Antiproliferative and proapoptotic effects of histone deacetylase inhibitors on gastrointestinal neuroendocrine tumor cells

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Abstract

Treatment options of advanced neuroendocrine tumors (NETs) are unsatisfactory. Hence, innovative therapeutic approaches are urgently needed. Inhibition of histone deacetylases (HDACs) is a promising new approach in cancer therapy. While several HDAC inhibitors have already entered clinical trials, the effect of HDAC inhibition on NET has not been investigated. Therefore, we evaluated the antineoplastic effects of three different HDAC inhibitors, trichostatin A (TSA), sodium butyrate (NaB), and MS-275, on growth and apoptosis of the gastrointestinal NET cell lines CM and BON. We could demonstrate that HDAC inhibition dose-dependently inhibited proliferation of both cell lines with IC₅₀ values varying from the millimolar (NaB) to the micromolar (MS-275) and the nanomolar range (TSA). Moreover, HDAC inhibition potently induced apoptosis, which was accompanied by DNA-fragmentation, an up to 12-fold caspase-3 activation and downregulated Bcl-2 expression. Furthermore, HDAC inhibition resulted in cell cycle arrest at the G₁–S-transition, which was associated with the suppression of cyclin D1 expression and induction of p21 and p27 expression. For BON cells, we observed an additional block in the G₂/M phase, which was aligned with a downregulation of cyclin B1. In addition, combined treatment with MS-275 and somatostatin or the synthetic somatostatin analog octreotide was evaluated. Neither somatostatin nor its stable analog octreotide augmented the antiproliferative effect of MS-275 in NET cells. To conclude, our data show that HDAC inhibition is a promising new approach in the treatment of NET disease, which should be evaluated in clinical studies.

Endocrine-Related Cancer (2006) 13 1237–1250

Introduction

Gastrointestinal neuroendocrine tumors (NETs) represent a rare and rather heterogeneous tumor entity. Metastatic NETs often release excessive amounts of biogenic amines and/or neuropeptides, thereby causing a characteristic hypersecretion syndrome. Even though the hypersecretion syndromes are generally well controlled by somatostatin analogs or interferon- α (Öberg 2001, Scherübl *et al.* 2003), further treatment options to control NET growth are urgently needed.

Recently, evidence has accumulated that inhibition of histone deacetylases (HDACs) is a promising new target in cancer therapy. Acetylation and

deacetylation of histones play an important role in the regulation of gene transcription and in the modulation of chromatin structure (Marks *et al.* 2001, Kouraklis & Theocharis 2002). The equilibrium of steady-state acetylation is tightly controlled by the antagonistic effects of histone acetyltransferases (HATs) and HDACs. Aberrant gene expression resulting in functional inactivation of HAT activity or overexpression of HDACs can mediate tumor cell proliferation (Kouzarides 1999) and deregulation of HDAC recruitment to promoters contributes to tumorigenesis (Monneret 2005).

So far, various HDAC inhibitors have been shown to exhibit potent anti-tumor activity *in vitro* and *in vivo*

(Kramer *et al.* 2001, Marks *et al.* 2001, Vigushin *et al.* 2001, Johnstone 2002), underlining their potency as novel anticancer agents. Several new substances have already entered clinical trials both in hematological malignancies and in solid tumors (Kramer *et al.* 2001, Acharya *et al.* 2005, Monneret 2005). HDAC inhibitors have been reported to be potent inducers of differentiation and cell cycle arrest. Furthermore, HDAC inhibitors have been demonstrated to induce apoptosis by activating both the death receptor and intrinsic apoptotic pathway (Acharya *et al.* 2005, Monneret 2005, Lin *et al.* 2006).

Till date, four chemical classes of HDAC inhibitors have been developed: short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides, and benzamides (Kramer *et al.* 2001, Johnstone 2002). Despite their shared capacity to increase histone acetylation, individual HDAC inhibitors exert diverse actions on cell cycle regulation, signal transduction, and the expression of survival-related proteins, which may account for their disparate actions.

