Introduction

Glioblastoma multiforme carries a poor prognosis, and although radiation therapy prolongs patient survival, cures remain discouragingly elusive. The clear need for new, biologically effective agents has propelled investigations into histone deacetylase (HDAC) inhibitors (HDACI) that in turn have emerged as promising anticancer therapies (1–12). HDACIs display unique antineoplastic properties most likely through increased nuclear histone acetylation and modulation of gene expression. A dynamic equilibrium between histone acetylation and HDAC controls levels of acetylated histones in nuclear chromatin (13). Through regulation of gene expression and through potential additional unknown mechanisms, HDACIs play key roles in growth arrest, differentiation, and apoptosis of tumor cells (2–11, 14). Several HDACIs are in preclinical and clinical development, including a prodrug of butyric acid [pivaloyloxymethyl butyrate (AN-9); refs. 12, 15], hydroxamic acids (suberoylanilide hydroxamic acid and trichostatin A; refs. 16–19), benzamid derivatives (MS-275 and CI-994; refs. 20–23), cyclic peptides (trapoxin, apicidin, and depsipeptide; refs. 24–27), and valproic acid (28). Recent studies have revealed radiosensitizing capacity of various HDACIs, including MS-275 (29), suberoylanilide hydroxamic acid (30), valproic acid (31), and trichostatin A (30, 32), indicating potential advantages to incorporating HDACIs into multimodality therapy for gliomas.

Butyric acid, a HDACI, possesses low efficacy in vivo due to its rapid metabolism (33); to overcome this limitation, acyloxymethyl esters of butyric acid were synthesized and characterized (4, 5). Acyloxymethyl esters of butyric acid efficiently deliver butyric acid, resulting in higher intracellular concentrations. Among these butyric acid prodrugs, AN-9, which on hydrolysis releases butyric acid, formaldehyde, and pivalic acid, is the best characterized (Fig. 1). AN-9 has been evaluated in a phase I clinical study that revealed very limited toxicities (12). In phase II clinical trials, AN-9 improved the well-being and survival of patients with non–small cell lung carcinoma (15). Antineoplastic activity has been shown for additional butyric acid prodrugs, including butyroyloxymethyl butyrate (AN-1) that releases two equivalents of butyric acid and one equivalent of formaldehyde (11, 34).

We sought to assess the antineoplastic activity of this family of HDACIs in the treatment of high-grade gliomas. AN-1 and AN-9 exhibited cytotoxic effects against all tested glioma cell lines, potentiation of radiation therapy, and in vivo inhibition of tumor growth in an in vivo flank model of glioblastoma multiforme.

Butyric acid prodrugs are histone deacetylase inhibitors that show antineoplastic activity and radiosensitizing capacity in the treatment of malignant gliomas

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Abstract

Histone modification has emerged as a promising approach to cancer therapy. We explored the efficacy of a novel class of histone deacetylase inhibitors in the treatment of malignant gliomas. Treatment of glioma cell lines with two butyric acid derivatives, pivaloyloxymethyl butyrate (AN-9) and butyroyloxymethyl butyrate (AN-1), induced hyperacetylation, increased p21cip expression, inhibited proliferation, and enhanced apoptosis. Histone deacetylase inhibitor–induced apoptosis was mediated primarily by clonal survival curves revealed marked reductions in cell proliferation, and enhanced apoptosis. Clonal—irradiation potentiated—irradiation. Preliminary in vivo experiments using human glioma cell lines grown as xenografts in mouse flanks suggest in vivo efficacy of AN-9. The data suggest that novel butyric acid prodrugs provide a promising treatment strategy for malignant gliomas as single agents and in combination with radiation therapy.

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Materials and Methods

Cell Culture and Irradiation

U87 MG, U251 MG, and SF188 glioma cell lines as well as primary human astrocytes (35) were grown in a humidified incubator at 37°C and 8% CO2 in DMEM supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA) and 100 units/mL penicillin-100 g/mL streptomycin (Life Technologies). Cells were irradiated at room temperature in a Mark I-68 Cesium 137 irradiator (J.L. Shepherd & Associates, San Fernando, CA) at a dose rate of 2.26 Gy/min.

