**Therapeutics, Targets, and Chemical Biology**

**Autophagic Survival in Resistance to Histone Deacetylase Inhibitors: Novel Strategies to Treat Malignant Peripheral Nerve Sheath Tumors**

Gonzalo Lopez1,2,3, Keila Torres3,4, Juehui Liu3,4, Belinda Hernandez4, Eric Young2,4, Roman Belousov3,4, Svetlana Bolshakov3,4, Alexander J. Lazar2,3,5, John M. Slopis6, Ian E. McCutcheon7, David McConkey2,8, and Dina Lev1,2,3

**Abstract**

Histone deacetylase inhibitors (HDACi) show promise as cancer therapeutics; however, the full scope of their utility remains unknown. Here we report findings that strongly rationalize clinical evaluation of HDACis in malignant peripheral nerve sheath tumors (MPNST), a class of highly aggressive, therapeutically resistant, and commonly fatal malignancies that occur sporadically or in patients with the inherited neurofibromatosis type-1 (NF1) syndrome. We evaluated the effects of the chemical HDACis PCI-24781, suberoylanilide hydroxamic acid, and MS-275 on a panel of human NF1-associated and sporadic MPNSTs *in vitro* and *in vivo*. A subset of MPNSTs was found to be highly sensitive to HDACis, especially to PCI-24781. All cell lines in this group were NF1-associated. Significant proapoptotic effects were noted *in vitro* and *in vivo* and were independent of p53 mutational status. In contrast, as a group the sporadic –MPNST cells were markedly resistant to HDACi treatment. HDACis were found to induce productive autophagy in MPNST cells. Genetic and/or pharmacologic autophagy blockade resulted in significant HDACi-induced apoptosis in cells defined as resistant or sensitive, leading to abrogated growth of primary tumors and lung metastases in tumor xenograft assays. Among autophagy-associated genes expressed in response to HDACi, the immunity-related GTPase family, M was validated as a critical target in mediating HDACi-induced autophagy and enhanced apoptosis. Taken together, our findings strongly support the evaluation of HDACi currently in clinical trials as an important new therapeutic strategy to treat MPNST, including in combination with autophagy blocking combination regimens in particular for patients with sporadic MPNST. Cancer Res 71(1): 185–96. ©2010 AACR

**Introduction**

Malignant peripheral nerve sheath tumors (MPNST) are rare malignancies originating from Schwann lineage cells and arising proximate to peripheral nerves (1, 2). MPNSTs account for 3% to 10% of all soft tissue sarcomas (STS) and are highly aggressive histologic subtype (3–5). More than 50% of these occur in patients with the inherited neurofibromatosis type-1 (NF1) syndrome; approximately 8% to 12% of NF1 patients will develop an MPNST in their lifetime, commonly arising within a preexisting deep, plexiform neurofibroma (6, 7); the remainder develop sporadically (6). In adults with NF1, MPNSTs are the most common malignancy, the major source of morbidity, and the leading cause of NF1-related mortality (8, 9). Complete surgical resection, frequently not feasible due to local invasiveness and/or uncontrollable metastases, is the only potentially curative option; radio- and chemotherapy have not demonstrably affected survival, underlying 20% to 50% 5-year survival rates (1, 8, 9). Lack of effective systemic therapies is the major unresolved MPNST clinical problem; new therapeutic approaches are urgently needed.

Recently, attention has focused on potentially reversible alterations in chromatin structure that modulate gene expression during malignant transformation (10). Histone deacetylases (HDAC) play an important role in the epigenetic regulation of gene expression by catalyzing the removal of acetyl groups from histone and non-histone proteins, stimulating chromatin condensation, and promoting transcriptional repression and other molecular processes (11). The emerging delineation of HDAC-driven alterations that coincide with tumorigenicity and malignant progression has provided impetus for development of HDAC inhibitors (HDACi) as novel cancer therapeutics (12, 13). Such initiatives are prompted by broad growth-inhibitory and cytotoxic HDACi effects observed in cultured cancer cells (with normal cell...
clinical trials have documented HDACi potential efficacy in xenograft models (14). To date, more than 15 early-phase preclinical setting. We have recently shown in vitro and in vivo efficacy for broad spectrum hydroxamic acid-based HDACis [suberoylanilide hydroxamic acid (SAHA) and PCI-24781] against a range of genetically complex STS, especially when administered in combination with doxorubicin (17). MPNSTs were not included in these original investigations; to the best of our knowledge, the effect of HDACi on these tumors has not been assessed. The goal of this study was to bridge this investigational gap and to evaluate the effects of HDACis on MPNST is a preclinical setting.

