Preclinical Development

Suppression of Neurotensin Receptor Type 1 Expression and Function by Histone Deacetylase Inhibitors in Human Colorectal Cancers

Xiaofu Wang3, Lindsey N. Jackson3, Sara M. Johnson3, Qingding Wang1,2, and B. Mark Evers1,2

Abstract

Neurotensin, a gut peptide, stimulates the growth of colorectal cancers that possess the high-affinity neurotensin receptor (NTR1). Sodium butyrate (NaBT) is a potent histone deacetylase inhibitor (HDACi) that induces growth arrest, differentiation, and apoptosis of colorectal cancers. Previously, we had shown that NaBT increases nuclear GSK-3β expression and kinase activity; GSK-3β functions as a negative regulator of extracellular signal-regulated kinase (ERK) signaling. The purpose of our current study was to determine: (a) whether HDACi alters NTR1 expression and function, and (b) the role of GSK-3β/ERK in NTR1 regulation. Human colorectal cancers with NTR1 were treated with various HDACi, and NTR1 expression and function were assessed. Treatment with HDACi dramatically decreased endogenous NTR1 mRNA, protein, and promoter activity. Overexpression of GSK-3β decreased NTR1 promoter activity (> 30%); inhibition of GSK-3β increased NTR1 expression in colorectal cancer cells, indicating that GSK-3β is a negative regulator of ERK and NTR1. Consistent with our previous findings, HDACi significantly decreased phosphorylated ERK while increasing GSK-3β. Selective MAP/ERK kinase/ERK inhibitors suppressed NTR1 mRNA expression in a time- and dose-dependent fashion, and reduced NTR1 promoter activity by ~70%. Finally, pretreatment with NaBT prevented neurotensin-mediated cyclooxygenase-2 and c-myc expression and attenuated neurotensin-induced interleukin-8 expression. HDACi suppresses endogenous NTR1 expression and function in colorectal cancer cell lines; this effect is mediated, at least in part, through the GSK-3β/ERK pathway. The downregulation of NTR1 in colorectal cancers may represent an important mechanism for the anticancer effects of HDACi. Mol Cancer Ther; 9(8); OF1–10. ©2010 AACR.

Introduction

Neurotensin, a tridecapeptide synthesized by enterochromaffin cells (N cells) and potently released into the circulation following the ingestion of fats, is a growth factor for normal intestinal mucosa (1). Typical physiologic functions for neurotensin include stimulation of pancreatic and biliary secretions, inhibition of small bowel and gastric motility, and facilitation of fatty acid translocation (2–4). In addition to its trophic effects on normal gastrointestinal tissues, neurotensin stimulates the proliferation, migration, and invasion of various cancers bearing neurotensin receptors (NTR), including pancreatic, colorectal, prostate, lung, and breast cancers (5–9). For example, Maoret et al. (10) showed stimulation of the growth of multiple colorectal cancer cell lines (SW480, SW620, HT29, and HCT116) by neurotensin acting through its high-affinity receptor, which is consistent with our previous findings as well (8, 9).

The effects of neurotensin are mainly mediated by the high-affinity NTR1, a member of the G-protein coupled receptor family (11). NTR1 is expressed in a majority of colorectal cancers and many colorectal cancer cell lines (3, 12, 13). Signaling through neurotensin/NTR1 stimulates various signal transduction pathways, including the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), c-jun-NH2-kinase, RhoGTPase, and focal adhesion kinase, among others, leading to the activation of various transcription factors and altering the expression of a number of tumor-promoting genes, such as cyclooxygenase-2 (COX-2) and interleukin-8 (IL-8; refs. 14, 15). Our group has recently reported that neurotensin stimulates IL-8 expression and colorectal cancer cell migration; this effect was predominantly through NTR1 and subsequent activation of Ca2+-dependent protein kinase C, NF-κB, and MAP/ERK kinase (MEK)/ERK-dependent activator protein-1 (8). Therefore, it is important to characterize the regulation of extracellular signal-regulated kinase 1.
of NTR1 in human colorectal cancer cells, which may identify potential targets for therapeutic interventions.