Reagents

The butyric acid derivatives AN-9 and AN-1 were prepared as described previously (7, 36). For in vitro studies, compounds were solubilized in DMSO followed by dilution in medium to a final DMSO concentration of ≤0.2%. Caspase inhibitors included the pan-caspase peptide inhibitor Z-Ala-Asp-fluoromethylketone (Z-VAD-fmk; FK-009) and a more caspase-8 specific inhibitor Z-Ile-Glu(OMe)-Thr-Asp(OMe)-Asp-fluoromethylketone (Z-IETD-fmk; FK-012; Enzyme Systems Products, Livermore, CA). Antibodies included rabbit anti-acetylated histone H4 (Lys12, Lys16, Lys24, and Lys29), rabbit anti-histone H3 (Lys20, Lys36), rabbit anti-total H4, rabbit anti-cleaved caspase-3, mouse anti-p21Cip1, mouse anti–poly(ADP-ribose) polymerase (PARP; all from Cell Signaling Technology, Beverly, MA), mouse anti-Ki-67 (DakoCytomation California Inc., Carpinteria, CA; clone MIB1), mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO), biotinylated horse anti-mouse, and biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA).

Histone Acetylation

Three million cells per 100-mm plate were seeded, allowed to adhere for 24 hours, and then treated with HDACi or control medium. At specified times following treatment, cells were collected by trypsinization, washed with PBS, and lysed in 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L DTT, and 1.5 mmol/L phenylmethylsulfonyl fluoride. Histones were acid extracted with H2SO4 (0.2 mol/L) for 1 hour and dialyzed against 0.1 mol/L acetic acid for 2 hours and twice against water (2 hours each). A third overnight dialysis was carried out against water containing 50% glycerol. A similar procedure was carried out to assess in vivo histone acetylation using homogenized brain tissue harvested at specified times following treatment.

Western Blot Analysis

Whole-cell lysates or acid-extracted histones were solubilized in sample buffer and equal amounts of protein were separated on SDS-PAGE. Purified histones were transferred to nitrocellulose membranes and whole-cell lysates to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were probed with primary antibody and then with horseradish peroxidase–conjugated species-specific secondary antibody (Amersham Biosciences, Piscataway, NJ). Band intensities, visualized by enhanced chemiluminescence, were measured using Scion Image software (Scion Corp., based on NIH image for macintosh, Frederick, MD).

HDAC Activity Assay

Triplicate samples of 25 μg brain extract or 3 μg cell extract were loaded per well of 96-well plates and HDAC activity was assayed following the manufacturer’s instructions (Biomial, Plymouth Meeting, PA). HDAC activity was reflected in increased fluorescence that was not emitted by the deacetylated substrate. Each experiment was carried out four times starting with different sets of animals or cells.

Cell Proliferation

U251 MG and U87 MG (3,000 cells per well), SF188, and primary human astrocytes (5,000 cells per well) were seeded in 96-well plates. Sixteen hours later, medium was aspirated, and fresh medium (100 μL) containing escalating drug doses was added. Cells were then incubated for 48 hours following addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) reagent (Promega, Madison, WI) and further incubation for 1 to 3 hours. Light absorbance, which reflects cellular viability, was measured using a plate reader at A490 nm.

Colony Formation Assays

For clonogenic survival assays, exponentially growing U251 MG cells were treated with escalating doses of AN-1 for 24 hours and harvested by trypsinization. Specified numbers of cells were plated in six-well plates already containing 20,000 cells per well of lethally irradiated feeder SF188 cells to maximize plating efficiency. For colony formation assays assessing interactions with radiation, specified numbers of cells were plated in medium containing AN-1 (35 μmol/L). Following a 16-hour incubation, cells were irradiated with specified doses and colony-forming efficiency was determined in the continued presence of AN-1. Cultures were then incubated for 8 days and colonies of ≥50 cells were scored. Cell survival measurements were fitted to a linear quadratic mathematical model using the FIT 2.5 program (37, 38). Within each of at least two independent experiments, two to four different dilutions were made per radiation dose and each dilution was plated in triplicates.
Butyric Acid Prodrugs as HDACI Therapy for Gliomas

initiated with two different sets of cells in culture, were done. In each experiment, three independent measurements were taken for each sample. The mean ± SE values were generated from the two mean values obtained for each sample in the two individual experiments.