Materials and Methods

Cell lines

Human NF1-related MPNST cell lines ST88-14, T265, and S462, and non-NF1 sporadic human MPNST cell lines STS26T and MPNST724 were maintained and propagated as previously described (18). Primary cultured normal human Schwann cells (NSC) served as controls. The NF1-associated cell line MPNST642 was established by us (Supplementary Data); DNA fingerprinting (short tandem repeat; Supplementary Data) was conducted for all cell lines less than 6 months prior to the conduct of the studies, confirming that no cross-contamination has occurred. STS26T and MPNST724 cells were stably transfected to express GFP-LC3; overexpressing cells were FACs-sorted on the basis of green fluorescent protein (GFP) expression. HDAC inhibitors included PCI-24781 (Pharmacies), SAHA, and MS-275 (Cayman Chemical). Bafilomycin and chloroquine were obtained from Sigma. Commercially available antibodies were used for immunoblot or immunohistochemical detection of acetylated H3, acetylated H4 (Millipore); acetylated tubulin (Sigma); caspase-3, LC3B (Cell Signaling); GFP, beclin, p53, actin (Santa Cruz); immunity-related GTPase family, M (IRGM), PARP (Abcam); Ki-67 (MIB-1), vim (Dako) and S-100 (Biogenex).

Cellular assays

MTS, clonogenicity, and soft agar colony formation assays were performed as previously described (19). Doses needed to inhibit growth by 50% (GI50) were determined. Western blot analyses were performed by standard methods (17). Apoptosis was measured using the Apoptosis Detection kit I (BD Biosciences) as per manufacturer’s recommendations. Further information is available as Supplementary Data.

Transfection procedures

siRNAs and p53 construct transfections procedures are described in Supplementary Data.

Gene Expression Assays

Gene expression profiling was conducted using the Autophagy RT2 Profiler PCR Array (SABiosciences). RT-PCR and qRT-PCR were conducted by standard methods. Additional information and primer sequences are provided in Supplementary Data.

Transmission Electron Microscopy and Quantification of acidic vesicular organelles

Assays were performed as previously described (20). Additional information is provided in Supplementary Data.

In vivo animal models

All animal procedures/care was approved by UTMDACC Institutional Animal Care and Usage Committee. Animals received humane care as per the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals." Animal models were utilized as previously described (17). Animal models, therapeutic schemas, drug doses, and immunohistochemical procedures are provided in Supplementary Data.

Statistics

Cell culture-based assays were repeated at least thrice; mean ± SD was calculated. Cell lines were examined separately. For outcomes that were measured at a single time point, 2-sample t tests were used to assess the differences. Differences in xenograft growth (tumor/metastases) in vivo were assessed using a 2-tailed Student’s t test. Significance was set at P ≤ 0.05.

Results

HDACis induce significant apoptosis in a subset of human MPNST

HDACi effects on human MPNST cell growth and clonogenicity were evaluated. Six MPNST cell lines were used including the NF1-associated MPNST642 cell line recently established by us (Supplementary Fig. S1); primary cultured (NSC served as controls. Three HDACis were tested: PCI-24781, SAHA, and MS-275. PCI-24781 induced a time- and dose-dependent growth inhibition that was most pronounced in a subset of cell lines tested independent of growth rate (see Supplementary Results). Figure 1A depicts GI50s at 48 hours; 4 MPNST cell lines were markedly sensitive to PCI-24781, with GI50s ranging between 0.1 and 0.35 μmol/L, whereas the 2 additional cell lines (STS26T and MPNST724) were relatively resistant exhibiting GI50s more than the clinically relevant dose (>1 μmol/L). NSCs were resistant to PCI-24781 growth inhibitory effects. Higher doses of SAHA and MS-275 were needed to achieve PCI-24781–equivalent MPNST growth inhibition; however, a similar response pattern was found for all these drugs, enabling MPNST cell designations to “sensitive” and “resistant” cohorts (Fig. 1A). A similar pattern of response was noted when the effect of HDACis on colony-forming capacity was evaluated (Fig. 1B). This pattern was also reflected in the induction of apoptosis by these compounds (Fig. 1C). Marked apoptosis (evaluated by Annexin-V/PI staining FACs analysis) was seen in "sensitive" cell lines whereas no significant apoptosis was induced in "resistant" cells. Similarly, an increase in cleaved caspase-3 was seen in "sensitive" but not in "resistant" cells (Fig. 1C). A time- and dose-dependent increase in target protein acetylation could be observed in all the cell lines regardless of the growth inhibitory effects
Figure 1. A subset of MPNST cell lines is highly sensitive to HDACis. A, growth-inhibitory effects (48 hours) were determined via MTS assays. GI50s are depicted. One MPNST cell-line subset was highly sensitive to the effects of all 3 drugs [highest sensitivity was seen to PCI-24781 (PCI)], whereas a second exhibited relative resistance. No significant effect on normal human Schwann cells (NSC) growth was noted in clinically relevant therapeutic doses. B, clonogenic assays showed a similar pattern of response. C, marked apoptosis was noticed in “sensitive” cell lines but not in “resistant” cell lines [Annexin-V (X axis)/PI (Y axis) FACS analysis (48 hours) and cleaved caspase-3 WB]. D, a time- and dose-dependent increase in target protein acetylation was shown after treatment with either of the compounds in all cell lines (including NSC) independent of growth inhibitory response (MS-275 is a selective HDAC1/HDAC2 inhibitor and does not affect tubulin acetylation). Graphs represent the average of 3 repeated experiments ± SD. *, statistically significant effects (P < 0.05).
can be recapitulated and in vivo. Together, these data suggest that a subset of tumors (induced antiproliferative, proapoptotic effects in the "sensitive"
(Ki67) and apoptosis (TUNEL) confirmed that PCI-24781 abrogated the growth of MPNST642 xenografts. A significant decrease in average tumor size and weight of treated tumors as compared to controls was noted at study termination (P = 0.00016 and 0.0004). In contrast, no significant growth inhibitory effects were seen in MPNST724 (Fig. 2A) and STS26T (data not shown) xenografts, although a higher dose of PCI-24781 was tested (50 mg/kg/d). In accordance, immunohistochemical analysis of tumor sections for cell proliferation (Ki67) and apoptosis (TUNEL) confirmed that PCI-24781 HDACi on tumor growth in vivo. PCI-24781 was selected because of its enhanced efficacy in vitro. As shown in Figure 2A, a relatively low (25 mg/kg/d) PCI-24781 dose markedly abrogated the growth of MPNST642 xenografts. A significant decrease in average tumor size and weight of treated tumors as compared to controls was noted at study termination (P = 0.00016 and 0.0004). In contrast, no significant growth inhibitory effects were seen in MPNST724 (Fig. 2A) and STS26T (data not shown) xenografts, although a higher dose of PCI-24781 was tested (50 mg/kg/d). In accordance, immunohistochemical analysis of tumor sections for cell proliferation (Ki67) and apoptosis (TUNEL) confirmed that PCI-24781 induced antiproliferative, proapoptotic effects in the "sensitive" tumors (P = 0.0008 and 0.0008), but not in the MPNST724 xenografts (Fig. 2B). Together, these data suggest that a subset of MPNSTs is highly sensitive to HDACi monotherapy in vitro and in vivo. In addition, HDACi "resistance" observed in vitro can be recapitulated in vivo, offering a novel model for further investigation of HDACi tumor response mechanisms.

