Experimental therapy of malignant gliomas using the inhibitor of histone deacetylase MS-275

Ilker Y. Eyüpoğlu,1 Eric Hahnen,2,3 Christian Tränkle,4 Nicolai E. Savaskan,5 Florian A. Siebzehnrübl,2 Rolf Buslei,2 Dieter Lemke,6 Wolfgang Wick,6 Rudolf Fahrbusch,1 and Ingmar Blümcke2

Departments of 1Neurosurgery and 2Neuropathology, University of Erlangen-Nuremberg, Erlangen-Nuremberg, Germany; 3Institute of Human Genetics, Institute of Genetics, and Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany; 4Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, Bonn, Germany; 5Division of Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam, the Netherlands; and 6Department of General Neurology, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany

Abstract

Inhibitors of histone deacetylases are promising compounds for the treatment of cancer but have not been systematically explored in malignant brain tumors. Here, we characterize the benzamide MS-275, a class I histone deacetylase inhibitor, as potent drug for experimental therapy of glioblastomas. Treatment of four glioma cell lines (U87MG, C6, F98, and SMA-560) with MS-275 significantly reduced cell growth in a concentration-dependent manner (IC50, 3.75 μmol/L). Its antiproliferative effect was corroborated using a bromodeoxyuridine proliferation assay and was mediated by G0-G1 cell cycle arrest (i.e., up-regulation of p21/WAF) and apoptotic cell death. Implantation of enhanced green fluorescent protein–transfected F98 glioma cells into slice cultures of rat brain confirmed the cytostatic effect of MS-275 without neurotoxic damage to the organotypic neuronal environment in a dose escalation up to 20 μmol/L. A single intratumoral injection of MS-275 7 days after orthotopic implantation of glioma cells in syngeneic rats confirmed the chemotherapeutic efficacy of MS-275 in vivo.

Furthermore, its propensity to pass the blood-brain barrier and to increase the protein level of acetylated histone H3 in brain tissue identifies MS-275 as a promising candidate drug in the treatment of malignant gliomas. [Mol Cancer Ther 2006;5(5):1248–55]

Introduction

Acetylation and deacetylation of core histones are key regulatory mechanisms of gene expression (1). Whereas histone acetylation seems to relax the chromatin structure, thereby promoting gene transcription, histone deacetylation induces a repressive environment mediated by chromatin condensation. In addition, normal cell differentiation and adjustment of metabolic activity require coordinated gene transcription and balanced activity of histone acetyltransferases versus histone deacetylases (HDAC; ref. 2). This assumption is supported by the finding that deletions or inactivating mutations of histone acetyltransferase are associated with tumor progression in humans (3, 4). The propensity to block HDAC activities by specific inhibitors provides the intriguing opportunity to pharmacologically modulate gene transcription by epigenetic regulation and may counteract the described dysbalance. Various HDAC inhibitors have been identified as promising compounds for the treatment of cancer, either alone (5, 6) or in combination with other agents (6–8). These novel compounds induce growth arrest and apoptotic cell death in a variety of transformed cells (9, 10). Molecular pathways induced by HDAC inhibitors and which either accelerate cellular maturation or cell death remain to be determined. However, epigenetic modifiers of gene expression exert their beneficial effect in the treatment of central nervous system tumors (e.g., malignant gliomas) by up-regulation of cell cycle regulator proteins (i.e., p21/WAF and gelsolin; refs. 5, 11, 12) or initiation of apoptosis mechanisms in glioma cells (12–14). Moreover, in vitro experiments with leukemia cells have shown that HDAC inhibitors are able to induce expression of death receptors, which result in enhanced activity of caspase-8 or cleavage of Bid (15, 16). Furthermore, HDAC inhibitors are able to disrupt cellular redox state (e.g., reactive oxygen species; refs. 9, 17, 18), damage function of heat shock proteins (e.g., acetylation of heat shock protein 90; ref. 19), and down-regulate survival signaling pathways (15, 20).

Despite multimodal therapy regimens, including radical neurosurgical resection, radiotherapy, and polychemotherapy, the response of malignant gliomas remains poor (21) and patients experience a median survival of 12 months from the time point of diagnosis (22, 23). Recently, the DNA alkyllating drug temozolomide seems to increase survival in ~20% of patients (24, 25). This success will foster the identification of novel chemotherapeutic compounds,
which would then rapidly translate into clinical perspectives. HDAC inhibitors may offer this opportunity. MS-275 has entered clinical phase I trial for the treatment of solid tumors and lymphoma (26, 27). It potently triggers the early release of reactive oxygen species and induces mitochondrial damage in various cancer cell lines (9, 17). Here, we analyze the therapeutic potential of MS-275 to reduce glioma progression in monolayer cultures, an organotypic glioma invasion model using entorhino-hippocampal slice cultures of rat brain, as well as in vivo following single intrathecal injection of MS-275 7 days after implantation of glioma cells into rat brain.