Apoptosis Assay
Glioma cells were seeded in six-well plates (100,000 per well), allowed to adhere, and incubated with AN-1 or AN-9 for 48 hours. Adherent and nonadherent cells were collected and double-stained with Annexin V-FITC and propidium iodide according to the manufacturer’s instructions (Calbiochem, San Diego, CA). Apoptotic cells were quantitated by flow cytometry using a FACSCalibur cytometer (Becton Dickinson, Rockville, MD).

In vivo Flank Xenografts and Drug Administration
Xenografts of human glioma cell lines were established by s.c. inoculation of 3 × 10⁶ U251 MG cells into flanks of BALB/c nu/nu athymic mice (Charles River Laboratories, Wilmington, MA). Mice were monitored according to the protocol approved by the Institutional Animal Care and Use Committee. BALB/c nu/nu athymic mice were inoculated with 3 × 10⁶ U251 MG cells, and drug administration began when xenografts reached 30 mm³ in size. Mice were randomly assigned to one of two groups: mineral oil vehicle alone as control or 200 mg/kg AN-9 diluted in mineral oil. Agents were given by oral gavage using 20-gauge curved needles (Popper and Sons, New Hyde Park, NY). Oral drug administration continued for the entire duration of the experiment, consisting of 150 days after initiation of treatment, unless the tumor reached 2.5 cm, at which point mice were euthanized in accordance with the protocol approved by the Institutional Animal Care and Use Committee.

H&E Staining and Immunohistochemical Analysis of Ki-67 and Cleaved Caspase-3
Formalin-fixed, paraffin-embedded sections, each 5 μm thick, were cut from tumors excised from euthanized mice. For H&E staining, deparaffinized slides were stained with hematoxylin for 5 minutes, washed with acetalcohol (differentiation solution) for 30 seconds, and then stained with eosin of the cyclin-dependent kinase inhibitor p21⁰Cip1 is a hallmark of HDACI activity (2). Elevation in p21⁰Cip1 expression is associated with cell cycle arrest, induction of differentiation, and/or apoptosis (8), all cellular responses induced by HDACI treatment. Consistent with these roles of p21⁰Cip1, a dose-dependent increase in p21⁰Cip1 expression in all glioma cell lines was observed following AN-1 and AN-9 treatment. As predicted by their p53 status, basal p21⁰Cip1 expression levels were high in primary astrocytes and U87 MG that express wild-type p53 and low in U251 MG and SF188 that harbor p53 mutations.

Effect of Butyric Acid Prodrugs on Proliferation and Colony Formation of Gliomas and Primary Astrocytes
We investigated the biological consequences of AN-1 and AN-9 treatment, focusing first on cellular viability and proliferation. To establish drug concentrations resulting in at least three independent dose-response titrations. Comparisons of drug activity were done by two-sided t tests or two-way ANOVA (Microsoft Excel). Survival was estimated by Kaplan-Meier curves, and differences in survival between the groups were analyzed by the Cox Mantel test (Statistica, Tulsa, OK, StatSoft Derived Comparisons, Inc., 1984-2001).

Results

HDAC Activity and p21⁰Cip1 Expression
We explored whether AN-9 and AN-1 inhibit HDAC activity and increase histone acetylation, as their purported mechanism of action would predict. Treatment of U251 MG in vitro with 100 μmol/L AN-1 or AN-9 increased histone H4 and H3 acetylation, with a histone H4 acetylation peak of 5 to 30 hours for both AN-1 and AN-9, and histone H3 acetylation peaks of 1.5 to 18 and 3 to 5 hours for AN-1 and AN-9, respectively (Fig. 2A). In vivo experiments using histones purified from brains of mice that were treated with AN-9 (100 mg/kg given orally) exhibited a broad peak of histone H3 and H4 acetylation 1.5 to 5 hours after drug administration followed by a decline in acetylation 18 hours after treatment. Of note, acetylated histone H4 levels were still higher than control levels even at this later time point (Fig. 2B). Consistent with the observed elevation in histone acetylation, HDAC activity in extracts of U251 MG and mouse brains exhibited peak inhibition 3 to 5 hours following treatment with either AN-1 or AN-9, with gradual recovery thereafter (Fig. 2C).

The data show that these butyric acid–releasing prodrugs efficiently cross the blood-brain barrier and effectively increase acetylation, prompting us to investigate their utility in the treatment of malignant gliomas.