The histone deacetylase (HDAC) family of transcriptional corepressors has emerged as important regulators of intestinal cell differentiation and transformation. Expression of several HDAC isoforms is upregulated in the majority of colorectal cancers relative to adjacent normal mucosa, implicating a role of these HDACs in tumor promotion (16). Downregulation of specific HDACs with pharmacologic inhibitors leads to differentiation, growth arrest, and apoptosis of colorectal cancer cells in vitro and inhibits intestinal tumorigenesis in vivo (16–18). In addition to histones, HDACs have multiple protein substrates involved in the regulation of gene expression; inhibition of HDACs can also cause accumulation of acetylated forms of these proteins, altering their function (19). HDAC inhibitors (HDACi) are also potent sensitizers for radiation therapy in multiple cell types including colorectal cancer cells (20). A number of HDACi, such as vorinostat, belinostat, entinostat, and valproic acid, have or are presently being evaluated in clinical trials for the treatment of various cancers, including colorectal cancer (16, 18, 21). Despite increasing literature supporting a role of NTR1 in human colorectal cancer cells, which may play an important role in HDACi-mediated cellular processes, including embryonic development, cell differentiation, and apoptosis (22–24). Previously, we found that NaBT increased nuclear glycogen-synthase kinase-3β (GSK-3β) expression and activity, but decreased ERK1/2 in colorectal cancer cells (25, 26). Additionally, we reported that inhibition of GSK-3 significantly induced the phosphorylation of ERK1/2 in HT29 and Caco-2 cells, and increased the expression of COX-2 and IL-8, which are two important downstream targets of ERK1/2 activation, indicating that GSK-3β functions as a negative regulator of MAPK/ERK1/2 in colorectal cancer cells (24). We speculate that GSK-3β/ERK signaling may play an important role in HDACi-mediated cellular events, including gene regulation.

GSK-3 and ERK are critical downstream signaling proteins for the phosphatidylinositol 3-kinase (PI3K)/Akt and Ras/Raf/MEK-1 pathway, respectively, and regulate diverse cellular processes, including embryonic development, cell differentiation, and apoptosis. In our current study, we sought to determine the role of the GSK-3/ERK1/2 signaling cascade on the regulation of NTR1 by HDACi in human colorectal cancer cells. Here, we show that HDACi suppresses endogenous NTR1 expression and function in NTR1-positive colorectal cancer cell lines. Additionally, we found that overexpression of GSK-3β decreased NTR1 promoter activity, whereas inhibition of GSK-3β increased NTR1 expression in colorectal cancer cells, indicating that GSK-3β is a negative regulator of ERK and NTR1. HDACi significantly decreased phosphorylated ERK; selective MEK/ERK inhibitors suppressed NTR1 mRNA expression in a time- and dose-dependent fashion. Our results suggest that HDACi alters NTR1 expression through, at least in part, the GSK-3β/ERK pathway.

Materials and Methods

Materials

The GSK-3β inhibitor SB-216763 was purchased from Tocris. Protein synthesis inhibitors cycloheximide and anisomycin were purchased from Sigma Chemical Co. Nontargeting control small interfering RNA (siRNA) SMARTpool and the SMARTpool for GSK-3α and GSK-3β were purchased from Dharmacon Inc. 32P-UTP (3,000 Ci/mmole) was from PerkinElmer. The GSK-3β overexpression plasmid was a kind gift from Dr. James Woodgett (Samuel Lunenfeld Research Institute, Toronto, Canada). The human NTR1 promoter-luciferase reporter plasmid was a gift from Dr. PatriciaForgez (Sanofi-Aventis, Paris, France). The CCK (A) and CCK (B) receptor plasmids were gifts from Dr. Mark Hellmich (University of Texas Medical Branch, Galveston, TX). Ultraspec RNA reagent for RNA isolation was from Biotec Laboratories. Formaldehyde loading dye, positively charged nylon membranes, T7/SP6 MAXIscript labeling kit, and RPA III Ribonuclease Protection kit were purchased from Ambion. The hCK-5 multiprobe template set was purchased from BD PharMingen. Rabbit polyclonal anti-human NTR1 antibody was purchased from Santa Cruz Biotechnology. Uo126 and the dual lucerase assay system were purchased from Promega. Cell lysis buffer (10×), rabbit antiphosphorylated ERK1/2 and total ERK1/2 antibodies were purchased from Cell Signaling. Lipotectamine Plus and NuPAGE bis-Tris gel were purchased from Invitrogen. Other reagents were purchased from Calbiochem.