HDAC inhibition has not been investigated for the growth control of NETs so far. Hence, in the present study, we investigated the potency of the short-chain fatty acid sodium butyrate (NaB), the hydroxamic acid trichostatin A (TSA) and the synthetic benzamide-derivative MS-275 to inhibit the growth of gastrointestinal NET cells.

NaB is a non-toxic short-chain fatty acid formed by the flora of the gastrointestinal tract. Millimolar concentrations of NaB, comparable with those encountered within the colonic lumen, affect colonocyte phenotype *in vitro*, causing cell cycle blockade, differentiation, or apoptosis in a number of transformed cell lines (Staiano-Coico *et al.* 1990, Hague *et al.* 1993, McBain *et al.* 1997, Schwartz *et al.* 1998). In addition, an inverse association between tumor size and luminal concentration of NaB has been observed in chemically induced rat colon cancers (McIntyre *et al.* 1993).

The hydroxamic acid TSA, originally isolated from *Streptomyces hygroscopicus* as an antifungal antibiotic, induces cell differentiation and exerts potent antiproliferative activities at nanomolar concentrations. Since the discovery of its HDAC-inhibiting abilities, TSA has demonstrated strong antineoplastic effects in various tumor cell lines, including breast, bladder, colon, gastric, and hepatic cancers (McBain *et al.* 1997, Saito *et al.* 1999, Eickhoff *et al.* 2000, Suzuki *et al.* 2000, Herold *et al.* 2002).

In addition to the natural compounds, several synthetic substances with HDAC-inhibiting properties are being developed. Among these, the orally available benzamide MS-275 is the most developed and has

already entered clinical trials both for mono and combination therapies in solid and hematological malignancies (www.clinicaltrials.gov).

In the present study, we demonstrate for the first time that HDAC inhibition by TSA or NaB or MS-275 potently inhibits growth of NET cells in a dose-dependent manner. Moreover, we provide an insight into the underlying mechanisms of HDAC-induced growth inhibition as we could demonstrate the induction of apoptosis and cell cycle alterations due to HDAC inhibitor treatment in NET cells.

As somatostatin and its stable analogs are well known to effectively control the hypersecretion syndromes of NET disease, we studied the combination therapy of MS-275 with somatostatin (analogs).

Materials and methods

Materials

NaB was bought from Biomol (Hamburg, Germany). MS-275(*N*-(2-Aminophenyl)-4-[*N*-(3-pyridineyl-methoxycarbonyl)aminomethyl]-benzamide) and TSA were purchased from ALEXIS Biochemicals (Lausen, Switzerland). Stock solutions were prepared in DMSO. Octreotide (SMS 201-995) and somatostatin (somatostatin 14) were from Sigma. Stock solutions were prepared in H₂O. All the drugs were diluted in a fresh medium before each experiment. To evaluate the effects of the drugs, cells were incubated with either control medium or a medium containing increasing concentrations of the respective drugs. In all the experiments, the final DMSO concentration was <0.5%, not affecting cell growth. Cell culture material was from Biochrom (Berlin, Germany); all other chemicals were from Sigma, if not stated otherwise.

Cell lines

Human pancreatic carcinoid BON cells, which were established from a human pancreatic carcinoid tumor, are a useful model to study the biology of NETs *in vitro* (Evers *et al.* 1994). BON cells were grown in a 1:1 mixture of DMEM, and Ham's F-12 medium containing 10% FCS (Biochrom) and 1% L-glutamine. The human insulinoma cell line CM (Baroni *et al.* 1999), kindly provided by Professor P Pozzilli (University La Sapienza of Rome, Italy), was cultured in RPMI 1640 supplemented with 5% FCS (Biochrom) and 1% L-glutamine. Both cell lines were kept at 37 °C in a humidified atmosphere (5% CO₂).