Previous studies have indicated that increased expression of the cyclin-dependent kinase inhibitor p21⁰Cip1 is a hallmark of HDACI activity (2). Elevation in p21⁰Cip1 expression is associated with cell cycle arrest, induction of differentiation, and/or apoptosis (8), all cellular responses induced by HDACI treatment. Consistent with these roles of p21⁰Cip1, a dose-dependent increase in p21⁰Cip1 expression in all glioma cell lines was observed following AN-1 and AN-9 treatment. As predicted by their p53 status, basal p21⁰Cip1 expression levels were high in primary astrocytes and U87 MG that express wild-type p53 and low in U251 MG and SF188 that harbor p53 mutations (Fig. 3A).

Effect of Butyric Acid Prodrugs on Proliferation and Colony Formation of Gliomas and Primary Astrocytes
We investigated the biological consequences of AN-1 and AN-9 treatment, focusing first on cellular viability and proliferation. To establish drug concentrations resulting in 50% growth inhibition (IC₅₀), MTS assays were done 48 hours after drug treatment. IC₅₀ values were calculated by linear regression and averages of three to five independent experiments are shown in Table 1. Both AN-1 and AN-9
displayed inhibitory activity in all three glioma cell lines tested as well as in human primary astrocytes. AN-1 was somewhat more potent than AN-9, reflected in more pronounced decreases in MTS values following AN-1 treatment \((P < 0.05; \text{two-sided } t \text{ test}).\)

Clonogenic survival assays confirmed the sensitivity of U251 MG to AN-1 treatment (Table 1). As expected, the IC\(_{50}\) value for clonogenic survival (39 \(\mu\)mol/L) was lower than the corresponding value generated by MTS assay (118 \(\mu\)mol/L) because sustained clonogenic capacity is a more rigorous measure of cell survival than persistent proliferation measured by MTS.

Decreases in MTS values may reflect cell death, growth arrest, or a combination thereof. To determine the mechanism of AN-9- and AN-1-induced cytotoxicity, cellular viability was quantitated using flow cytometry to detect Annexin V-FITC and propidium iodide staining. Following 48 hours of drug treatment, adherent and nonadherent cells were pooled and viable cells were identified as those stained with neither Annexin V-FITC nor propidium iodide. Percentages of viable cells are plotted in Fig. 3B and show that primary astrocytes underwent little to no apoptosis following treatment, whereas all three glioma cell lines displayed significant apoptotic responses. Whereas MTS assays did not show substantial differences among the cell lines, flow cytometry revealed significant differences in susceptibility to AN-9-induced apoptosis. Primary human astrocytes displayed only 20% cell death at 200 \(\mu\)mol/L AN-9, indicating that inhibition observed in MTS assays reflected mostly growth arrest. After identical treatments, virtually no SF188 cells remained viable, indicating that in this pediatric glioma cell
line AN-9-induced decreases in MTS values reflected chiefly cell death. U87 MG and U251 MG displayed an intermediate susceptibility to AN-9-induced cell death, 49% and 58% cell death at 200 μmol/L AN-9, respectively. The above data indicate that following AN-9 treatment the dominant cellular response is growth arrest in normal astrocytes and cell death in glioma cell lines, reflecting some selective toxicity of AN-9 for malignant cells. Of note, different mechanisms contribute to the efficacy of AN-9 in various glioma cell lines, for example, predominantly apoptosis in SF188 and a combination of apoptosis and growth arrest in U251 MG and U87 MG.

**Figure 3.** Induction of cell death by acyloxy methyl esters of butyric acid. A, PARP cleavage and p21<sup>Cip1</sup> expression. Glioma cells and primary astrocytes were treated with escalating doses of AN-1 for 24 h. Cell lysates (20 μg) were subjected to Western blot analysis using p21<sup>Cip1</sup> and PARP antibodies as well as α-actin as loading control. B, primary astrocytes and glioma cells were treated with AN-9 for 48 h. Adherent and nonadherent cells were pooled, double stained with Annexin V-FITC and propidium iodide, and analyzed by flow cytometry. Columns, mean of three independent experiments; bars, SE. C, activities of caspase-8 and caspase-9 in U251 MG and SF188 treated with AN-1 for designated periods of time. Treatment with tumor necrosis factor (TNF; 20 ng/mL) and cycloheximide (CHX; 30 μg/mL) was used as a positive control for caspase-8 activity. UV irradiation (400 J) was used as a positive control for caspase-9 activity. Y axes, ratios of activity in treated samples versus untreated controls. Columns, mean of two independent experiments; bars, SE. D, rescue of U251 MG and SF188 cells from apoptosis by 200 μmol/L Z-VAD or Z-IETD was evaluated by MTS assays. Y axis, percent proliferation in treated samples versus untreated controls. Columns, mean of three independent experiments; bars, SE.
Table 1. Effects of AN-1 and AN-9 on cell proliferation and colony formation