Cell culture

Human colorectal cancer cell lines HCT116, HT29, SW480, and SW620 cells were obtained from the American Type Culture Collection. HT29 and HCT116 were maintained in McCoy’s 5A supplemented with 10% of fetal bovine serum. SW480 and SW620 were maintained in DMEM/Leibovitz (1:1) supplemented with 10% fetal bovine serum. The human colon cancer cell line KM20 was obtained from Dr. Isaiah Fidler (M.D. Anderson Cancer Center, Houston, TX) and grown in minimum Eagle’s medium supplemented with 10 ml/L of sodium pyruvate, 10 ml/L of nonessential amino acids, 20 ml/L of minimum Eagle’s medium essential vitamin mixture, and 10% fetal bovine serum.

Northern blot

RNA was isolated from cultured cells using Ultraspec RNA reagent according to the manufacturer’s protocol. Total RNA (35 μg) was dissolved in formaldehyde loading dye, resolved on a 1.2% agarose/formaldehyde gel,
and transferred to positively charged nylon membranes. After baking in a vacuum oven at 80°C for 2 hours, membranes were prehybridized in hybridization buffer at 65°C for 2 hours, then incubated for 8 to 12 hours with a 32P-UTP-labeled cRNA probe containing 1,087 bp (471/1558 from start site) of human NTR1 cDNA subcloned into the XhoI/SmaI sites of the pGEM-7zf vector, or a labeled cRNA probe of human COX-2 (8), or a labeled cDNA probe of human c-myc (a gift from Dr. Brad Thompson, University of Texas Medical Branch, Galveston, TX) utilizing the T7/SP6 MAXIscript labeling kit. Membranes were then washed in 2× SSC (1× SSC = 0.15 mol/L NaCl, 0.015 mol/L Na citrate, 0.1% SDS) at room temperature, followed by two washes in 0.1× SSC at 65°C for 1 hour. Results were detected by autoradiography. All membranes were reprobed with GAPDH as an internal control.

RNase protection assay
RNA was isolated from cultured cells as described above. A 32P-UTP-labeled antisense RNA probe was prepared using the hCK-5 multiprobe template set and T7SP6 MAXIscript kit. RNase protection assays were done using labeled probes and the RPA III Ribonuclease Protection Kit according to the manufacturer’s recommendations, and as we have described previously (8, 27). Finally, samples were analyzed by electrophoresis on a 5% denaturing polyacrylamide gel and detected by autoradiography.

Transient transfection and luciferase assay
HCT116 or SW480 cells were seeded in 24-well plates one day prior to transfection. Cells were transfected with 1 μg NTR1 promoter-luciferase plasmid with or without 1 μg of GSK-3 expression plasmids or HDAC expression vectors (28) and 20 ng pRL-TK (internal control) using Lipofectamine Plus; 24 hours following transfection, cells were treated with or without reagents for various times and harvested for analysis. Luciferase was assayed with the dual luciferase assay system, suggesting transcriptional regulation.

Protein preparation and Western blotting assay
Protein preparation and Western blotting were done as described previously (2). In brief, cells were lysed with 1× cell lysis buffer. Equal amounts of protein were resolved on NuPAGE bis-Tris gel and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight with primary antibodies in Tris-Tween Buffered Saline followed by a horse-radish peroxidase-labeled secondary antibody for 1 hour. Membranes were developed using the enhanced chemiluminescence detection system (GE Heathcare).

Statistical analysis
The luciferase activity, measured in experiments where only two treatment groups were used (Figs. 1D, 4C, and 5C), was analyzed using the two-sample t test. Luciferase activity measured in an experiment with five treatment groups (vector and four HDACs; Fig. 2D) was analyzed using one-way classification ANOVA. All tests were assessed at the 0.05 level of significance. All statistical computations were conducted using the SAS system, release 9.1 (30).