**HDACi sensitivity/resistance pattern is recapitulated in vivo**

Human MPNST xenografts (Supplementary Fig. S1), including HDACi "sensitive" (MPNST642) and "resistant" (MPNST724 and STS26T) tumors, were used to evaluate the effects of HDACi on tumor growth in vivo. PCI-24781 was selected because of its enhanced efficacy in vitro. As shown in Figure 2A, a relatively low (25 mg/kg/d) PCI-24781 dose markedly abrogated the growth of MPNST642 xenografts. A significant decrease in average tumor size and weight of treated tumors as compared to controls was noted at study termination (P = 0.00016 and 0.0004). In contrast, no significant growth inhibitory effects were seen in MPNST724 (Fig. 2A) and STS26T (data not shown) xenografts, although a higher dose of PCI-24781 was tested (50 mg/kg/d). In accordance, immunohistochemical analysis of tumor sections for cell proliferation (Ki67) and apoptosis (TUNEL) confirmed that PCI-24781 induced antiproliferative, proapoptotic effects in the "sensitive" tumors (P = 0.0008 and 0.0008), but not in the MPNST724 xenografts (Fig. 2B). Together, these data suggest that a subset of MPNSTs is highly sensitive to HDACi monotherapy in vitro and in vivo. In addition, HDACi "resistance" observed in vitro can be recapitulated in vivo, offering a novel model for further investigation of HDACi tumor response mechanisms.

**HDACis induce autophagy in MPNST cells in vitro and in vivo**

To further evaluate HDACi-induced structural changes in "resistant" cells, transmission electron microscopic (TEM) evaluation was conducted: a large number of cytoplasmic autophagosomes was noticed after treatment [24 (Fig. 3A), 48, and 72 hours] but no signs of apoptosis were seen. Although the noted autophagosome accumulation suggests drug-induced autophagy, multiple assays are needed to confirm this observation (24). Acidrine-orange staining showed increased acidic vesicular organelles in PCI-24781 (0.5 μmol/L per 24 hours) treated compared to control dimethyl sulfoxide (DMSO)-treated cells, as was confirmed via FACS analysis (Fig. 3B). Furthermore, LC3B conversion and LC3B-II expression (normalized to actin) both increased after treatment with all 3 HDACis tested (Fig. 3C). Because the experiments described earlier may represent either enhanced autophagosomal turnover due delayed trafficking or reduced autophagosome–lysosome fusion (24), cells were pretreated (1 hour) with low doses of the autophagy inhibitors Bafilomycin A1 (1 nmol/L) or chloroquine (1 μmol/L) prior to PCI-24781 treatment (24 hours). PCI-24781 treatment produced increased LC3B-II expression even in the presence of these inhibitors, providing evidence of efficient autophagic flux (Fig. 3C). Furthermore, cells stably transduced to express LC3-GFP, exhibited increased GFP puncta in response to HDACi treatment (Fig. 3D). Western blot (WB) showed increased GFP cleavage following HDACi treatment that was blocked by pretreatment with chloroquine, further supporting HDACi-induced productive autophagy (Fig. 3D). Similarly, HDACi-induced autophagy could be observed in vivo. Animals bearing STS26T tumors were treated with PCI-24781 for 4 days, and on day 5 tumors were harvested 2, 4, and 6 hours after final dose. WB analysis showed a time-dependent increase in LC3B-II expression (Fig. 3D). Similarly, MPNST724/ GFP-LC3 xenografts were treated with PCI-24781, chloroquine, or their combination (Fig. 3D). A marked increase in free GFP expression was noted in response to PCI-24781. Chloroquine blocked HDACi-induced increases in free GFP.

