Enhancement of Xenograft Tumor Radiosensitivity by the Histone Deacetylase Inhibitor MS-275 and Correlation with Histone Hyperacetylation

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ABSTRACT

Purpose: Histone deacetylase (HDAC) inhibitors are undergoing clinical evaluation in cancer therapy. Because HDAC modulation has been shown to enhance the radiosensitivity of tumor cells in vitro, we investigated the effects of the HDAC inhibitor MS-275 on the radiosponse of DU145 prostate carcinoma xenografts.

Experimental Design: As an indicator of HDAC inhibition in vivo, the histone acetylation status in tumor lysates was determined after two, four, and six injections of MS-275 delivered at 12-hour intervals, as well as 24 and 48 hours after the last injection. Tumor growth delay studies were then performed using this DU-145 xenograft model with radiation administered to leg tumors after the fourth dose of MS-275, which corresponded to the time of maximum histone hyperacetylation.

Results: An increase in histone hyperacetylation was detected in each tumor after two injections of MS-275 with a maximum hyperacetylation occurring after four to six injections. In tumor growth delay studies, the combination of MS-275 and radiation resulted in a greater than additive inhibition of tumor growth as compared with the individual modalities. As alternative sources for an indicator of drug radiosensitizing activity, histone hyperacetylation was determined in a series of normal tissues, including lymphocytes. Each of the normal tissues also had a maximal histone hyperacetylation after four to six injections of MS-275.

Conclusions: These studies show that MS-275 enhances the radiosensitivity of DU145 xenografts and suggest that histone hyperacetylation status can serve as a useful marker for drug radiosensitizing activity.

INTRODUCTION

Whereas oncogenesis has been associated with histone acetyl transferase inactivation, it is aberrant histone deacetylase (HDAC) activity that is considered a potential target for cancer therapy (1). HDAC inhibition, putatively via modulation of gene expression, has been reported to induce tumor cell differentiation, apoptosis, and/or growth arrest, depending on the experimental system (2). However, for most cell lines generated from solid tumors, the primary effect of HDAC inhibition is one of cytostasis, as is the case for most target directed chemotherapeutic agents. Regarding a potential application in the treatment of solid tumors, previous studies have shown that sodium butyrate and trichostatin, two structurally disparate compounds with the common action of inhibiting HDAC activity, enhanced the in vitro sensitivity of tumor cells to radiation (2–4). Those reports suggested that HDACs might serve as a target for radiosensitizers. However, a significant impediment to the targeting of HDAC has been the lack of a clinically applicable HDAC inhibitor. Because of its very short half-life and low achievable serum concentration, sodium butyrate has limited clinical applicability (5–8). Similarly, trichostatin A has excessive cytotoxicity apparently because of actions not involving histone acetylation and is unstable under in vivo treatment conditions (9–11).

Recently, a number of HDAC inhibitors with in vivo activity have been developed. One such compound is the benzamide derivative MS-275. It is a potent HDAC inhibitor and has been reported to have in vivo antitumor activity in a number of preclinical models (11). As an initial step in identifying a clinically applicable HDAC inhibitor for combination with radiotherapy, we investigated the effects of MS-275 on the in vitro radiosensitivity of the two human tumor cell lines DU145 (prostate carcinoma) and U251 (glioma) cells (12). Exposure of DU145 and U251 cells to MS-275 before and after irradiation resulted in a significant increase in radiosensitivity, whereas exposure of cells to MS-275 only before or only after irradiation had essentially no effect. In these studies, HDAC inhibition was evaluated in terms of histone hyperacetylation. Maximal hyperacetylation was achieved by 24 to 48 hours of exposure; after removal of MS-275, the degree of acetylation rapidly decreased by 6 hours, approaching control levels by ~16 hours. The failure of MS-275 administered only before or only after irradiation to significantly enhance radiosensitivity suggests that the hyperacetylation must be present at the time of and for at least 6 hours after radiation exposure. These data suggested a correlation between MS-275-induced histone hyperacetylation and the enhancement of radiosensitivity. Moreover, they suggested that histone acetylation could be used as a marker in the design of in vivo antitumor protocols combining MS-275 and radiation.
To further evaluate the antitumor potential of an MS-275/radiation combination, we have extended these in vitro results to an in vivo xenograft model. Mice bearing DU-145 tumors were exposed to an increasing number of MS-275 injections and the level of tumor histone acetylation determined. Radiation was then delivered in a single exposure at the time of maximum histone acetylation, which resulted in a significant enhancement in tumor response. Moreover, a similar histone hyperacetylation response was detected in lymphocytes, suggesting an approach for the rational design of a clinical protocol combining MS-275 and radiotherapy.