Results
NaBT downregulates NTR1 expression in colorectal cancer cell lines
To examine the role of NaBT, a potent HDAC inhibitor, on the expression of NTR1 in colon cancer cell lines, HT29 cells were treated with different dosages of NaBT; NTR1 expression was then examined over a time course following treatment of cells with NaBT (2.5 mmol/L). We found that NaBT inhibited NTR1 mRNA expression in HT29 cells in both dose- and time-dependent fashions (Fig. 1A). A32P-labeled antisense RNA probe was prepared using the hCK-5 multiprobe template set and T7/SP6 MAXIscript labeling kit. Membranes were then washed in 2× SSC (1× SSC = 0.15 mol/L NaCl, 0.015 mol/L Na citrate, 0.1% SDS) at room temperature, followed by two washes in 0.1× SSC at 65°C for 1 hour. Results were detected by autoradiography. All membranes were reprobed with GAPDH as an internal control.

To further confirm the downregulation of NTR1 expression by NaBT, we next assessed the effect of NaBT on NTR1 protein expression by Western blot (Fig. 1C). Treatment with NaBT decreased NTR1 protein expression in both SW480 and HT29 cells. Finally, to assess whether the effects of NaBT are through regulation of gene transcription, SW480 and HCT116 cells were transiently transfected with the NTR1 promoter-luciferase plasmid and Renilla-luciferase plasmid (as an internal control; Fig. 1D). NaBT dramatically decreased NTR1 promoter activity, suggesting transcriptional regulation. Interestingly, as noted by RNase protection assays, NaBT treatment did not affect mRNA expression of other gastrointestinal hormone receptors, such as the cholecystokinin-B receptor (Fig. 1E), CCK-A receptor, and gastrin-releasing peptide receptor (data not shown).

NTR1 expression is inhibited by multiple HDAC inhibitors
To confirm that NaBT inhibits NTR1 mRNA expression through inhibition of HDAC, trichostatin A (TSA), valproic acid, entinostat, and apicidin, all structurally distinct HDACi compounds, were tested. These inhibitors suppressed NTR1 in all NTR1-positive cell lines tested, including HT29 and HCT116 (Fig. 2A and B), SW480, SW620, and KM20 (data not shown).

We did not detect any appreciable decrease of NTR1 mRNA expression using HDAC1, 2, or 3 siRNA transfected into SW480, HCT116, and HT29 cells (data not shown). One possible explanation for the absence of
an effect by the siRNA may be the fact that other HDAC isoforms are present, which then substitute for the absence of one isoform as we had previously noted with knockdown of HDAC3 leading to HDAC2 activation in colorectal cancer cells.4

NTR1 may be a direct target gene of HDACi

HDACi-regulated genes can be separated into direct and indirect targets (16). Direct targets, such as p21\textsuperscript{waf1}, are altered within a few hours of HDACi treatment, occurring despite the presence of protein synthesis inhibitors (31). We had previously noted that, after treatment with NaBT for 4 hours, the expression of NTR1 is significantly reduced, suggesting that NTR1 may be a direct target of HDACi (31). To confirm this finding, we next

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4 Q.D. Wang, unpublished data.
treated HT29 cells with the HDACi TSA and apicidin, and harvested cells at different time points. Treatment with both TSA and apicidin for 4 hours led to significantly decreased NTR1 mRNA expression (Fig. 3A). These results were confirmed using SW620 cells (Fig. 3B). Next, treatment of HT29 cells with the protein synthesis inhibitor cycloheximide, in combination with either NaBT or apicidin, minimally altered the effects of HDACi treatment (Fig. 3C); similar results were noted with anisomycin, another protein synthesis inhibitor. Taken together, these results indicate that NTR1 may be a direct target gene for HDACi.