HDACi-induced autophagy was not exclusive to sporadic MPNST cells and in a series of experiments as depicted earlier we found autophagy to occur in response to PCI-24781 in all NF1-associated HDACi "sensitive" cells (Supplementary Fig. S3). However, in contrast to the MPNST "resistant" cells, morphologic features of apoptosis were also present. Together, our data strongly support that HDACis induce productive autophagy in MPNST cells in vitro and in vivo.

**Autophagy blockade enhances HDACi proapoptotic effects in MPNST cells**

It is currently unclear whether drug-induced autophagy contributes to cell death or possibly represents a mechanism of therapeutic resistance (25, 26). To that end, we evaluated the impact of autophagy blockade using complementary genetic and pharmacologic manipulations on HDACi-induced effects. siRNA knockdown of beclin, ATG5, and ATG7 were conducted (Fig. 4A–C). Autophagy blockade was assessed via LC3B-II WB; knockdown of any of the 3 targets decreased both baseline and PCI-24781–induced LC3B-II expression. Most importantly, enhanced apoptosis in response to PCI-24781 pretreatment of cells was found after target knockdown, as determined via PARP cleavage and Annexin-V/PI FACS analysis (Fig. 4A–C). Pharmacologic autophagy inhibition was achieved using bafilomycin and chloroquine (Fig. 4D). The clonogenic capacity of STS26T or MPNST724 cells was not affected by any of the compounds when used alone, but was significantly affected by chloroquine or bafilomycin pretreatment of cells (1 hour) followed by PCI-24781 for 24 hours (P < 0.05). The combination also resulted in increased apoptosis. Together, these data suggest that autophagy blockade sensitized “resistant” MPNST cells to the proapoptotic effects of HDACi in vitro.

Next, we evaluated the impact of autophagy blockade on "sensitive" MPNST cell lines where autophagy in response to some synthesis and productive autophagy or reduced autophagosome–lysosome fusion (24), cells were pretreated (1 hour) with low doses of the autophagy inhibitors Bafilomycin A1 (1 nmol/L) or chloroquine (1 μmol/L) prior to PCI-24781 treatment (24 hours). PCI-24781 treatment produced increased LC3B-II expression even in the presence of these inhibitors, providing evidence of efficient autophagic flux (Fig. 3C). Furthermore, cells stably transduced to express LC3-GFP, exhibited increased GFP puncta in response to HDACi treatment (Fig. 3D). Western blot (WB) showed increased GFP cleavage following HDACi treatment that was blocked by pretreatment with chloroquine, further supporting HDACi-induced productive autophagy (Fig. 3D). Similarly, HDACi-induced autophagy could be observed in vivo. Animals bearing STS26T tumors were treated with PCI-24781 for 4 days, and on day 5 tumors were harvested 2, 4, and 6 hours after final dose. WB analysis showed a time-dependent increase in LC3B-II expression (Fig. 3D). Similarly, MPNST724/ GFP-LC3 xenografts were treated with PCI-24781, chloroquine, or their combination (Fig. 3D). A marked increase in free GFP expression was noted in response to PCI-24781. Chloroquine blocked HDACi-induced increases in free GFP.
HDACi occurs in parallel with apoptosis. Using an experimental approach as described earlier we found that blocking autophagy significantly enhanced apoptosis both when low doses (0.1 μmol/L per 24 hours) and high doses (1 μmol/L per 24 hours) of PCI24781 were used (Supplementary Fig. S3). These data suggest that even in HDACi sensitive cells autophagy is a potential survival mechanism opposing apoptosis.