Measurement of growth inhibition

Drug-induced changes in cell numbers of BON and CM cells were evaluated by crystal violet staining, as described by Gillies *et al.* (1986). In brief, cells in 96-well plates were fixed with 1% glutaraldehyde. Then, cells were stained with 0.1% crystal violet in PBS. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 0.2% Triton-X-100 in PBS. Light extinction, which increases linearly with the cell number, was analyzed at 570 nm using an ELISA-Reader.

Determination of cytotoxicity

Cells were seeded at a density of 8000 cells/well into 96-well microtiter plates and incubated with increasing concentrations of TSA, NaB, or MS-275 for 24 or 48 h. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH), indicating cytotoxicity, was measured by using a colorimetric kit from Roche Diagnostics as described elsewhere (Decker & Lohmann-Matthes 1988).

Detection of apoptosis

Preparation of cell lysates and determination of caspase-3 activity was performed as described by Sutter *et al.* (2004). The activity of caspase-3 was calculated from cleavage of the fluorogenic substrate DEVD-AMC (Calbiochem, Bad Soden, Germany). Cell lysates were incubated with substrate solution (caspase-3 substrate AC-DEVD-AMC 20 µg/ml, HEPES 20 mM, glycerol 10%, dithiothreitol 2 mM, pH 7.5) for 1 h at 37 °C, and substrate cleavage was measured with a VersaFluor fluorometer (excitation, 360 nm; emission, 460 nm) from Bio-Rad.

DNA fragmentation was determined by using the DNA laddering kit (Roche Diagnostics) according to the manufacturers' instructions. In brief, HDAC inhibitor-treated cell samples were lysed and subsequently poured into filter tubes containing glass fiber fleece, which binds DNA in the presence of a chaotropic salt, and guanidine HCl. After additional washing steps, the DNA was released with a low salt buffer. Purified DNA (2 µg) was mixed with loading buffer and analyzed on agarose–DNA gel by ethidium bromide staining followed by u.v.-illumination.

The proportion of apoptotic cells was determined by quantifying the percentage of sub-G₁ (hypodiploid) cells after flow cytometric analysis of propidium iodide-stained isolated nuclei.

Cell cycle analysis

Cell cycle analysis was performed by the method of Vindelov and Christensen, as described previously (Maaser *et al.* 2001). Cells were trypsinized, washed, and the nuclei were isolated using the CycleTest PLUS DNA reagent kit (Becton Dickinson, Heidelberg, Germany). DNA was stained with propidium iodide according to the manufacturers' instructions. The DNA content of the nuclei was measured by flow cytometry and analyzed using *ModFit* software (Becton Dickinson).

Western blotting

Western blotting was performed as described by Höpfner *et al.* (2004). In brief, whole-cell extracts were prepared by lysing cells. Lysates containing 30 µg protein were subjected to gel electrophoresis. Proteins were then transferred to PVDF membranes by electroblotting for 90 min. The blots were blocked in 5% non-fat dry milk in TBS–Tween solution for 1 h at room temperature, and then incubated at 4 °C overnight with antibodies directed against anti-human Bax (1:1000), Bcl-2 (1:500), cyclin B1 (1:200), cyclin D1 (1:200), p27 (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or p21 (1:1000; NEB, Ipswich, MA, USA). Anti-β-actin (1:5000) from Sigma served as loading control. After incubation with horseradish peroxidase-coupled anti-IgG antibodies (1:10 000, Amersham) at room temperature for at least 1 h, the blot was developed using enhanced chemiluminescent detection (Amersham) and subsequently exposed to Hyperfilm ECL film (Amersham).

Statistical analysis

If not stated otherwise, means of at least three independent experiments $\pm$ s.e.m. are shown. Significance between controls and treated samples was calculated by Student's two-sided *t*-test. Caspase-3 measurements were evaluated using the two-sided Welch *t*-test. The *P* values were considered to be significant at <0.05 .

