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Valproic Acid Activates Notch1 Signaling and Induces Apoptosis in Medullary Thyroid Cancer Cells

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Abstract

Objective—To examine the effects of valproic acid (VPA) on Notch1 expression and cell proliferation in medullary thyroid cancer (MTC) cells.

Background—Besides surgery, there are no effective treatments for MTC. We have previously shown that over-expression of Notch1 in MTC cells inhibits cell growth and hormone production. The histone deacetylase inhibitor VPA, long used for the treatment of epilepsy, has been identified as a potential Notch1 activator. We hypothesized that VPA might activate Notch1 signaling in MTC cells with antiproliferative effects.

Methods—Human MTC cells were treated with VPA (0–5 mM) and Western blotting was performed to measure levels of Notch1 pathway proteins and neuroendocrine tumor markers. The MTT assay was used to assess the effect of VPA treatment on MTC cell proliferation. To determine the mechanism of growth inhibition, protein levels of various markers of apoptosis were measured. Enzyme-linked immunosorbent assays were used to confirm induction of apoptosis and measure calcitonin expression after VPA treatment.

Results—Notch1 was absent in MTC cells at baseline. VPA treatment resulted in an increase in both full-length and active Notch1 protein. Notch1 activation with VPA down-regulated two neuroendocrine tumor markers, ASCL1 and chromogranin A, and suppressed calcitonin expression. Importantly, VPA inhibited the growth of MTC cells in a dose-dependent manner and induced apoptosis.

Conclusions—VPA activates Notch1 signaling in MTC cells and inhibits growth by inducing apoptosis. As the safety of VPA in human beings is well-established, a clinical trial using this drug to treat patients with advanced MTC could be initiated in the near future.

Keywords

Valproic acid; Medullary thyroid cancer; Neuroendocrine tumors; Notch1; Achaete-scute complex-like 1 (ASCL1); Chromogranin A; Calcitonin; Histone deacetylase (HDAC) inhibition; Apoptosis

Introduction

Medullary thyroid cancer (MTC) is a neuroendocrine malignancy that arises from the calcitonin-secreting parafollicular C cells of the thyroid gland. MTC is the third most common

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type of thyroid cancer, accounting for approximately 3% of all cases¹. The natural history of MTC is characterized by early lymph node metastasis². In advanced cases, MTC can invade local structures such as the trachea and jugular vein, and metastasize to distant organs such as the liver, lungs, and bone^{3, 4}. Surgery is potentially curative, but complete resection is often not possible due to widespread disease. Furthermore, conventional anti-cancer treatments such as external beam radiation and cytotoxic chemotherapy are ineffective in MTC. Therefore, new therapeutic approaches to MTC are needed.

We have previously reported that ectopic expression of Notch1 in MTC cells resulted in suppression of neuroendocrine tumor markers and inhibition of cancer cell growth⁵. These findings suggested that Notch1 activation is an attractive therapeutic target for MTC. Until recently, however, pharmacologic activators of Notch1 signaling have not been available.

Valproic acid (VPA) is a branched-chain fatty acid that has been used for decades in the treatment of patients with epilepsy, bipolar disorder, and other neuropsychiatric diseases⁶. In addition to other properties, VPA is a histone deacetylase (HDAC) inhibitor and is currently in clinical trials for various malignancies. Stockhausen and colleagues reported the ability of VPA to increase Notch1 protein levels in neuroblastoma cells⁷. We subsequently demonstrated that VPA treatment of carcinoid cancer cells resulted in Notch1 activation, decreased production of neuroendocrine tumor markers, and dose-dependent suppression of cancer cell proliferation⁸. Based on these findings, we hypothesized that VPA may also activate Notch1 signaling in MTC cells, with similar anti-tumor effects. In this study, we describe for the first time the effects of VPA on Notch1 signaling, neuroendocrine tumor marker expression, and cancer cell growth in human MTC cells.

Methods

Cell Culture and Cell Proliferation Assay

Human MTC cells (TT) were obtained from American Type Culture Collection (Manassas, VA) and maintained as previously described^{5, 9}. MTC cell proliferation was measured by the MTT (methylthiazolyldiphenyl-tetrazolium bromide; Sigma-Aldrich, St. Louis, MO) rapid colorimetric assay as previously described¹⁰. Briefly, cells were seeded in quadruplicate on 24-well plates and incubated for 24 hours under standard conditions. The cells were then treated with the HDAC inhibitors valproic acid (VPA; 2-propylpentanoic acid; Sigma-Aldrich; 0–5 mM) or trichostatin A (TSA; Sigma-Aldrich; 0–50 nM), and incubated for up to 6 days. The MTT assay was performed every 2 days by replacing the standard medium with 250 μ L of serum-free medium containing MTT (0.5 mg/mL) and incubated at 37°C for 3 hours. After incubation, 750 μ L of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well and mixed thoroughly. The plates were then measured at 540 nm using a spectrophotometer (μ Quant; Bio-Tek Instruments, Winooski, VT).