<table>
<thead>
<tr>
<th>Cell type</th>
<th>AN-1 (IC50, μmol/L)</th>
<th>AN-9 (IC50, μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTS proliferation assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary astrocytes</td>
<td>147 ± 16</td>
<td>219 ± 10</td>
</tr>
<tr>
<td>U251 MG</td>
<td>118 ± 13</td>
<td>177 ± 4</td>
</tr>
<tr>
<td>SF188</td>
<td>94 ± 5</td>
<td>141 ± 8</td>
</tr>
<tr>
<td>U87 MG</td>
<td>88 ± 10</td>
<td>134 ± 7</td>
</tr>
<tr>
<td>Colony formation assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U251 MG (plating efficiency, 60%)</td>
<td>39 ± 4</td>
<td>Not done</td>
</tr>
</tbody>
</table>

To further assess apoptosis after HDACI treatment, PARP cleavage was evaluated by Western blot analysis. The data (Fig. 3A) are consistent with those obtained by flow cytometry (Fig. 3B) and show that PARP cleavage was most prominent in SF188, somewhat less apparent in U251 MG, and absent in U87 MG and primary astrocytes. These data complement the flow cytometry results in showing the range of HDACI-induced susceptibility to apoptosis seen in gliomas. Our observation that primary astrocytes express exceedingly low levels of PARP proform is consistent with previously published reports (39).

**Mechanism of HDACI-Induced Apoptosis in Gliomas**

The mechanism of AN-1-induced apoptosis was investigated by asking which caspase pathways were activated in gliomas following HDACI treatment. By using caspase-specific fluorescent substrates, the activities of caspase-8 and caspase-9 were measured following treatment with 100 μmol/L AN-1 (Fig. 3C). In SF188, AN-1 treatment induced activities of both caspase-8 and caspase-9, suggesting that both death receptor- and mitochondria-mediated pathways of apoptosis triggered cell death. In U251 MG, however, AN-1 exposure induced only caspase-8 activity, indicating that only the death receptor–mediated pathway executed apoptosis in this cell line. Levels of caspase-8 activity were lower in treated U251 MG than in treated SF188 cells, consistent with higher susceptibility to apoptosis of SF188 exposed to HDACIs.

We further examined the roles of caspases in HDACI-induced apoptosis by using a pan-caspase inhibitor, Z-VAD, and a more specific caspase-8 inhibitor, Z-IETD. Incubation with Z-VAD partially overcame AN-1-induced inhibition of cell proliferation (50-60%) for both U251 MG and SF188. In U251 MG, Z-IETD mimicked the protection afforded by Z-VAD, consistent with the dominant role of caspase-8 in AN-1-induced apoptosis of U251 MG (Fig. 3D). In SF188, Z-IETD enhanced cell proliferation by only 32% compared with 53% seen with Z-VAD, reflecting the dual roles of both caspase pathways in HDACI-induced apoptosis of this cell line.

**Combination Treatment of Radiation and Butyric Acid Prodrugs**

AN-1 and AN-9 exhibited promising antineoplastic activities as single agents in vitro, an important finding given the global resistance of gliomas to anticancer agents. Radiation has a proven role in the treatment of gliomas and we therefore sought to investigate the combined effects of ionizing radiation and HDACIs. To optimize this combined treatment, preliminary MTS assays were done to explore several treatment sequences. Pretreatment of cells with AN-9 followed by irradiation 24 hours later maximally enhanced growth inhibition (data not shown). Other sequencing approaches, such as irradiation before AN-9 or concurrent treatments, did not enhance growth inhibition.