MATERIALS AND METHODS

Materials. DU145 a human prostate tumor cell line was obtained from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) containing glutamate (5 mmol/L) and 5% FBS and maintained at 37°C in an atmosphere of 5% CO2 and 95% room air. MS-275, provided by the Developmental Therapeutics Program of the National Cancer Institute, was dissolved in 5 mL of 0.5 N HCl. 45 mL of double-distilled water, and 500 μL of Tween 80 and stored at −20°C.

In vivo Tumor Model. Four to 6-week-old male SCID mice (Fredrick Labs, Frederick, MD) were used in these studies. Mice were caged in groups of five or less, and all animals were fed a diet of animal chow and water ad libitum. For the in vivo passageing of tumors, animals with tumors 1000 mm3 were sacrificed and the skin overlying the tumor cleaned with betaine and ethanol. A single cell suspension of tumor cells in 0.9% sodium citrate, from C57BL/6 mice. The whole blood was diluted with an equal volume of PBS, layered on lymphoprep (sodium citrate) from whole blood, which was collected in tubes containing sodium citrate, from C57BL/6 mice. The whole blood was diluted with an equal volume of PBS, layered on lymphoprep reagent (Axis-Shift, Oslo, Norway) at a reagent to blood volume ratio of 1:2.5, and centrifuged at 800 × g at room temperature for 30 minutes. After centrifugation, the lymphocyte band was collected, washed three times with cold PBS, and prepared for immunoblot as above. Blood collected and pooled from five animals was the source for each lymphocyte sample.

Histone Acetylation Analysis. The acetylation status of histone H4 was determined by immunoblot analysis. Fresh tumor tissue was used to generate a single cell suspension as described above, which was then centrifuged at 2000 × g for 10 minutes at 4°C. Cells were resuspended in lysis buffer containing Tris (0.02 mol/L, pH 7.4), 1% Triton X-100, 0.02% 2-mercaptoethanol, and 2 ng/mL aprotinin. Proteins were solubilized by sonication and 20 mg subjected to SDS-PAGE using Novex NuPage 4 to 12% gels and the NuPage MES buffer system according to the manufacturer’s instructions (Invitrogen, San Diego, CA). After electrophoresis, the gel was electroblotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The nonspecific sites on the membrane were blocked at room temperature for 3 hours with 5% nonfat milk in Tris-buffered saline supplemented with 0.1% Tween 20 (TBS-T). Membranes were probed with antibodies to acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY) or actin (Chemicon International, Inc., Temecula, CA) diluted in blocking solution overnight at 4°C. Membranes were then washed three times in TBS-T and incubated with the appropriate FITC-conjugated secondary antibody at a 1:500 dilution in blocking solution for 1 hour at room temperature. Membranes were again washed three times in TBS-T and incubated with an anti-FITC alkaline phosphatase-conjugated antibody (1:1000) for 1 hour at room temperature. Enhanced chemiluminescence Western blotting detection reagents (Amersham/Pharmacia) were used to detect the alkaline phosphatase tertiary antibody. Visualization was performed using the Typhoon scanner (Molecular Dynamics, Sunnyvale, CA).

Tumor Biopsies. Mice were anesthetized by inhaling isoflurane via a nose cone, and a 20-mg section of tumor was removed using a 14-gauge biopsy gun. The incision was sutured with 4-0 PDS and mice returned to their individual cages. The second biopsy was obtained, 6 hours after the sixth dose of drug, from the opposite side of the tumor followed by sacrificing of the mouse, after which, tissue from normal organs was obtained. Single cell suspensions were prepared from the ex vivo material as described above and immunoblot analysis performed.