The negative role of GSK-3β in NTR1 regulation by NaBT

Protein synthesis inhibitors minimally altered the effects of HDACi treatment, suggesting that other factors may be involved in NTR1 regulation. It has been reported that NTR1 mRNA is increased by LiCl, a nonspecific inhibitor of GSK-3β, in breast cancer cells (32). Previously, we showed that nuclear GSK-3β expression and kinase activity were increased by treatment with NaBT and other HDACi (26). Because GSK-3 plays an important role in the growth and differentiation of cancer cells, we next determined if GSK-3β is involved in NTR1 regulation. We first treated HT29 and SW480 cells with the selective GSK-3β inhibitor SB-216763, and examined NTR1 mRNA expression over a time course. Treatment with SB-216763 significantly upregulated NTR1 mRNA expression in both cell lines (Fig. 4A). SW480 cells were then transfected with GSK-3α siRNA, GSK-3β siRNA, or control siRNA, and were analyzed by Northern blot assay. GSK-3β siRNA, but not GSK-3α siRNA, increased NTR1 expression (Fig. 4B), indicating that GSK-3β negatively regulates NTR1. To confirm these results, the NTR1 promoter plasmid was cotransfected with the GSK-3β-overexpressing plasmid in both SW480 and HCT116 cell lines. Overexpression of GSK-3β decreased NTR1 promoter activity by >30% (Fig. 4C). Furthermore, treatment with the GSK-3β inhibitor SB-216763 partially attenuated the NTR1 mRNA suppression resulting from NaBT treatment (Fig. 4D). Taken together, these results suggest that GSK-3β is involved, at least in part, in NTR1 regulation by NaBT.
The MAPK/ERK pathway is required for NTR1 regulation by HDACi/GSK-3β signaling

We had previously reported that GSK-3β functions as a negative regulator of ERK in NTR1-positive HT29 cells and NTR1-negative Caco-2 cells (24), and NaBT decreases ERK activity in Caco-2 cells (25), suggesting that HDACi may increase GSK-3β activity, subsequently suppressing ERK in NTR1-positive colorectal cancer cells. We propose that this alteration of GSK-3β/ERK signaling may be involved in NTR1 regulation by HDACi.

To investigate this supposition, HT29, SW480, and HCT116 cells were treated with the selective MEK/ERK inhibitor U0126, and NTR1 expression was analyzed by Northern blot. Treatment with U0126 suppressed NTR1 mRNA expression in a time- and dose-dependent fashion in HT29 cells (Fig. 5A); similar results were noted with SW480 and HCT116 cells studies (Fig. 5B), as well as SW620 and KM20 cells (data not shown). We confirmed these results by treating cells with another MEK/ERK inhibitor, PD98059; the results were identical to those using U0126. In contrast, treatment with a selective p38 inhibitor, SB-203580, did not affect NTR1 expression (Fig. 5B). Next, SW480 cells were transfected with the NTR1 promoter-luciferase plasmid; after a 24-hour transfection,

Figure 4. The role of GSK-3β in NTR1 regulation by NaBT. A, HT29 and SW480 cells were treated with GSK-3β inhibitor SB-216763 or vehicle (DMSO) over a time course, and RNA was isolated and analyzed by Northern blots using hNTR1 and GAPDH probes. B, SW480 cells were transfected with GSK-3α or GSK-3β siRNA or control siRNA (representative of three independent experiments). RNA was isolated and analyzed by Northern blots for NTR1 mRNA expression. Whole proteins were extracted and knockdown of GSK-3α and GSK-3β was confirmed by Western blotting using anti-GSK-3α/β. C, SW480 and HCT116 cells were cotransfected with NTR1 promoter plasmid and HA-GSK-3β overexpression plasmid or empty vector (pCDNA3.1); 40 hours after transfection, cells were harvested and luciferase activity measured (representative experiment shown from three separate experiments utilizing at least 3 wells/construct). The overexpression of hemagglutinin-tagged GSK-3β was confirmed by Western blotting using anti-hemagglutinin antibody. D, HT29 cells were pretreated with SB-216763 or DMSO for 30 minutes followed by combination treatment with NaBT or vehicle (PBS) for an additional 6 hours. RNA was isolated and analyzed by Northern blots using hNTR1 and GAPDH probes.
cells were treated with Uo126 (5 μmol/L) for another 16 hours (Fig. 5C). Treatment with the MEK/ERK inhibitor Uo126 resulted in an approximately 70% decrease in NTR1 promoter activity in SW480 cells (Fig. 5C). These results suggest that the MEK/ERK pathway is necessary for NTR1 mRNA expression.