To extend these analyses, gliomas and primary astrocytes were treated with AN-9 or AN-1 for 24 hours before irradiation and subjected to analysis by flow cytometry 48 hours later. Figure 4A and Table 2 show that combined HDACI and radiation treatments resulted in at least additive cell death in all cell lines tested. In SF188, AN-9 (100 μmol/L) caused 4% cell death, 3 Gy alone resulted in 16% mortality, but combination thereof enhanced cell death to 51% (Table 2).

To confirm the potentiation of radiation by HDACIs, clonogenic survival assays were done by pretreating U251 MG with AN-1 (35 μmol/L) followed 24 hours later by irradiation. Cell survival curves shown in Fig. 4B confirmed the efficacy and potentiation of combinatorial treatments with HDACI and radiation.

To examine the mechanism of cytotoxic potentiation of HDACI and radiation, caspase-8 activity assays were repeated, this time with AN-1 and radiation as single agents and in combination (Fig. 4C). In SF188, AN-1 (75 μmol/L) in combination with irradiation (2 Gy) significantly enhanced the apoptotic response compared with each treatment alone after 6 and 24 hours ($P < 0.05$, two-way ANOVA) but not after 48 hours ($P = 0.6$). In U251 MG, such potentiation between AN-1 (75 μmol/L) and irradiation (3 Gy) was statistically significant only when assayed 48 hours ($P < 0.03$) but not 6 or 24 hours after irradiation ($P = 0.6$ and 0.5, respectively). Potentiation of irradiation by HDACIs occurred at earlier time points in SF188 than in U251 MG, consistent with higher sensitivities of SF188 to both modalities. The data suggest that caspase-8 activity is responsible at least in part for the potentiation of cytotoxicity documented with combinations of HDACIs and radiotherapy.

**In vivo Efficacy of AN-9**

In view of the encouraging in vitro data, we sought to expand our studies to test the in vivo efficacy of AN-9 in mice bearing flank xenografts of human glioma cell lines. We concentrated our efforts on AN-9, rather than on AN-1, because completed and ongoing clinical trials are testing AN-9, and a maximum tolerated dose already exists for this drug, thus paving the way for clinical studies of AN-9 in glioma patients. In a preliminary experiment, BALB/c nu/nu athymic mice were inoculated with $3 \times 10^6$ U251 MG cells, and drug administration began when xenografts reached 30 mm$^3$ in size. Mice were randomly assigned to one of two groups: mineral oil vehicle as control or 200 mg/kg AN-9 diluted in the same vehicle. Agents were given by oral gavage thrice weekly for a complete 150-day course or until tumors reached 2.5 cm, at which point mice were euthanized.
The survival curves of the treated mice in this preliminary experiment are shown in Fig. 5A. All tumors in the control group (8 of 8) grew exponentially, showing no evidence of regression. In three of seven mice receiving 200 mg/kg AN-9, tumors did not grow beyond 150 mm³ for the entire duration of the experiment. Flank tumors in the remaining four mice receiving 200 mg/kg grew exponentially with no apparent growth delay. Survival analysis revealed a trend toward longer survival in mice receiving AN-9 (\( P = 0.1 \), Mantel-Cox comparison test). Of note, no constitutional signs or toxicities were observed in any of these mice for the entire duration of the experiment. Specifically, no changes were observed in any animal’s weight or activity and no pathologic signs

**Figure 4.** Effects of concurrent treatment with radiation and HDACIs. A, flow cytometry analysis of SF188 cells treated with AN-1 or AN-9 (100 \( \mu \)mol/L) and irradiation (3 Gy) as indicated. Percent viable cells for each sample are shown. B, colony formation assay of U251 MG treated with AN-1 (35 \( \mu \)mol/L) and irradiation. Clonogenic survival curves were generated after normalizing to cell killing by AN-1 alone. C, caspase-8 activities in U251 MG and SF188 treated with AN-1 (75 \( \mu \)mol/L), radiation (3 Gy for U251 MG and 2 Gy for SF 188), or combinations thereof. Results are presented as ratios of treated samples versus untreated controls. Points, mean of samples obtained from two independent experiments; bars, SE.
were documented. To further assess any potential toxicity, gross and microscopic examinations were done on livers and kidneys isolated from representative euthanized mice. These organs displayed normal architecture with no observed toxicities, except for rare calcifications observed in the kidney, most consistent with the age of the mice rather than treatment effects.