**Figure 2.** PCI-24781 inhibits the growth of MPNST xenografts exhibiting significant sensitivity in vitro. A, SCID mice bearing MPNST642 and MPNST724 xenografts were treated with PCI-24781 (PCI; 25 mg/kg/d and 50 mg/kg/d, respectively) or vehicle (10 mice per group). Tumor growth/weight curves are depicted showing that PCI-24781 abrogated the growth of MPNST642 tumors \( \left( P = 0.00016 \right) \) and \( 0.0004 \) for tumor size and weight, respectively), but not of MPNST724 tumors although treated with a higher drug dose; B, IHC analysis showed enhanced tumor necrosis in MPNST642 PCI-24781-treated tumors (hematoxylin and eosin; H&E), decreased tumor proliferation (Ki67; \( P = 0.008 \)), and increased apoptosis (TUNEL; \( P = 0.0008 \)). No significant differences were found for MPNST724 xenografts. *, statistically significant effects; \( P < 0.05 \).
Autophagy blockade enhances HDACi proapoptotic effects in vivo

Next, we evaluated the effects of autophagy blockade on HDACi treatment response in vivo. MPNST724 and STS26T xenografts were used and therapy was initiated when tumors reached a mean diameter of 0.5 cm (Fig. 5A and B). No major side effects were noted. No significant growth inhibition was seen with PCI-24781 or chloroquine as monotherapy. However, chloroquine/PCI-24781 combination significantly inhibited tumor growth in both animal models as compared to

Figure 3. HDACis induce productive autophagy in therapeutic resistant MPNST cells. A, TEM pictures showing ultrastructural changes in MPNST cells in response to PCI-24781 (0.5 μmol/L per 24 hours). A large number of autophagic vesicles were identified (arrows) without evidence of chromatin condensation, nuclear membranes remained intact further supporting lack of apoptotic effect. B, acridine-orange staining showed increased acidic vesicular organelles (AVO) in PCI-24781 (0.5 μmol/L per 24 hours) compared to control DMSO-treated cells, as was further confirmed via FACS analysis. C, increased LC3B-II expression (normalized to actin; densitometry values are depicted) was noticed after treatment with any of the HDACis (top). Cells were pretreated (1 hour) with low doses of the inhibitors Bafilomycin A1 (1 nmol/L) or chloroquine (1 μmol/L) prior to PCI-24781 treatment (24 hours; bottom). Additional increased LC3B-II expression in response to PCI-24781 was noticed in the presence of these inhibitors. D, cells stably transduced to express LC3-GFP exhibited increased GFP puncta in response to HDACis (top). GFP cleavage was found in response to HDACi and was blocked by pretreatment with chloroquine (middle). Furthermore, WB of STS26T xenografts' protein treated with PCI-24781 (50 mg/kg/d) harvested 2, 4, and 6 hours after final dose showed a time-dependent increase in LC3B-II expression (left, bottom). Similarly, MPNST724 GFP-LC3 xenografts were treated with PCI-24781, chloroquine, or their combination. Free GFP expression was found in response to PCI-24781 treatment. Chloroquine blocked HDACi-induced increases in free GFP (right, bottom).
control and either of the compounds alone (P < 0.05). A significant increase in apoptosis was observed in combination-treated tumors as determined by TUNEL staining (P < 0.05; Fig. 5C).

Next, an experimental MPNST lung metastasis model (Supplementary Fig. S1) was used; therapy was initiated a week after tail-vein injection and continued for 2 weeks (Fig. 5D). All control (n = 6), PCI-24781 (n = 7), and chloroquine (n = 7) treated mice exhibited extensive macroscopic lung metastases at study termination, whereas in 3 of 7 combination-treated mice there were no visible metastases, and less than 5 metastases could be found in the remaining 4 mice. A significantly lower average lung weight was found in combination-treated mice (P < 0.05). Together, these data suggest that autophagy blockade can sensitize MPNST to the proapoptotic effects of HDACi in vivo.

**HDACi-induced autophagy-related gene-expression changes**

Due to their mechanisms of function, one of the major consequences of HDACi therapy is gene expression modulation. Using a focused autophagy PCR array, we sought to identify potential autophagy-related genes in MPNST cells whose expression is modified secondarily to HDACi treatment. Results revealed 4 genes that were reproducibly overexpressed in both cell lines and 5 that were consistently downregulated (Supplementary Table S1). Three overexpressed genes (IRGM, CXCR4, and TMEM74) and 1 downregulated gene, NF-κB, were selected for validation. A concordant dose-dependent increase/decrease in corresponding RNA expression levels was identified after PCI-24781 treatment (24 hours; Fig. 6A). Similarly, IRGM, CXCR4, and TMEM74 mRNA expression was increased in

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**Figure 4.** Autophagy blockade sensitizes MPNST cells to the proapoptotic effects of HDACis. A, anti-Beclin siRNA (20 nmol/L pool) knockdown was confirmed via WB and autophagy blockade was validated using LC3B-II WB. Enhanced apoptosis in response to PCI-24781 (0.5 μmol/L per 24 hours) was determined via PARP cleavage and Annexin-V/PI FACS analysis. B, similar effects were noted after ATG5 knockdown. Knockdown was confirmed by RT-PCR. C, as earlier, ATG7 knockdown (confirmed by RT-PCR) resulted in increased PCI-24781 sensitivity. D, pharmacologic autophagy inhibition was achieved using bafilomycin and chloroquine. None of the compounds alone significantly affected the clonogenic capacity of STS26T or MPNST724; however, significant effects were noticed after chloroquine (1 μmol/L) or bafilomycin (1 μmol/L) pretreatment of cells (1 hour) followed by PCI-24781 for 24 hours (top; P < 0.05). Markedly increased apoptosis was also identified using these therapeutic combinations (bottom). *, statistically significant effects; P < 0.05. BFA, bafilomycin A1.
PCI-24781–treated xenograft tissues; NF-κB mRNA levels were decreased (Fig. 6B). Together, these experiments identified several targets modified by HDACi that may play a role in HDACi-induced autophagy and merit further investigation. Accordingly, IRGM, exhibiting the highest fold increased expression, was selected for additional study. To confirm that increased IRGM expression in MPNST is a common consequence of HDACi exposure, the effects of SAHA and MS-275 on IRGM mRNA expression were evaluated (Fig. 6C); both agents induced IRGM expression. A PCI-24781–induced increase in IRGM protein was also shown. IRGM siRNA knockdown showed inhibition of PCI-24781–induced autophagy as