Lymphocyte Preparation. Lymphocytes were harvested from whole blood, which was collected in tubes containing sodium citrate, from C57BL/6 mice. The whole blood was diluted with an equal volume of PBS, layered on lymphoprep reagent (Axis-Shift, Oslo, Norway) at a reagent to blood volume ratio of 1:2.5, and centrifuged at 800 × g at room temperature for 30 minutes. After centrifugation, the lymphocyte band was collected, washed three times with cold PBS, and prepared for immunoblot as above. Blood collected and pooled from five animals was the source for each lymphocyte sample.

Statistical Analysis. A two-way ANOVA was performed on the square-root transformed tumor volumes because the variances and SDs were approximately equal between the groups. A statistically significant P was define as <0.05.

RESULTS

The human prostate carcinoma cell line DU145, grown as xenograft tumors in the hind leg of male SCID mice, was used as an in vivo model for these studies. Previously, we showed a correlation between the HDAC inhibitory action of MS-275 and...
enhanced tumor cell radiosensitivity in vitro (12). Therefore, to rationally design an in vivo combination protocol, it was first necessary to determine the effects of MS-275 on the histone acetylation status of DU145 xenografts.

Animals were injected with MS-275 (12 mg/kg/day), a daily amount previously shown to induce a modest tumor growth delay in an Ewing’s sarcoma xenograft (14), given as a 6 mg/kg/injection, at 12-hour intervals for up to 3 days. Mice were sacrificed daily 6 hours after two, four, or six injections, and the level of acetylated histone H4 in each of DU145 tumors determined by immunoblot analysis. The result from a representative experiment is shown in Fig. 1A. An increase in the level of acetylated histone H4 was detected after two injections of MS-275 with the maximum level achieved after 4–6 injections. Previous in vitro studies indicated that histone hyperacetylation decreased rapidly after the removal of MS-275. To determine whether a similar loss in histone hyperacetylation occurs in vivo, mice bearing DU145 tumors were injected twice daily for 3 days with MS-275 (six injections of 6 mg/kg delivered at 12-hour intervals) to induce maximum acetylation; tumors were then analyzed for histone H4 acetylation at 24 and 48 hours after the last injection (Fig. 1B). Histone H4 hyperacetylation was significantly reduced by 24 hours after the last injection of MS-275 and returned to control levels by 48 hours. These data are consistent with the in vitro results in that there is a rapid loss of histone hyperacetylation after the cessation of MS-275 treatment.

The histone acetylation data presented in Fig. 1 were generated from tumors grown in individual animals. To investigate the potential of the following histone acetylation status in the same tumor in a given animal and potentially overcome some of the interanimal variability seen in Fig. 1, A and B, we performed serial biopsies on individual DU145 tumors before and 6 hours after six doses of MS-275. In these experiments, a 14-gauge biopsy gun was used to biopsy an area of tumor the day before initiation of MS-275 treatment. The wound was closed with 4–0 PDS and the second biopsy was performed on the opposite side of the tumor 6 hours after the sixth injection of MS-275. As shown in Fig. 2, tumor samples obtained from mice before treatment contained no detectable histone H4 acetylation. However, tumor samples obtained from the same mice after treatment with MS-275 contained significant levels of acetylated histone H4. Mice that were biopsied twice but received no MS-275 injections had no detectable increase in histone H4 acetylation (data not shown). These data suggest that analysis of serial tumor biopsies is an effective means of histone acetylation status in individual animals.