Of the three surviving mice, one tumor shrunk and was no longer detectable following treatment, precluding further biochemical tissue analyses. Tumors excised from the other two surviving animals following euthanization were assessed for morphology by H&E staining, for apoptosis by immunohistochemistry staining for the cleaved form of caspase-3, and for cellular proliferation by immunohistochemistry staining for Ki-67.

H&E staining revealed that tumors from control animals were highly cellular neoplasms with marked cellular pleomorphism, variable necrosis, and stromal desmoplasia. In contrast, tumors from the AN-9-treated animals were more heterogeneous; desmoplastic and less cellular zones adjacent to adipose tissue were admixed with cellular areas (Fig. 5B, top). In addition, tumors excised from the animals that were treated with AN-9 displayed a dramatic reduction in the proportion of proliferating cells as reflected by significantly reduced Ki-67 antigen staining (Fig. 5B, middle). Apoptotic cell populations in both control and drug-treated animals were low, with only a marginal increase in cleaved caspase-3 staining in treated animals (Fig. 5B, bottom). Our in vivo immunohistochemistry results indicate that, after oral administration of AN-9, growth arrest plays a key role in the cytotoxicity induced by these novel HDACIs.

Discussion

The promise of HDACIs for cancer treatment, as single agents and in combination with standard therapies, is supported by in vitro assays, preclinical experiments, and clinical studies. Recent studies show that butyric acid prodrugs that undergo intracellular esterase-catalyzed

Table 2. Effects of combined butyric acid–releasing HDACIs and irradiation on glioma cell viability

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF188</td>
<td></td>
</tr>
<tr>
<td>3 Gy</td>
<td>16</td>
</tr>
<tr>
<td>100 μmol/L AN-1</td>
<td>50</td>
</tr>
<tr>
<td>100 μmol/L AN-1 + 3 Gy</td>
<td>92.5*</td>
</tr>
<tr>
<td>100 μmol/L AN-9</td>
<td>4</td>
</tr>
<tr>
<td>100 μmol/L AN-9 + 3 Gy</td>
<td>51*</td>
</tr>
<tr>
<td>U251 MG</td>
<td></td>
</tr>
<tr>
<td>6 Gy</td>
<td>13.1</td>
</tr>
<tr>
<td>100 μmol/L AN-1</td>
<td>6.8</td>
</tr>
<tr>
<td>100 μmol/L AN-1 + 6 Gy</td>
<td>19.5*</td>
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<tr>
<td>100 μmol/L AN-9</td>
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<td>27.7*</td>
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<td>U87 MG</td>
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<tr>
<td>100 μmol/L AN-9</td>
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<tr>
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<td>20.0*</td>
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<tr>
<td>Primary human astrocytes</td>
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</tr>
<tr>
<td>6 Gy</td>
<td>0</td>
</tr>
<tr>
<td>200 μmol/L AN-9</td>
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<tr>
<td>200 μmol/L AN-9 + 6 Gy</td>
<td>21.1*</td>
</tr>
</tbody>
</table>

*Cellular viability for combined treatment modalities are shown in bold.

Figure 5. In vivo effects of AN-9. A, survival of nude mice treated with AN-9. BALB/c nu/nu athymic mice bearing 30 mm³ flank tumors were randomly assigned to one of two treatment groups: control vehicle or 200 mg/kg AN-9. The agents were given orally thrice weekly and tumor sizes were recorded. B, analyses of tumor sections from control (left) and AN-9-treated (right) BALB/c nu/nu athymic mice bearing xenografts of U251 MG. Top, H&E staining; middle, Ki-67 staining; bottom, cleaved caspase-3 staining.
hydrolysis are orally bioavailable, reduce tumor growth, and inhibit metastases in xenograft models of prostate cancer (34). Furthermore, AN-9 in intralipid formulation (Pivanex) increases survival and improves the well-being of patients in phase II clinical studies (12, 15).

In this study, we have shown that, whereas the dominant effects of AN-1 and AN-9 on normal astrocytes are chiefly cell cycle arrest, malignant gliomas display marked apoptotic responses. Of note, prodrug hydrolysis produces not only butyric acid but also one equivalent of formaldehyde, which has also been shown to induce apoptosis and inhibit proliferation of cancer cells (40). Thus, both butyric acid and formaldehyde have antineoplastic activity, the former acid by inhibiting HDAC and the latter aldehyde by an unknown mechanism.