Figure 5. PCI-24781/chloroquine combination results in superior local and metastatic MPNST growth inhibition in vivo. A, tumor growth curves/weight bars showing a significant impact of PCI-24781/chloroquine combination on MPNST724 growth (10 mice/treatment group; $P = 0.04$). No significant effect was noted after either compound alone. B, a similar superior effect of combination therapy was found after the treatment of STS26T xenografts ($P = 0.03$). C, a significant increase in apoptosis (TUNEL staining) was observed in combination-treated xenografts ($P < 0.05$). D, STS26T lung metastases bearing mice were treated with PCI-24781, chloroquine, or their combination. A significant decrease in average lung weight and number of visible metastasis were found in the combination therapy group ($P < 0.05$) but not after treatment with either agent alone. *, statistically significant effects; $P < 0.05$. 

PCI-24781–chloroquine combination on MPNST724 growth (10 mice/treatment group; $P = 0.04$). No significant effect was noted after either compound alone. B, a similar superior effect of combination therapy was found after the treatment of STS26T xenografts ($P = 0.03$). C, a significant increase in apoptosis (TUNEL staining) was observed in combination-treated xenografts ($P < 0.05$). D, STS26T lung metastases bearing mice were treated with PCI-24781, chloroquine, or their combination. A significant decrease in average lung weight and number of visible metastasis were found in the combination therapy group ($P < 0.05$) but not after treatment with either agent alone. *, statistically significant effects; $P < 0.05$. 

PCI-24781–choloroquine combination on MPNST724 growth (10 mice/treatment group; $P = 0.04$). No significant effect was noted after either compound alone. B, a similar superior effect of combination therapy was found after the treatment of STS26T xenografts ($P = 0.03$). C, a significant increase in apoptosis (TUNEL staining) was observed in combination-treated xenografts ($P < 0.05$). D, STS26T lung metastases bearing mice were treated with PCI-24781, chloroquine, or their combination. A significant decrease in average lung weight and number of visible metastasis were found in the combination therapy group ($P < 0.05$) but not after treatment with either agent alone. *, statistically significant effects; $P < 0.05$. 

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suggested by decreased LC3B-II expression (Fig. 6D). Most importantly, IRGM knockdown resulted in enhanced PCI-24781–induced apoptosis in both cell lines. Together, our results implicate HDACi-induced IRGM expression and autophagy in therapeutic resistance in sporadic MPNST cells.

Discussion

There is a crucial need for improved anti-MPNST therapeutic strategies. Studies here show that a subset of human MPNSTs is highly sensitive to HDACis antiproliferative, proapoptotic effects in vitro and in vivo; importantly, NSCs are resistant to these effects. Both broad spectrum hydroxamic acid-based agents (PCI-24781 and SAHA; ref. 17) and the more selective HDAC1/HDAC2 inhibitor (MS-275; ref. 27) result in a similar anti-MPNST response pattern. However, in support of our previous observations in STS (17), PCI-24781 exhibited growth inhibitory effects at nanomolar versus micromolar doses needed for SAHA/MS-275. Of note, a phase I/II clinical study examining the effects of PCI-24781 in combination with doxorubicin on advanced sarcomas following failure of anthracycline therapy has recently been initiated (28); results support the inclusion of MPNST patients in this or other HDACi-based clinical investigations.

With the limitations of a small testable MPNST cell-line cohort, it is intriguing and of possible major biological and

![Figure 6](https://example.com/figure6.png)