To confirm that the MEK/ERK pathway is regulated by HDACi/GSK-3β signaling, which subsequently alters NTR1 expression, we next assessed the effects of NaBT and apicidin on ERK1/2 protein expression in SW480 and HCT116 cells by Western blot (Fig. 5D). Treatment with NaBT and apicidin significantly decreased phospho-ERK1/2 levels in both SW480 and HCT116 cells; in contrast, emodin, which is an inhibitor of protein kinase CK2, did not affect ERK1/2 levels. Collectively, these results indicate that the MEK/ERK pathway is necessary for NTR1 expression in NTR-positive colorectal cancer cells.

NaBT attenuates neurotensin-induced hCOX-2, c-myc, and IL-8 expression

We next determined the effects of NaBT treatment on neurotensin-mediated gene expression in colorectal cancer cells. Neurotensin increases the expression of IL-8, COX-2 (8, 33), and c-myc (Fig. 6A), all of which contribute to colon cancer cell growth and metastasis (8, 16, 24). To evaluate the effects of NaBT on neurotensin-induced cellular events, HT29 and HC116 cells were pretreated with NaBT for 6 hours, then incubated with neurotensin over a time course. As shown in Fig. 6A, neurotensin increased c-myc expression in a time-dependent fashion; NaBT prevented neurotensin-stimulated c-myc induction. Pretreatment with NaBT blocked neurotensin-induced upregulation of c-myc, indicating that NaBT may be a potential therapeutic target for colon cancer treatment.
COX-2 (Fig. 6B) and IL-8 (Fig. 6C) expression in HT29 and HCT116 cells, respectively. Interestingly, NaBT alone slightly increased IL-8 expression in HCT116 cells, which has been reported in other cells (34, 35). Collectively, these results indicate that HDACi, such as NaBT, are important regulators for neurotensin/NTR1 signaling; this effect seems to be predominantly through the suppression of NTR1 expression.

Discussion

The chemotherapeutic potential of HDACi, including a number of dietary factors with HDAC inhibitory activity and antitumor effects in the colon, has been extensively described (36). Based upon these preclinical findings, several HDACi are currently being evaluated in clinical trials for the treatment of a variety of hematologic and solid tumors including colorectal cancers (18). In our present study, the effect of HDACi on NTR1 gene expression was determined. NaBT and other HDACi suppressed endogenous NTR1 expression in all NTR1-positive colorectal cancer cell lines examined through, at least in part, the GSK-3β/ERK pathway. Furthermore, NaBT prevented neurotensin-mediated induction of genes (i.e., c-myc, COX-2, and IL-8), which can promote colorectal cancer proliferation and invasion.

The effects of various hormones on tumor growth are well established. For example, altering hormone levels or blocking ligand binding to receptors has become part of the adjuvant treatment strategy for certain breast and prostate cancers (3, 7, 37). Similarly, cancers can also express receptors for gastrointestinal hormones that can alter the growth of these receptor-positive cancers. Studies from our laboratory and others have shown that ~50% of colorectal cancers possess NTR1 (12). HDACi suppresses NTR1, whereas we did not detect decreases in the expression of CCK (A) or CCK (B) receptors or gastrin-releasing peptide receptor in the colorectal cancers examined. The importance of NTR1 on colorectal cancers has been clearly delineated (3, 32); however, the significance of CCK and GRP receptors in colorectal cancer proliferation and prognosis is more controversial (38). Together, the effect of HDACi on NTR1 expression may reflect the importance of NTR1 on colorectal cancer proliferation and may also represent an important mechanism for the anticancer effects of HDACi.

