptosis at 48 and 72 hours. However, these results warrant further investigations of this novel therapeutic approach. Taken together, these results provided strong evidence to support the notion that irreversible epigenetic reprogramming has taken place and were responsible for the reduced tumorigenicity. Because long-term non-specific HDAC inhibitor valproic acid administration in children is well tolerated (Eiris *et al.* 1995; Guo *et al.* 2001), these results suggest that acute treatment with a novel HDAC selective inhibitor may be maintained in children with medulloblastomas after chemotherapy and radiotherapy, which may possibly decrease recurrence and enhance survival. The findings of this study may lay the groundwork for further studies using specific genetically engineered models to establish the causal relationship between MI-192 antitumor activity and specific genetic pathways and to identify molecular markers that will predict drug responsiveness and guide the development of future clinical therapies.

In conclusion, selective inhibitors of HDACs are a promising prospect for the future cure of cancers. The cure of high risk embryonal human cancers especially of the CNS of childhood by inhibiting tumour activities is a yet unaccomplished task. Inhibitors of HDACs may well play a role in achieving this goal. Our findings show that the novel selective HDAC inhibitor MI-192 possesses potent *in vitro* antimedulloblastoma activities by inhibiting cell expansion and proliferation with minimal apoptosis. In addition, this compound could have further promise as therapeutic agents in other forms of cancers. This study warrants further studies and may help in the design of new protocols geared at the cure of high risk cancers.

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