**Figure 6.** HDACis modulate autophagy-related gene expression; a potential role for IRGM in drug-induced autophagy and therapeutic resistance. A, a focused autophagy PCR array identified several genes to be reproducibly deregulated in response to PCI-24781 (0.5 μmol/L per 24 hours; Supplementary Table S1). A concordant dose-dependent increase/decrease in corresponding RNA expression levels was identified (qRT-PCR) in both cell lines after treatment with PCI-24781 (24 hours). B, similarly, an increase in IRGM, CXCR4, and TMEM74 mRNA and a decrease in NF-κB mRNA levels were identified by qRT-PCR and RT-PCR in xenograft tissues retrieved from experiments described earlier. C, an increase in IRGM mRNA was also identified by in response to SAHA and MS-275 (1 μmol/L per 24 hours; left). Furthermore, a PCI-24781–induced increase in IRGM protein was shown (WB; right). D, IRGM siRNA knockdown (pool, 20 nmol/L) resulted in PCI-24781–induced autophagy blockade (decreased LC3B-II expression) and in enhanced PCI-24781–induced apoptosis.
Clinical importance that all NF1-associated cells exhibited marked HDACi sensitivity. The molecular hallmark of NF1-associated MPNSTs is the loss of the GTPase activating protein, Nf1, a rat sarcoma (RAS)–negative regulator leading to constitutive activation of the RAS pathway (29, 30). Previous data suggest that HDACis induce cell death selectively in cells exhibiting enhanced RAS signaling (31, 32). Molecular deregulations such as elevated reactive oxygen species activity and decreased STAT1 expression and/or function, operative in cells exhibiting activated RAS, have been proposed to underlie the increased HDACi susceptibility of these tumor cells (31, 32). HDACis were found to exert some of their proapoptotic effects through the induction, acetylation, and/or activation of p53 (22), p53 gene and its protein product are frequently deleted, mutated, and/or inactivated in MPNSTs (23); this molecular deregulation is thought to be one of the major driving forces for NF1-associated neurofibroma transformation and progression into its malignant MPNST counterpart (23). It is thus pertinent that as we have shown previously for other STS cells, no significant HDACi response differences were seen in wild-type p53 versus p53-mutated cells (17). With the increased interest in HDACi as anticancer therapy, our data suggest that the NF1-associated MPNST preclinical model can be used to establish mechanisms of action driving sensitivity, and especially the role of NF1 loss and RAS activation in this process.

We found sporadic MPNST cell lines to exhibit relative resistance to HDACis. It is important to note that the diagnosis of sporadic MPNSTs, occurring outside the context of NF1, can be difficult (1). Sporadic MPNSTs are generally not associated with preexisting neurofibromas and diagnosis is mainly one of the exclusion. Strict criteria must be applied to ensure diagnostic consistency and at least one of the following conditions must be met: association with a peripheral nerve and ultrastructural, histologic, and/or immunohistochemical features characteristic of Schwannian differentiation. As described earlier, germline NF1 deactivating mutations are the hallmark of NF1-associated MPNSTs. Several studies have identified somatic NF1 mutations to occur in a subset of sporadic MPNSTs, although not uniformly (33). The exact prevalence and importance of NF1 loss in sporadic MPNST tumorigenesis is currently unknown. Both cell lines studied here were shown to retain expression of Nf1 protein (Supplementary Figs. S1 and S2). Further studies are needed to determine the role of NF1 loss in HDACi sensitivity. If such a role is confirmed, it is possible that future treatment studies for sporadic MPNST and other malignancies where somatic NF1 mutations commonly occur would benefit from the use of NF1 mutation status or protein expression as biomarkers for patients stratification.

A major objective of this study was to identify potential mechanisms of HDACi resistance. Such knowledge will facilitate appropriate patient selection for treatment and enhance development of effective combination strategies to maximize HDACi cytotoxic effects. Enhanced antioxidant expression (34), retinoic acid signaling deregulation (35), and multidrug resistance (36) are several potential molecular mechanisms previously proposed as contributory to HDACi resistance. This study highlights the potential role of autophagy as a mechanism of therapeutic resistance.

Described more than 50 years ago, interest in autophagy in tumorigenesis and cancer progression has recently emerged, suggesting a complex molecular and functional interplay (26, 36, 37). Autophagy activation has been reported in response to diverse anticancer therapies (radiation, chemotherapy, targeted therapies; i.e., ER inhibitors and imatinib; refs. 26, 38). Of note is that a large number of studies show autophagy induction based on autophagosome accumulation and LC3 conversion; however, such findings can reflect either increased autophagic activity (productive autophagy) or reduced turnover of autophagosomes and autophagy blockade (24). This has major relevance to HDACis because of the recent finding that HDAC6 controls autophagosome maturation and autophagosome–lysosome fusion, and that its inhibition might in turn induce autophagy blockade (39). Taking this into account, here we have utilized a large panel of assays to test both autophagy steady state and flux, and have evaluated the impact of a selective HDACi (MS-275), which does not block HDAC6.

HDACi-induced autophagy has previously been described (40). The impact of drug-induced autophagy is currently being debated. Pro-death effects via a possible (albeit not yet fully substantiated) autophagic death mechanism (programmed cell-death type-II), or apoptosis enhancement have been described (26). In contrast, pro-survival, antiapoptotic effects have also been reported, consonant with a role for drug-induced autophagy in tumor chemoresistance (37). For example, Shao et al. (41) found both apoptosis and autophagy to occur in Henrietta Lacks cells in response to HDACis; apoptosis blockade did not diminish therapeutic-induced cell death, highlighting the role of autophagy and/or necrosis in this process. Similarly, Hrzenjak and colleagues (42) observed that HDACi treatment mediated autophagic, caspase-independent cytotoxicity in endometrial sarcoma cells. In contrast, Carew et al. (43) found that autophagy blockade significantly enhanced HDACi-mediated apoptosis in chronic myelogenous leukemia cells. These seemingly contradictory data suggest that consequences of drug-induced autophagy may be compound-type, tumor-type, or even molecular context–dependent and depict a complex cross talk between autophagy and apoptosis (44).