Results

Growth-inhibitory effects of HDAC inhibitors on neuroendocrine gastrointestinal tumor cells

The effects of HDAC inhibition on the growth of CM insulinoma and BON carcinoid cells were studied by crystal violet staining. TSA (CM, 0–500 nM; BON, 0–1000 nM), NaB (CM, 0–5 mM; BON, 0–10 mM), and MS-275 (CM, 0–5 µM; BON, 0–10 µM) inhibited

the growth of both cell lines in a dose-dependent manner (Fig. 1). The sensitivity of the NET cell lines towards the three chemical classes of HDAC inhibitors differed as reflected by IC_{50} values varying from the nanomolar to the millimolar range: after 3 days of continuous exposure to the respective drug, the IC_{50} values were 240 ± 20 nM (CM) and 380 ± 30 nM (BON) for TSA, 0.8 ± 0.1 mM (CM) and 2.4 ± 0.3 mM (BON) for NaB, and 0.6 ± 0.1 μ M (CM) and 3.3 ± 0.3 μ M (BON) for MS-275 treatment.

Combination treatment of MS-275 with somatostatin or octreotide

We evaluated whether combined treatment of somatostatin or its analog octreotide with the HDAC inhibitor MS-275 might potentiate growth-inhibitory effects of MS-275 on NET cells. When treating either CM or BON cells with a sub- IC_{50} concentration of MS-275 plus somatostatin (0–1000 nM) or octreotide (0–1000 nM), we observed that neither somatostatin nor octreotide were able to increase the antiproliferative effect of MS-275 (Fig. 2).

LDH release from NET cells after HDAC treatment

In the next step, we compared the cytotoxicity of TSA, NaB, and MS-275 by determining the release of LDH into the cell culture supernatant of CM and BON cells (Fig. 3). Incubating NET cells with increasing concentrations of the respective HDAC inhibitors for 24 h resulted in only slight increases of LDH release in both cell lines. This indicates that the HDAC inhibitors investigated do not directly affect cell membrane integrity and do not have immediate necrotic effects even at higher concentrations. On the other hand, after 48 h of continuous exposure to the drugs, LDH release dose-dependently increased up to nearly 30% above the control levels (Fig. 3A). Most probably, this was due to the loss of cell membrane integrity of cells in late apoptotic stages. This is an *in vitro* phenomenon, since *in vivo* apoptotic cells are rapidly eliminated by macrophages.

Induction of apoptosis by HDAC inhibition in NET cells

To study the potency of HDAC inhibitors to induce apoptosis in NET cells, we investigated HDAC inhibitor-induced activation of caspase-3, a key enzyme of the apoptotic pathway.

Again, cells were treated with increasing concentrations of the respective HDAC inhibitors and caspase-3 activity was determined after 24 h (Fig. 4).

Figure 1 Antiproliferative effects of TSA, NaB, and MS-275. TSA, NaB, or MS-275 treatment led to a dose-dependent growth inhibition of neuroendocrine gastrointestinal tumor cells. The HDAC inhibitors TSA or NaB or MS-275 were added to the cell culture medium in increasing concentrations. After 3 days of incubation, cell numbers were determined by crystal violet staining. Data are given as percentage of controls (mean $\pm$ S.E.M. of 3–5 independent experiments). *Significant difference ($P < 0.05$) compared to controls.

Figure 2 Combination treatment of MS-275 with somatostatin or octreotide. CM and BON cells were treated for 3–6 days with increasing concentrations of somatostatin or octreotide and cell numbers were determined by crystal violet staining. Combination treatments of either somatostatin or octreotide with a sub-IC₅₀ concentration of MS-275 were performed and cell numbers were determined after 3 days of incubation. Data are given as percentage of untreated controls (mean $\pm$ S.E.M. of three independent experiments).

In both cell lines investigated, HDAC inhibition resulted in a dose-dependent increase of caspase-3 enzyme activity. In CM cells, 500 nM TSA led to a sixfold increase (Fig. 4A), while 5 mM NaB or 5 μ M MS-275 resulted in an approximately 12-fold increase of enzyme activity as compared with the control (Fig. 4C and E). Caspase-3 induction by HDAC inhibitors in BON cells showed a comparable dose dependency, but a less marked increase of enzyme activity (Fig. 4B, D and F).