Materials and Methods

Monolayer Culture

The rat glioma cell line F98 was established from ethylnitrosourea-induced carcinogenesis in CDF Fisher rats (28). The human glioma cell line U87MG (29) and the rat glioma cell line C6 (30) were obtained from the American Type Culture Collection (Manassas, VA). SMA-S60 mouse glioma cells (31) were kindly provided by D.D. Bigner (Durham, NC). All glioma cell lines were cultured in a humidified atmosphere at 37°C and 5% CO2 using culture conditions and medium as recommended by the supplier.

Chemicals

N-(2-aminophenyl)-4-[[N-(pyridine-3-ylmetoxycarbonyl)amino)methyl]benzamide (MS-275) was obtained from Calbiochem (Bad Soden, Germany). Temozolomide was supplied by Schering-Plough (Kenilworth, NJ). Both compounds were dissolved in DMSO (Sigma-Aldrich, Munich, Germany) and diluted in medium before experimental use.

Microscopic Evaluation

Morphometric analysis was done using high-power optical fields digitized with a CCD camera (Color View II, Soft Imaging System, Münster, Germany) equipped to a BX51 microscope (Olympus, Tokyo Japan) and respective imaging software (analySIS, Soft Imaging System, Stuttgart, Germany). Fluorescence-labeled tumor cells transplanted to organotypic brain slice cultures as well as propidium iodide (PI) staining intensities (see below) were analyzed by an Olympus microscope (IX 70) equipped with a TRITC (excitation filter 520–550 nm, barrier filter 580 nm) and FITC (excitation filter 450–490 nm, band filter 520–550) narrow band filter, a CCD camera (F-View II), and respective image software (analySIS). Statistical significance was calculated with Student’s t test (StatView II, Abacus).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide and Bromodeoxyuridine Proliferation Assays

Viable cell numbers were estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells (8,000/mL) were seeded in a 96-well plate (final volume, 250 µL/well). One hour after seeding, the cells were treated with either MS-275, temozolomide, or solvent. Medium was changed after 48 hours. At 96 hours after incubation, 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-

zolium bromide (Sigma-Aldrich) dissolved in medium was applied for 2 hours. Subsequently, the medium was discarded and each well was incubated with 100 µL of an isopropanol stock solution containing 165 µL hydrochloric acid/50 mL isopropanol. The absorbance of each well was subsequently determined using a microplate reader (Tecan, Crailsheim, Germany) set to 550 nm (wavelength correction set to 690 nm). The proliferation of F98 glioma cells was analyzed using the Bromodeoxyuridine Labeling and Detection Kit III according to the manufacturer’s protocol (Roche, Mannheim, Germany).

Measurement of HDAC Activity

HDAC activity was determined by applying a BioVision fluorimetric assay (BioCat, Heidelberg, Germany; ref. 32). Fluorescence signal was measured with a NOVOstar plate reader (BMG Labtech, Offenburg, Germany) at excitation wavelength of 380 nm and emission wavelength of 460 nm. Rat liver HDAC was purchased from Alexis Biochemicals (Grünberg, Germany); 30 µL of the stock preparation were applied per well; its specific HDAC activity amounted to ~100 pmol substrate/min at 37°C. Trichostatin A (1 mmol/L) was used as a positive control.

Data Analysis

Concentration-effect curves for the inhibition of cell viability and HDAC activity following drug treatment were calculated by nonlinear regression analysis using GraphPad Prism version 4.00 for Windows (GraphPad, San Diego, CA). Data were fitted to a four-variable logistic equation comprising the top plateau, bottom plateau, inflection point IC50, and curve slope nH. The variables IC50 and nH and “bottom” were set as variables and “top” was the control value of cell viability or HDAC activity, respectively, and was set constant at 100%. It was tested successively, whether the slope nH or the bottom plateau was different from unity or zero (F test). Cell viability approached zero in the presence of high concentrations of the test compounds and extended incubation times. Therefore, the variable “bottom” was set at constant = 0 in the respective nonlinear regression analyses.