Our observations suggest that in MPNST HDACi-induced autophagy increases cell survival by possibly opposing apoptosis and show that autophagy blockade enhances HDACi proapoptotic effects. This occurs not only in cells resistant to HDACi but also in MPNSTs showing relative sensitivity where autophagy blockade further increases HDACi proapoptotic effects. Chloroquine is known for its ability to block autophagy and is currently being evaluated in human glioblastoma and lung cancer clinical trials; initial studies already confirm its safety (45). Our studies support the investigation of HDACi/chloroquine combinations for MPNST treatment.

Currently, autophagy-specific inhibitors are lacking; consequently, the majority of studies, including ours, have been conducted with pharmacologic agents with known off-target...
effects beyond autophagy (46). Development of autophagy-specific inhibitors may help determine the role of autophagy in cancer treatment and can form the basis for novel effective therapeutic combinations. It is therefore pertinent to establish an understanding about selective control of autophagy in specific therapeutic contexts. Our studies have identified and validated several molecular targets that potentially contribute to HDACi-induced autophagy in MPNST including IRGM, CXXC4, and TMEM74. A role in autophagy was recently attributed to the protein products of all 3 genes (see supplementary information; refs. 47–49). However, the mechanisms by which they activate autophagy and possibly contribute to drug-induced autophagy and chemoresistance are yet to be fully elucidated. Accordingly, and as a proof of principle, knockdown of IRGM was found to block HDACi-induced autophagy and enhance apoptosis. Additional studies are currently ongoing and will hopefully lead to findings of clinical relevance. For example, CXXC4 inhibitors are available and are undergoing human cancer clinical trials (50); pending further supportive data HDACi/CXXC4 inhibitor therapeutic combinations could be evaluated as a novel approach for the treatment of MPNST.

Disclosure of Potential Conflicts of Interest

Studies were partially supported through funds gifted to the Sarcoma Research Laboratory by Pharmaceuyics, Inc.

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References

Correction: Autophagic Survival in Resistance to Histone Deacetylase Inhibitors: Novel Strategies to Treat Malignant Peripheral Nerve Sheath Tumors

In this article (Cancer Res 2011;71:185–96), which appeared in the January 1, 2011, issue of Cancer Research (1), there are several errors in the figures. As soon as they became aware of these issues, for sake of accuracy, the authors repeated the related experiments demonstrating the very same experimental results as the original investigations. The corrected figures are as follows:

Figure 1D. This panel includes multiple Western blot analyses in which different cell lines were treated with HDAC inhibitors demonstrating increase in histone and tubulin acetylation. Actin loading controls of the S462 and STS26T cell line blots were accidentally duplicated. All the panels for the S462 and STS26T cell lines have been replaced with new data in the corrected figure below; all other Western blots in panel D and the original data in panels A, B, and C remain the same.

Figure 3. There was a recurring typographical error in the marking of the Western blots. Original data remain the same in panel A.

Figure 3B. Acridine orange staining FACS analysis histograms were erroneously duplicated. The histograms for both STS26T and MPNST724 (DMSO and PCI) have been replaced with new data; original data for the cell images remain the same.

Figures 3C and 4C. Actin loading controls of the bottom gels of Fig. 3C (in which STS26T and MPNST724 cells were treated with PCI-24781, chloroquine or bafilomycin, or their combination) and those of Fig. 4C were erroneously duplicated. For Fig. 3C, original data remain the same for the upper Western blots of STS26T and MPNST724 treated with PCI, SAHA, MS-275. The lower Western blots of STS26T (PCI and BFA) and MPNST724 (PCI and CQ) have been replaced with new data.

Figure 3D. The labels of the three Western blots using MPNST724 (LC3-GFP) have been corrected; the data remain the same. Original data remain the same for the cell images with LC3-GFP puncta and the bottom right Western blot of STS26T treated with PCI (V, 2, 4, and 6 hours).

Figure 4D. The treatment for MPNST724 is chloroquine and not BFA, as was originally indicated. The Western blots on the lower part of Fig. 4D have been replaced with new data; the upper clonogenic images and graph are original data and remain the same. Panels A, B, and C remain the same with original data.

The corrected figures appear below. These errors do not change either the results or the conclusions of the article. The authors regret these errors.

Reference

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Figure 1
Figure 3
Figure 4
Autophagic Survival in Resistance to Histone Deacetylase Inhibitors: Novel Strategies to Treat Malignant Peripheral Nerve Sheath Tumors

Gonzalo Lopez, Keila Torres, Juehui Liu, et al.


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