To further substantiate HDAC inhibitor-induced apoptosis in NET cells, we determined DNA

fragmentation as a biochemical hallmark of apoptosis. After 24 h of incubation with TSA (CM, 500 nM; BON, 1000 nM) or MS-275 (CM, 5 μ M; BON, 10 μ M), DNA fragmentation was more pronounced in CM versus BON cells (Fig. 5).

HDAC inhibition and cell cycle regulation in NET cells

To test whether induction of cell cycle arrest contributed to the antiproliferative potency of HDAC inhibitors in NET cells, we performed flow cytometric cell cycle analyses. Incubating CM and BON cells with

Figure 3 HDAC inhibitor-induced cytotoxicity. LDH release into the supernatant of CM and BON cells was determined after 24 and 48 h of continuous incubation with increasing concentrations of TSA, NaB, or MS-275. Only slight increases of LDH release were observed within 24 h of cell treatment. Data of at least three independent experiments $\pm$ S.E.M. for each cell line are shown. *Statistical significance ($P < 0.05$) compared to controls, which were set at 100%.

escalating doses of TSA (CM, 0–500 nM; BON, 0–1000 nM) or NaB (CM, 0–5 mM; BON, 0–10 mM), or MS-275 (CM, 0–5 μ M; BON, 0–10 μ M) for 24 h resulted in an arrest of the NET cells in the G₁ phase of

the cell cycle, thereby decreasing the proportion of cells in the S phase (Fig. 6). Moreover, treatment with high HDAC inhibitor concentrations led to an additional G₂/M phase arrest in BON cells, which was

Figure 4 HDAC inhibitor-induced activation of caspase-3. Caspase-3 activity was evaluated in CM and BON cells after 24 h of continuous exposure to drugs. HDAC inhibition resulted in a dose-dependent increase of enzyme activity, which was more pronounced in CM cells. Data are given as percentage of untreated control (mean $\pm$ S.E.M. of four independent experiments for each cell line). *Statistical significance ($P < 0.05$) compared to controls.

Figure 5 MS-275-induced DNA fragmentation in neuroendocrine tumor cells. DNA isolated from CM cells, treated for 24 h with 500 nM TSA or 5 μ M MS-275 and BON cells, treated for 24 h with 1000 nM TSA or 10 μ M MS-275, was subjected to agarose gel electrophoresis and stained by ethidium bromide. Representative tracings of three independent experiments are shown. (A) 1, CM cell control; 2, TSA treated CM cells; +, positive control; M, molecular weight marker (100 bp); 3, BON cell control; 4, TSA treated BON cells. (B) 1, CM cell control; 2, MS-275 treated CM cells; M, molecular weight marker (100 bp); +, positive control; 3, BON cell control; 4, MS-275 treated BON cells.

accompanied by a proportional decrease of cells in the S and/or the G_0/G_1 phase. A distinct G_2/M arrest was observed for TSA (Fig. 6B) and NaB treatment (Fig. 6D), whereas the MS-275-induced G_2/M arrest was less pronounced (Fig. 6F).

Moreover, an apoptosis-specific increase of the sub- G_1 peak (hypodiploid cells) was observed for high concentrations of each HDAC inhibitor. At 500 nM TSA, 5 mM NaB, or 5 μ M MS-275 for CM cells and at 1000 nM TSA, 10 mM NaB, or 10 μ M MS-275 respectively for BON cells, we detected a hypodiploid population of approximately 10% in CM and 5% in BON cells (data not shown).

HDAC inhibitors modulate the expression of apoptosis-specific and cell cycle-regulating proteins in NET cells

To further characterize the effects of HDAC inhibition concerning apoptosis and cell cycle regulation, we performed western blotting to reveal the underlying molecular mechanisms. Treating CM and BON cells for 24 h with up to 10 μ M MS-275 resulted in a dose-dependent decrease of mitochondrial antiapoptotic Bcl-2 protein, whereas the expression of the proapoptotic Bax remained unaffected in both NET cell lines (Fig. 7A and B).