Protein Preparation and Immunoblotting

F98 glioma cells were treated with either MS-275 or solvent. After 24 hours, cells were pelleted by centrifugation at 175 × g for 10 minutes and the supernatant fraction was decanted. Cells were washed with PBS and suspended in lysis buffer [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L DT, 1.5 mmol/L phenylmethylsulfonyl fluoride]. Sulfuric acid was added to a final concentration of 0.2 mol/L. The cells were incubated for 30 minutes on ice and centrifuged at 10,080 × g for 10 minutes at 4°C. The supernatant fraction was dialyzed against 200 mL of 0.1 mol/L acetic acid for 2 hours and dialyzed again thrice against 200 mL H2O (1 and 3 hours and overnight). Supernatant was subjected to SDS-PAGE on a 12% polyacrylamide gel. The soluble fraction (10 µg) was loaded per lane. The separated proteins were electroblotted onto nitrocellulose (Sigma-Aldrich). Equal loading amounts of the probes were estimated using immunostaining with an anti-mouse β-actin monoclonal antibody.
Slice cultures were prepared and maintained as described (33). Seven-day-old Wistar rats were used for explantation. After decapitation, the brains were removed and placed into preparation medium containing HBSS (Life Technologies, Karlsruhe, Germany) with 10% normal horse serum (BD Biosciences, Heidelberg, Germany). After preparation and every second day thereafter, Neurotoxicity in Organotypic Entorhino-Hippocampal Slice Cultures

Slice cultures were incubated with 1 μg/mL PI for 20 minutes followed by complete medium exchange to visualize irreversibly damaged cell bodies. Neurotoxicity was evaluated using an inverse fluorescence microscope (see details above) and respective documentation software (analySIS).
relationship between drug concentration and tumor cell growth was observed in all cell lines, indicating that MS-275 is highly effective at low micromolar ranges (Fig. 1A). The DNA alkylating agent temozolomide was employed to corroborate our experimental model. This drug is a clinical well-established compound in the treatment of patients suffering from malignant gliomas. To provide a proof-of-concept for the therapeutic efficacy of MS-275 in our ex vivo model, we compared the antiglioma efficacy of MS-275 with that of temozolomide (Fig. 1B).

In F98 cells, the equieffective concentration to reduce cell viability by 90% (IC_{90}) was 688 μmol/L for temozolomide and 3.75 μmol/L for MS-275. These results were confirmed by bromodeoxyuridine proliferation assays (Fig. 1C). We continued to characterize the HDAC-inhibiting action of MS-275 and quantified its potency to reduce HDAC activity in a cell-free system. Interestingly, MS-275 showed an incomplete inhibition of HDAC activity even at excessive concentrations (>100 μmol/L; Fig. 1D). However, the capacity of MS-275 to induce an accumulation of acetylated histones at low concentrations was shown by immunoblotting of acetylated histone H3 (data not shown). Treatment of F98 cells with IC_{90} doses of MS-275 caused a prominent increase of acetylated histone H3 protein levels after 24 hours. Moreover, incubation of F98 glioma cells with MS-275 (IC_{90} for 48 hours) induced a switch of the morphologic phenotype with outgrowth of cellular processes (data not shown). Flow cytometry of F98 glioma cells treated with MS-275 (IC_{90}) for 48 hours revealed a significant G_{1} cell cycle arrest (data not shown). Following an incubation period of 72 hours, the majority of tumor cells accumulated in the sub-G_{1} phase, including apoptotic DNA fragmentation (Fig. 2A and B). As shown in previous studies on HDAC inhibitor treatment in malignant tumor cell lines (9, 37), the cell cycle control protein p21/WAF was up-regulated also in F98 glioma cells after MS-275 treatment (data not shown).

Neurotoxicity of MS-275 and Temozolomide in Brain Slice Cultures

In the next set of experiments, the cytotoxic effect of MS-275 was evaluated in organotypic brain environment ex vivo. PI uptake, which indicates irreversible cell damage, was not detectable at calculated IC_{90} concentrations for MS-275 and temozolomide. Dose escalation experiments corroborated the good tolerance of the benzamide, whereas significant cell damage was detected after administering >20-fold IC_{90} doses (80 μmol/L; Fig. 3A). In comparison, a 2-fold IC_{90} escalation of temozolomide significantly increased PI uptake in organotypic brain slice cultures (Fig. 3A and B).