These histone hyperacetylation data were then used to design an in vivo combination protocol aimed at determining whether MS-275 enhances the radioreponse of DU145 tumor xenografts. Because our previous in vitro study suggested that histone hyperacetylation had to be present before and maintained after irradiation, the in vivo protocol involved delivering MS-275 for 2 days before and 1 day after irradiation. Specifically, animals with s.c. DU-145 leg tumor xenografts (≈500 mm3) were randomized into four groups: vehicle; MS-275 alone; radiation alone (6 Gy); or the combination of MS-275 and radiation (n = 8). Treatment with MS-275 (6 mg/kg, i.p., every 12 hours for 3 days for a total of six doses) began the day of randomization. Radiation was delivered 6 hours after the fourth dose of MS-275 (time of maximum histone acetylation), which was followed by two more injections of MS-275. As shown in Fig. 3, tumor growth rate, measured as the mean tumor volumes per group, in groups receiving MS-275 or 6 Gy only was only marginally reduced as compared with control animals. However, the combination treatment resulted in a slower tumor growth rate than the individual treatments. The tumor growth delays, calculated for the individual mice in a treatment group as the mean number of days for a tumor to reach 1500 mm3 minus the mean number of days for the control tumors to reach 1500 mm3, from this experiment were 2.9 ± 2.7 and 1.9 ± 2.3 for the
MS-275 and 6 Gy treatment groups, respectively, whereas the tumor growth delay induced by the combination treatment was 8.3 ± 3.9. The results were statistically significant (ANOVA) comparing the combination of MS-275/radiation to drug alone (P < 0.001) or radiation alone (P < 0.005). Similar results were obtained in a repeat of this experiment. Thus, consistent with previous in vitro experiments, these data indicate that MS-275 enhances the radiosensitivity of DU145 xenografts.

In a clinical setting, it is not always possible to obtain tumor biopsies before, during, and after treatment. The availability of a surrogate tissue that could provide relevant information regarding a putative marker would be of considerable benefit in protocol design. In a treatment situation, one of the most easily obtained cell types is the lymphocyte, which would be an ideal candidate as a surrogate tissue for evaluating the effects of MS-275 on histone acetylation. However, in the studies described here, DU145 xenografts were grown in SCID mice, which lack a significant lymphocyte population. Therefore, to investigate the potential of lymphocytes to serve as readily available tissue for evaluation of histone acetylation status we used non–tumor-bearing C57BL/6 mice. These mice were injected with four doses of MS-275 delivered at 12-hour intervals and lymphocytes collected 6 hours after the last injection. This protocol resulted in the maximal increase in histone H4 acetylation in the DU145 xenografts after treatment of SCID mice (Fig. 1). As shown in Fig. 4, lymphocytes isolated from C57BL/6 mice treated with MS-275 had a significantly greater level of histone H4 acetylation than lymphocytes isolated from vehicle-injected mice. In an attempt to control for the change in mouse strain, we determined whether other normal tissue from SCID mice responded in a similar manner to MS-275 as did the lymphocytes from C57BL/6 mice. SCID mice received injections of MS-275 at 12-hour intervals for up to 3 days; they were sacrificed at 6 hours after the last injection, and liver and spleen tissue were then evaluated for histone H4 acetylation (Fig. 5). Histone H4 hyperacetylation was clearly evident after four injections of MS-275 treatment in both the liver and spleen of SCID mice. Thus the data presented in Figs. 1, 4, and 5 indicate that tumor tissue, liver, and spleen from SCID mice, as well as lymphocytes from C57BL/6 mice, all had similar temporal responses in histone H4 acetylation status after MS-275 treatment. These data suggest that in the clinical design of MS-275/radiotherapy combination protocols, patient lymphocytes may serve as a surrogate marker for tumor hyperacetylation after MS-275 treatment.