Mechanisms of HDACi on gene regulation involve both direct and/or indirect effects (16). The findings in our current study suggest that the effects of HDACi on NTR1 are likely through both direct and indirect mechanisms. The indirect effects of HDACi-mediated gene regulation involve many different pathways and factors. For example, HDACi can alter expression patterns through effects on signal transduction pathways, notably, the PI3K and MEK/ERK pathways (39, 40). In our current study, we found that inhibition of GSK-3β led to increased expression of NTR1 mRNA in colorectal cancer cells; overexpression of GSK-3β led to a decrease in NTR1 promoter activity, suggesting that GSK-3β is a negative regulator of ERK and NTR1. We next confirmed that treatment with MEK/ERK inhibitors U0126 and PD98059 suppressed NTR1 expression and promoter activity in colorectal cancer cell lines. Treatment with NaBT and apicidin significantly reduced phosphorylation of ERK1/2 in NTR1-positive colorectal cancer cells, which is consistent with findings of Tatebe et al. (41) showing that treatment of human hepatocellular carcinoma cells with the HDACi valproic acid led to decreased activation of ERK.
GSK-3 is an evolutionarily conserved signaling molecule that plays an important role in diverse biological processes (42). More recent studies indicate a role for GSK-3 in the control of cell proliferation and survival in mammalian cells and in the identification of GSK-3 as a component of the Wnt signaling pathway, which controls development in invertebrates and vertebrates (43, 44). Several kinases have been shown to phosphorylate GSK-3 inhibitory sites in vitro (42). For example, protein kinase B (PKB/Akt), a serine/threonine kinase located downstream of PI3K, phosphorylates these sites in vitro and in vivo (45), and certain protein kinase C isoforms have been shown to phosphorylate and inactivate both isoforms of GSK-3 (46). These findings suggest that GSK-3 represents an important convergence point that integrates signals from multiple signaling cascades. Therefore, the regulation of NTR1 by GSK-3 signaling suggested that additional signaling pathways and/or factors may be involved in NTR1 regulation by HDACi. Indeed, it has been reported that the NTR1 promoter is activated by the Wnt/β-catenin pathway in Cos-7 cells, and NTR1 mRNA is upregulated by LiCl, a nonspecific inhibitor of GSK-3β, in human breast epithelial cells (32). Together, our results indicate that the HDACi/GSK-3β/ERK signaling cascade is involved in NTR1 regulation in colorectal cancer cells.

Neurotensin/NTR signaling contributes to tumorigenesis, cancer cell migration, and metastasis, likely through the induction of downstream proteins that mediate these effects (5, 32, 37). We next evaluated the effect of NTR1 suppression by HDACi on neurotensin-induced cancer-promoting genes. COX-2, the rate-limiting enzyme for prostaglandin synthesis, plays a critical role in the inflammatory response and in colon tumorigenesis (47). COX-2 expression is induced by a number of factors, including neurotensin and other hormones (33). From our results, HDACi suppressed NTR1 expression and significantly inhibited neurotensin-induced COX-2 expression in HT29 cells, suggesting that this effect is mediated through NTR1 inhibition. In addition to COX-2, we found that treatment with HDACi attenuated neurotensin-stimulated IL-8 expression. IL-8, a potent chemotactic factor, has been implicated in the pathogenesis of inflammatory gastrointestinal diseases (48) and in the growth of colorectal cancers as an autocrine growth factor (49). The expression of IL-8 is regulated by numerous factors and pathways. Pretreatment with NaBT significantly attenuated neurotensin-stimulated IL-8 expression, suggesting this inhibitory effect is mediated through NTR1 suppression by HDACi. Next, c-myc has been reported to be commonly upregulated in certain human cancers, leading to cell proliferation, arrested differentiation, and malignant transformation (50). Our data showed that neurotensin increased c-myc expression in HCT116 cells; NaBT decreased basal c-myc expression and blocked neurotensin-stimulated c-myc activity. Taken together, our results indicate the significant inhibitory role of HDACi in neurotensin-induced expression of cancer-promoting genes.

In conclusion, our results show, for the first time, that HDACi suppresses endogenous NTR1 expression and function in colorectal cancer cell lines. Additionally, HDACi blocks neurotensin-mediated COX-2 and c-myc expression and attenuates neurotensin-mediated IL-8 expression. The downregulation of NTR1 may represent a possible mechanism for the anticancer effects of HDACi in colorectal cancers.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Xiaofu Wang, Lindsey N. Jackson, Sara M. Johnson, et al.

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