Moreover, we investigated the expression of cell cycle-regulating proteins. Cyclin D1, which is an essential promoter for the transition from the G_1 to the S phase, was downregulated in CM and BON cells by MS-275 treatment. At the same time, expression of the cyclin-dependent kinase inhibitors (CDKIs), p21 and

p27, markedly increased. The increase of p21 protein expression was dose-dependent, whereas p27 expression could not be enhanced by augmenting MS-275 concentration above 0.1 μ M (CM) and 1 μ M (BON) respectively (Fig. 7A and B).

Since we observed an additional G_2/M arrest in BON cells, we looked further for the expression of cyclin B1, which is known to promote G_2 -M transition. Treating BON cells with increasing concentrations of TSA or NaB led to a distinct decrease of cyclin B1 at 500–1000 nM TSA and at 5–10 mM NaB (Fig. 7C).

Discussion

Growth and spread of NET is often not well controlled by chemotherapy or biotherapy. Therefore, novel therapeutic options that are both effective and well tolerated are urgently needed.

HDAC inhibitors have already been demonstrated to inhibit the growth of several tumors *in vitro* and *in vivo* (Kramer *et al.* 2001, Marks *et al.* 2001, Vigushin *et al.* 2001, Johnstone 2002). Importantly, they are relatively nontoxic to nontransformed cells or tissue, but exhibit selective cytotoxic effects against a wide range of cancer cells (Byrd *et al.* 1999, Rosato & Grant 2003). Consequently, HDAC inhibitors are recognized as promising new anticancer drugs, and several investigational new drugs are currently undergoing clinical trials (Kramer *et al.* 2001, Acharya *et al.* 2005, Monneret 2005).

Since the effects of these agents have not yet been investigated in NETs, we explored the antiproliferative effects of three HDAC inhibitors TSA, NaB, and MS-275, on gastrointestinal NET cell lines. The growth pattern of NE gastrointestinal tumors exhibits a wide spectrum ranging from very slow- to fast-growing aggressive types of tumors (Öberg 1994). Therefore, we performed our studies on a carcinoid and an insulinoma cell line, which represent these different growth patterns. As a model for fast-growing NET cells, we chose the human insulinoma cell line CM with a doubling rate of less than 1 day (21 ± 1 h), while slower growing cells were represented by pancreatic carcinoid BON cells with a doubling time of 34 ± 4 h.

All the three HDAC inhibitors, investigated in this study, exerted strong antiproliferative effects in both gastrointestinal NET cell lines. Although TSA, NaB, and MS-275 were very effective in either cell line, fast-growing CM cells seemed to be more responsive towards HDAC inhibition. This is underlined by the IC_{50} values of TSA, NaB, and MS-275 (determined after 3 days of continuous incubation), which were significantly higher in BON than in CM cells.

Figure 6 Induction of cell cycle arrest by HDAC inhibition in neuroendocrine tumor cells. Incubation of CM and BON cells with increasing concentrations of TSA, NaB, or MS-275 for 24 h resulted in an altered distribution of G₀/G₁, S and G₂/M phases. Means of at least three independent experiments are shown. *Statistical significance ($P < 0.05$).

A proposed mechanism of action of HDAC inhibitors is the accumulation of acetylated histones, which leads to activation of the transcription of various genes whose expression causes inhibition or repression of tumor cell growth. This is supported by gene expression profilings of cells cultured in the presence of HDAC inhibitors, visualizing an altered expression

of a small number of genes, which are associated with apoptosis, differentiation, and cell cycle regulation (Van Lint *et al.* 1996, Della Ragione *et al.* 2001, Butler *et al.* 2002, Suzuki *et al.* 2002).