MS-275 Reduces Glioma Progression in the Organotypic Glioma Invasion Model

We employed brain slice cultures also to monitor glioma proliferation and invasion within the organotypic environment (35) by implantation of enhanced green fluorescent protein–labeled F98 cells into the entorhinal cortex.
Continuous increase of the bulk tumor mass was observed in untreated control experiments at all periods (Figs. 4 and 5). At day 5 after implantation, this increase amounted to 235% compared with the initial tumor implantation site at day 1 (defined as 100%). MS-275 and temozolomide caused a significant reduction of tumor growth 5 days after implantation as measured by fluorescence microscopy (Fig. 5A and B), whereas equieffective concentrations of temozolomide or MS-275, dissolved in fresh medium, were added every second day. Long-term follow-up of MS-275-treated brain slices revealed a 40% decrease of bulk tumor mass at 12 days after implantation (Fig. 5C) compared with day 1. Tumor mass observed in MS-275-treated slices at day 12 after implantation was >6-fold smaller than in non-treated controls at day 12. Dose escalation to the 5-fold IC50 of MS-275 aggravated tumor reduction from 375% in time-matched controls to 32% after treatment compared with day 1 (Fig. 5C). Interestingly, anatomic landmarks within the slice culture, which are helpful to assess its viability (i.e., CA1 pyramidal cell layer and hippocampal fissure), differed between treatment and control paradigms. Severe degeneration occurred only in the untreated group (Fig. 4B), whereas MS-275-treated slice cultures appeared anatomically intact until 12 day ex vivo (Fig. 4D).

Blood-Brain Barrier Passage of MS-275

To evaluate whether MS-275 crosses the blood-brain barrier, adult mice were treated with 2 and 4 mg MS-275 by i.p. injection. At 2 and 4 hours after treatment, an accumulation of acetylated H3 core histones was observed in total brain as shown by immunoblot analysis of acetylated versus total histone H3 (Fig. 6A).

MS-275 Blocks Glioma Growth In vivo

To confirm the promising results obtained from the organotypic glioma invasion model and to further evaluate the therapeutic efficacy of MS-275, we analyzed the antiglioma action of MS-275 in a syngeneic tumor transplantation model. Whereas control animals bear a median tumor volume of 96 mm3, tumor masses were reduced significantly to 38 mm3 in MS-275-treated rats (Fig. 6B and C).

Discussion

Malignant gliomas are common tumors of the central nervous system, but only a minority of patients responds satisfactorily to chemotherapeutic and radiotherapeutic treatment modalities. Progression of malignant gliomas from their ancestor glial precursors might rely on cell-autonomous escape mechanisms for growth arrest and apoptotic cell death (38). These pathogenetic pathways are targeted by mutational hits leading to inactivation of tumor suppressors or activation of oncogenes (39, 40). However, recent evidence also highlights epigenetic dysregulation as important pathomechanisms of cancer (i.e., affecting cell repair circuitries and pathways of programmed cell death; ref. 41). The availability of pharmacologic modifiers for ‘‘epigenetic’’ regulation of gene expression (i.e., inhibitors of HDAC) opens new avenues to chemotherapeutic treatment modalities of malignant brain tumors, such as glioblastoma multiforme (WHO grade IV; refs. 5, 13, 42).

Figure 2. MS-275 induces cell cycle arrest and apoptosis. A, flow cytometric analysis of F98 glioma cells after 48- and 72-h exposure to MS-275 using calculated IC50 concentration. Sub-G1, G0-G1, S, and G2-M phases of the cell cycle. B, a significant accumulation in the sub-G1 phase is observed for MS-275 treatment at 72 h (P < 0.05, t test) compared with time- and solvent-matched controls (data are given as percentage of cells in the different cell cycle phases).