As prodrugs of HDACIs, AN-1 and AN-9 augment the acetylation of H3 and H4 in glioma cell lines and in murine brain extracts. Consistent with the induction of histone acetylation are decreased HDAC activity and increased p21Cip1 expression in vitro and in vivo. p21Cip1 elevation is shown in all tested cell lines, including those with mutant p53, in which basal p53 levels are predictably much lower than in wild-type p53-expressing cells. These data are consistent with previous reports documenting p53-independent increases in p21Cip1 (8).

We explored the mechanism of HDACI-induced cytotoxicity in glioma cell lines and found that caspase-regulated pathways of apoptosis, both receptor-mediated (caspase-8) and mitochondria-mediated (caspase-9) components, participate in HDACI-induced cell death. However, experiments using caspase inhibitors suggest that additional caspase-independent mechanisms of cell death may come to bear as well. Such caspase-independent mechanisms of cell death may include nuclease, nuclease activators, and serine proteases (41).

Radio sensitizing functions have been attributed to butyric acid, the hydrolytic derivative of AN-1 and AN-9, two decades ago (42, 43). The prominent role of radiation therapy in standard treatments of many neoplasms has focused attention on radiosensitizing effects of various HDACIs. Our data indicate that butyric acid–delivering prodrugs may serve as effective radiosensitizing agents. Further in vivo experiments are now required to confirm enhanced effects of combined treatments compared with each modality alone. Potentiation of combined radiation and HDACI treatments is reflected not only in reduced cell viability but also in caspase-8 activation. This observation is consistent with our previous experiments in which caspase-8 successfully mediated apoptosis triggered by either HDACI or ionizing radiation alone.5 The data highlight the importance of engaging receptor-mediated apoptotic pathways in glioma treatment.

Preliminary in vivo experiments reported herein suggest that in vivo efficacy of AN-9 is coupled with a safe toxicity profile. The human nuclear antigen Ki-67 reflects cell proliferation because it is present in the nucleus during late G1, S, G2, and M phases (44). Reduced Ki-67 staining in tumor sections from AN-9-treated animals indicates that growth arrest plays a key role in the antineoplastic effects of these novel HDACIs in vivo. Of note, significant variability in responses of individual animals is evident among AN-9-treated mice. This unexpected variation among syngeneic mice may be due to differences among flank tumors in subpopulations of U251 MG that formed tumors or to differences in proximity to blood supply and other extracellular components.

Our plans for in vivo experiments include comparisons of oral and i.v. modes of drug delivery. Although the ease of oral administration offers obvious benefits for patients, the maximum tolerated dose is already established for i.v. given Pivanex, allowing a more expeditious, direct route to clinical use. In addition, the treatment regimen that proves most effective in the flank model will be further tested in an orthotopic intracranial model (45, 46). Furthermore, because in vitro data presented herein indicate that butyric acid–delivering HDACIs enhance the efficacy of ionizing radiation, combinations of AN-9 and clinically relevant radiation doses will be the focus of our next study.

Preliminary in vivo data reported herein suggest promising efficacy of AN-9 in the treatment of gliomas. Our in vivo results, coupled with additional preclinical studies of acyloxy methyl esters of butyric acid (34), show exceedingly low toxicities of these novel agents and set them apart from other HDACIs, such as suberoylanilide hydroxamic acid and MS-275 (19, 23). Thus, the promise of butyric acid prodrugs for cancer treatment in general and for highly morbid radioresistant malignant gliomas in particular, rests on their low toxicity, oral bioavailability, ability to cross the blood-brain barrier, and potential synergy with chemotherap y and radiation. Indeed, the above shown differential effects of the butyric acid prodrugs AN-1 and AN-9 on high-grade glioma cell lines versus their normal cellular counterparts, primary human astrocytes, shed light on their potential clinical use for glioma therapy. We have shown in vitro and in vivo efficacy of novel butyric acid–delivering prodrugs in the treatment of gliomas, and given their radiosensitizing function and oral bioavailability, we hope the potential utility of these agents is realized clinically.

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