In this context, HDAC inhibitors have been reported to exert their antineoplastic effects by an induction of apoptosis in a wide variety of cancers, including breast and prostate cancers, as well as neuroblastoma, hepatoma, and some types of hematologic malignancies (Marks *et al.* 2000, Herold *et al.* 2002, Henderson *et al.* 2003). In accordance with these reports, we could demonstrate that treatment with TSA, NaB, or MS-275 strongly induced apoptotic cell death, which was accompanied by a dramatic increase of caspase-3 activity and DNA fragmentation.

The mechanisms involved in HDAC inhibitor-induced apoptosis are complex and differ among cell types (Duan *et al.* 2005). Treatment with HDAC inhibitors triggers both the intrinsic pathways and sensitizes tumor cells to the death ligands that initiate the extrinsic pathway of apoptosis. NaB, TSA, and MS-275 have been reported to induce mitochondrial permeability transition with a subsequent release of proapoptotic cytochrome *c* into the cytosol, resulting in activation of caspase-9 and caspase-3, thereby executing apoptosis (Rosato *et al.* 2001, Maggio *et al.* 2004, Roh *et al.* 2004). Additionally, HDAC inhibitor treatment has been shown to upregulate proapoptotic Fas, a member of the tumor necrosis factor receptor superfamily and the tumor necrosis factor-related apoptosis-inducing ligand receptors/death receptors DR4 and DR5, thereby triggering the extrinsic pathway, and further downregulate FLICE (caspase-8)-inhibitory protein c-FLIP, leading to caspase-8 and subsequently to caspase-3 activation (Bhalla & List 2004, Natoni *et al.* 2005).

Furthermore, the altered expression of several pro- and antiapoptotic intracellular genes by HDAC inhibitors has been reported. Upregulation of proapoptotic Bak and induction of conformational changes

Figure 7 Modulation of the expression of apoptosis- and cell cycle-relevant proteins. CM and BON cells were incubated with indicated concentrations of MS-275 for 24 h. Total cellular proteins were prepared and subjected to western-blot analyses with antibodies specific for the corresponding proteins. The antiapoptotic Bcl-2 was dose-dependently downregulated in both cell lines, whereas no change of proapoptotic Bax was observed by MS-275 treatment (A and B). Moreover, cell cycle promoter cyclin D1 was downregulated by MS-275 treatment, while the cell cycle inhibitors p27 and p21 were upregulated (A and B). BON cells were incubated with increasing concentrations of TSA or NaB for 24 h. Changes in the expression of the cell cycle regulator cyclin B1 were analyzed by western blotting. TSA and NaB induced a dose-dependent decrease of cyclin B1 in BON cells (C). Representative results out of three independent experiments are shown.

of the proapoptotic protein Bax may be some of the HDAC inhibitor-induced upstream events that trigger the mitochondrial pathway of apoptosis (Herold *et al.* 2002, Fuino *et al.* 2003, Johnstone & Licht 2003). In contrast, also antiapoptotic proteins such as Bcl-XL, Bcl-2, XIAP, Mcl-1, and surviving can be upregulated by many HDAC inhibitors (Guo *et al.* 2004, Maggio *et al.* 2004), but it is apparently the balance between the proteins that strongly favors apoptosis.

Differences in the expression pattern of apoptosis-related proteins seems to be cell-type dependent. For example, in hepatoma cells, TSA decreased the expression of Bcl-2, while expression of proapoptotic Bax was increased (Herold *et al.* 2002), no change of Bax and Bcl-2 expression was found in glioma cells with either TSA or NaB treatment (Sawa *et al.* 2001). MS-275 has been described to downregulate Bcl-2 and upregulate Bax expression in breast cancer cells (Singh *et al.* 2005). In the present study, we also found a downregulation of Bcl-2 expression upon MS-275 treatment, but expression of Bax remained unaffected in both NET cell lines. However, the exact mechanistical networks by which HDAC inhibitors regulate genes involved in apoptosis need further investigations.