Figure 3. Neurotoxicity of MS-275 and temozolomide. A, organotypic entorhino-hippocampal slice cultures were treated with various concentrations of temozolomide or MS-275 for 5 d and neuronal cell damage was scored by PI staining. Significant increase of PI staining was identified for temozolomide at 2-fold IC50 concentration, whereas significant PI uptake was registered for MS-275 at 20-fold IC50 (P < 0.01, t test). B, neurotoxicity within brain environment was visualized by fluorescence microscopy. Color images were coded into a gray scale in which higher levels of PI uptake appear darker. Bar, 1 mm.
Recent data obtained from clinical trials using MS-275 for the treatment of cancer show reasonable tolerance after oral application with only moderate side effects (26, 27). Here, we systematically addressed the effect of the benzamide MS-275 to reduce malignant glioma progression in vitro (monolayer assay), ex vivo (organotypic slice culture), and in vivo (tumor transplantation model). Moreover, we identified the propensity of MS-275 to successfully pass the blood-brain barrier. I.p. injection of MS-275 increased the level of acetylated histone H3 protein in brain tissue. Our study highlights, therefore, MS-275 as potent chemotherapeutic drug for the treatment of central nervous system malignancies. As proof-of-concept, the efficacy of MS-275 was compared with that of the DNA alkylating agent temozolomide, which has recently been introduced in the treatment of glioblastoma multiforme and which significantly increase survival time and life quality (24, 25). Calculated equieffective concentrations (IC90) of temozolomide and MS-275 were applied to treat the ex vivo organotypic glioma invasion model (35). Both compounds confirmed significant antiglioma properties, and a low neurotoxic potential was discovered for MS-275. Dose escalations were tolerated by the organotypic brain environment up to 20 μmol/L (5-fold IC90), whereas significant cytotoxicity was identified for temozolomide at 2-fold IC90 (calculated to be 1.38 mmol/L in our paradigm). Considering that clinical side effects of temozolomide are in a tolerable range (43, 44), MS-275 seems to corroborate a promising perspective in clinical trials. We further employed an in vivo model using orthotopic implantation of glioma cells in syngeneic rats followed by a single intratumoral injection of MS-275 7 days later. This experiment confirmed the antiglioma action of MS-275 as described in our in vitro and ex vivo assays.

Despite the potent antiglioma action in various experimental models, MS-275 was not able to completely inhibit HDAC activity in a cell-free enzymatic assay. Until today, three classes of HDACs have been identified, with only class I and II enzymes being molecular targets of MS-275 (41, 45). According to the Serial Analysis of Gene Expression (SAGE) data, MS-275 showed a high degree of selectivity towards class I and II enzymes.

**Figure 4.** Organotypic glioma invasion model. Enhanced green fluorescent protein–positive F98 glioma cells were transplanted into the entorhinal cortex. Fluorescence microscopic image superimposed onto a translucent image of the same slice culture. A and B, same slice culture 1 d (A) and 12 d (B) after tumor implantation. Severe morphologic alterations occurred in the slice architecture after 12 d. C and D, same slice culture 1 d (C) and 12 d (D) after tumor implantation and treatment with MS-275 (IC90, 3.75 μmol/L). The bulk tumor mass shrinks and the anatomic slice architecture remains well preserved until 12 d ex vivo. Bar, 1 mm.

**Figure 5.** MS-275 reduced tumor growth in the organotypic glioma invasion model. A, transplantation of enhanced green fluorescent protein–transfected F98 glioma cells into the entorhinal cortex of the slice culture model and repetitive fluorescent microscopic monitoring. DAI, days after implantation. Bar, 450 μm. Slice cultures were treated with equiefficient IC90 concentrations of either temozolomide or MS-275. Fluorescence images were not superimposed onto translucent images (see Fig. 4 legend). B, tumor infiltration area was measured by analySIS software. Columns, mean of nine slice cultures in each group; bars, SD. Both compounds significantly reduced tumor growth 5 d after implantation (P < 0.01, t test). C, tumor growth was monitored up to 12 d after implantation. Both concentrations of MS-275 (IC90 and 5-fold IC90) reduced tumor growth and reached statistical significance after 5 d after implantation (P < 0.05, t test).
Expression (46), all class I and II HDAC isoenzymes seem to be expressed in brain. These data are compatible with the notion that favorable antiglioma properties of MS-275 associate with specific inhibition of HDAC isoenzymes (45).

Whereas accumulation of acetylated histones occurs in both normal and transformed cells (47, 48), the low cytotoxicity of HDAC inhibitors in normal compared with neoplastic cells remains enigmatic. Recently, it has been shown that HDAC inhibitors cause an accumulation of reactive oxygen species in transformed but not in normal cells, which is an important resistance determinant observed in native cells and tissues (17, 18). The mechanism of this selectivity remains, however, awaits further studies.

With increasing knowledge that chemotherapy plays a beneficial role in glioma treatment (24, 25), promising data of novel agents, such as HDAC inhibitors, may rapidly translate into clinical trials. However, it is reasonable to suggest that genetic inactivation or activation of key players in neoplastic transformation by allelic loss or mutational hits may induce escape mechanisms also in the face of HDAC inhibitor treatment. To successfully predict chemotherapeutic responsiveness of HDAC inhibitors in vivo, molecular diagnostic work-up of tumor specimens will be warranted and remain an important prerequisite to further address the anticancer potency of HDAC inhibitors.

In conclusion, polychemotherapeutic treatment modalities may increase the propensity to pharmacologically challenge highly malignant brain tumors. Adjustment of different treatment modalities, such as combination of DNA alkylating agents with HDAC inhibitors, could be helpful in this respect.

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