DISCUSSION

Although the use of molecularly targeted agents has received considerable attention as a cancer treatment strategy, to date, most of these agents have been found to be primarily cytostatic when used against solid tumors (15, 16). However, in some cases, these agents are directed against molecules/targets that can also affect radiosensitivity, suggesting that advantages could be obtained through the combination with radiotherapy. One such class of agents is HDAC inhibitors. Sodium butyrate
and trichostatin A, HDAC inhibitors, have been reported to enhance radiosensitivity in \textit{in vitro} cell models, although a role of histone acetylation was not established for either compound. Importantly, because of pharmacokinetic parameters and/or toxicity, these agents are not readily applicable to \textit{in vivo} treatment situations. Over the last several years, a number of clinically applicable HDAC inhibitors have been developed and evaluated in preclinical tumor models. One of these new HDAC inhibitors, the benzamide derivative, MS-275, has been shown to induce differentiation and apoptosis of leukemia cells (17) and to slow the \textit{in vivo} growth of a number of human tumor xenografts (11, 14, 18). We recently reported that MS-275 also enhances the \textit{in vitro} radiosensitivity of cell lines initiated from solid human tumors and that this sensitization correlated with histone hyperacetylation. Recently, another clinically applicable HDAC inhibitor SAHA was reported to enhance the \textit{in vitro} cytotoxicity induced by a number of DNA-damaging anticancer drugs (19). Thus, it appears that HDAC inhibitors may have a general ability to enhance the cytotoxicity initiated by DNA damage. Whether the mechanisms involved in enhancing drug response and radioresponse are the same remains to be investigated.

To further evaluate the clinical potential of the MS-275/radiation combination, it was necessary to extend the \textit{in vitro} observations to an \textit{in vivo} human tumor xenograft model. Moreover, one of the putative advantages of the molecular targeting strategy is the existence of a marker indicative of drug action. Thus, in an attempt to reduce the empiricism involved in the design of combined modality protocols, in the studies described here, radiation was delivered at a time corresponding to MS-275-induced histone hyperacetylation, which is typically used as a marker for HDAC inhibition. Moreover, \textit{in vitro} studies suggested that it was necessary to maintain histone hyperacetylation after irradiation to obtain the optimal sensitizing effect. The maximum histone acetylation in DU145 xenografts was achieved after four to six injections of MS-275; after the last injection of MS-275, histone acetylation rapidly decreased by 24 hours. These data then suggested that to increase tumor radiosensitivity radiation should be delivered after four doses of MS-275 and followed by the two additional injections. When MS-275 and radiation were combined in this manner, the result in tumor growth delay was greater than additive. Whether histone hyperacetylation plays a specific mechanistic role in radiosensitization remains to be determined. However, the \textit{in vivo} results presented here combined with previous \textit{in vitro} data suggest that tumor histone acetylation can be used as a marker for the radiosensitizing actions of MS-275, which would aid in the design of clinical combination protocols.

Because multiple tumor biopsies are not always feasible in patients, the availability of surrogate tissues for evaluating the effects of molecularly targeted agents would be of benefit. The data presented here indicate that the effects of MS-275 on histone acetylation in DU145 tumor xenografts correlated with the changes detected in liver, spleen, and lymphocytes. Clearly, sampling liver or spleen is not readily translated to a clinical situation. However, the potential of using lymphocytes as an indicator of the radiosensitizing effects of MS-275 or other HDAC inhibitor would be advantageous in the design of clinical combination protocols. Whereas the MS-275-induced histone hyperacetylation patterns in normal tissue may provide pertinent pharmacodynamic information, it also suggests the potential for enhanced radiation-induced normal tissue injury. However, previous results using \textit{in vitro} systems have shown that, although HDAC inhibitors induce histone hyperacetylation in normal cells, the toxicity induced is considerably less than detected in tumor cells (20). This suggests that, consistent with the differences in histone acetylation patterns between normal and tumor cells, the response of these cell types to changes in acetylation may also differ. At present, this clearly remains speculation and requires additional investigations into the mechanism of MS-275-induced radiosensitization, as well as additional \textit{in vivo} studies specifically addressing normal tissue response.

The goal of this study was to extend previous \textit{in vitro} findings of the enhancement of tumor cell radiosensitivity by MS-275 to an \textit{in vivo} model and to determine whether histone hyperacetylation can be used in the design of the combined modality protocol. To our knowledge, this is the first study to report an increase in tumor radiosensitivity when combining an HDAC inhibitor and radiation \textit{in vivo}. Moreover, either tumor or lymphocyte histone hyperacetylation status appears to be a marker for the effective combination.

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