Induction of the cyclin-dependent kinase inhibitor p21 is one of the common phenomena observed after HDAC inhibitor treatment (Richon *et al.* 2000, Herold *et al.* 2002). Although p21 has been commonly associated with the G₁ checkpoint (Yamashita *et al.* 2003), an additional involvement in the regulation of the G₂/M phase has also been demonstrated (Noh & Lee 2003, Peart *et al.* 2003). Accordingly, HDAC inhibitors have been shown to arrest the cell cycle both at the G₀/G₁ and the G₂/M phases, depending on cell type (Fournel *et al.* 2002, Takai *et al.* 2004, Ryu *et al.* 2006). Our findings for the HDAC inhibitors and the NET cells are in line with these data. TSA, NaB, or MS-275 induced an arrest of the cell cycle in the G₀/G₁ phase in either NET cell line, while we observed an additional G₂/M arrest for high HDAC inhibitor concentrations only in BON cells, confirming the cell-type-specific action of HDAC inhibitors.

Other studies have reported a p21-independent G₁ cell cycle arrest (Vaziri *et al.* 1998, Kim *et al.* 2000), making it unlikely that p21 is the sole target gene responsible for cell cycle arrest. There are several cell cycle-controlling genes known to be affected by HDAC inhibitors (Marks *et al.* 2003). Thus, NaB and TSA decreased the levels of cyclin D1, a cell cycle promoter acting at the G₁-to-S transition, and increased the expression of the cyclin-dependent kinase inhibitor p27, both leading to a G₁ cell cycle arrest (Takai *et al.* 2004, Chen & Faller 2005).

Cyclin B1 is the major gatekeeper in the G₂ phase. After treatment with TSA and NaB, BON cells were arrested in the G₂/M phase, due to downregulation of cyclin B1 (Lallemand *et al.* 1999). A recent study found that TSA downregulates the expression of cyclin B1, thereby affecting G₂–M transition (Noh & Lee 2003). Accordingly, we demonstrated that 500–1000 nM TSA and 5–10 mM NaB downregulate the expression of cyclin B1 in BON cells, arguing for the involvement of cyclin B1 in the TSA- or NaB-induced G₂ cell cycle arrest in BON cells.

Compared to the well-described effects of NaB and TSA on the expression of cell cycle-regulating genes, little is known how MS-275 affects cell cycle-regulating genes. This prompted us to focus on MS-275 as to the protein expression of cell cycle regulators. We found a dose-dependent upregulation of the cell cycle kinase inhibitor p21 in both cell lines, and that the G₁ arrest is accompanied by downregulation of cyclin D1 and an increased expression of the cyclin-dependent kinase inhibitors p27 (and p21).

Somatostatin analogs have been the mainstay of symptomatic management of patients with neuroendocrine tumors for two decades. In clinical studies, somatostatin and its stable analog octreotide have been shown to reduce blood levels of tumor-produced transmitters and hormones and thereby to control the hypersecretion syndrome (Öberg 1996).

Previously, it has been reported that somatostatin analogs might potentiate the antiproliferative effects of various chemotherapeutic agents in a synergistic or additive manner (Weckbecker *et al.* 1996). In the present study, combination treatments of somatostatin or octreotide with the HDAC inhibitor MS-275 failed to increase the antiproliferative effects of MS-275 in NET cells.

Nevertheless, the combination of MS-275 with a stable somatostatin analog, such as octreotide, represents a promising approach for treating NET disease, since tumor growth control might be achieved by the strong antiproliferative action of MS-275, whereas the hypersecretion syndromes will be suppressed by the somatostatin analogs.

Conclusions

Our study provides evidence that HDAC inhibitors potently inhibit proliferation of NET cells *in vitro*, thereby causing both apoptosis and a block of the cell cycle in gastrointestinal NET cells. Thus, HDAC inhibition is a promising novel strategy for achieving tumor growth inhibition in NET cells that justifies further clinical studies.

Acknowledgements

We are indebted to Antje Krahn and Björn Hoffmann for expert technical assistance. Viola Baradari was supported by a scholarship from the Deutsche Forschungsgemeinschaft. Alexander Huether was supported by a scholarship from the Sonnenfeld-Stiftung, Berlin, Germany. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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