Western Blot Analysis

MTC cells were treated with VPA (0–5 mM) or TSA (0–50 nM) for 48 hours and whole cell lysates were prepared as previously described¹¹. Total protein concentrations were quantified with a bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL). Denatured cellular extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH), blocked in milk, and incubated with appropriate antibodies as previously described¹¹. The following primary antibody dilutions were used: 1:1,000 for ASCL1 (BD Pharmingen, San Diego, CA), active/cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), active/cleaved caspase-9 (Cell Signaling Technology), poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology), chromogranin A (Invitrogen, Carlsbad, CA), and cyclin D1 (Cell Signaling

Technology), 1:2,000 for p21 (Cell Signaling Technology) and p27 (Santa Cruz Biotechnology), and 1:10,000 for G3PDH (1:10,000, Trevigen, Gaithersburg, MD). Horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Pierce Biotechnology) were used depending on the source of the primary antibody. For visualization of the protein signal, Immunstar (Bio-Rad Laboratories, Hercules, CA) or SuperSignal West Femto (Pierce Biotechnology) kits were used, according to the manufacturer's instructions, and then the blots were exposed to X-ray films.

Calcitonin ELISA

An enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen) was utilized to quantify the amount of calcitonin in MTC cells, as previously described⁵. MTC cells were treated with or without VPA (3 mM) for 48 hours, and cell lysates were used as the antigen source in a standard sandwich ELISA, per the manufacturer's instructions. Samples were analyzed in quadruplicate.

Apoptosis ELISA

Apoptosis was measured by the quantitation of cytosolic mono- and oligonucleosome-bound fragmented DNA by using an ELISA kit (Roche Applied Biosciences), according to the manufacturer's instructions. Briefly, MTC cells were treated with or without VPA (3 mM) for 48 hours, cell lysates were harvested, and the cytosolic fraction was isolated. The lysate was used as an antigen source in a standard sandwich ELISA, which consisted of a primary anti-histone antibody and a secondary anti-DNA antibody coupled to peroxidase. Absorbance values were used to calculate the percentage of fragmentation in comparison to control, and all samples were measured in triplicate.

Statistical Analysis

Analysis of variance (ANOVA) with Bonferroni *post hoc* testing (SPSS software, version 10.0; SPSS Inc, Chicago, IL) was used for statistical comparisons. A *p*-value of < 0.05 was considered to be statistically significant.

Results

VPA induces Notch1 expression in MTC cells

We have previously reported that Notch1 signaling is absent at baseline in human MTC tumors, and that Notch1 overexpression with an inducible Notch1 construct inhibited MTC cell growth and hormone production⁵. Recent research has demonstrated the ability of VPA to increase Notch1 protein levels in neuroblastoma⁷ and carcinoid cancer⁸ cells. Based on these findings, we performed Western blot analysis on extracts of VPA-treated MTC cells to determine if Notch1 signaling is activated by VPA. There was no detectable Notch1 protein in untreated cells. Importantly, VPA treatment resulted in induction of both full-length Notch1 and the active, cleaved form of the protein (Figure 1). TSA, another HDAC inhibitor, also up-regulated expression of full-length and cleaved Notch1 (data not shown).

VPA suppresses neuroendocrine tumor markers & hormones

ASCL1 is a helix-loop-helix transcription factor that regulates the neuroendocrine phenotype^{12, 13} and is an established target of Notch1 signaling^{5, 14}. Active Notch1 silences *ASCL1* gene transcription and enhances ASCL1 protein degradation, leading to decreased levels of the protein¹⁵. At baseline, high levels of ASCL1 were present in MTC cells. However, treatment with VPA down-regulated ASCL1 protein expression (Figure 2). This suppression of ASCL1 provided further evidence that VPA activates Notch1 signaling in MTC cells.

Patients with advanced MTC often suffer from debilitating symptoms because the tumors secrete various bioactive amines and peptides. Chromogranin A is an acidic glycoprotein that is co-secreted with hormones such as calcitonin by MTC cells. Chromogranin A is ubiquitously expressed in neuroendocrine tumors, and the protein is thus a useful clinical marker for this class of malignancies. As shown by Western analysis, untreated MTC cells had high levels of chromogranin A, whereas treatment with VPA dramatically suppressed the tumor marker (Figure 2).

Calcitonin is a polypeptide hormone secreted by thyroid parafollicular C cells. Virtually all patients with MTC have elevated calcitonin, and measurement of the hormone has been used for screening of patients with hereditary MTC, diagnosis of MTC, and surveillance for disease recurrence after surgery. Interestingly, VPA treatment of MTC cells for 2 days resulted in a 23% decrease in intracellular calcitonin, as measured by ELISA (Figure 3). This change in calcitonin was statistically significant. Taken together, these changes in ASCL1, chromogranin A, and calcitonin expression in MTC cells with VPA treatment indicate an alteration of the neuroendocrine phenotype.

VPA inhibits MTC cell proliferation

After demonstrating that VPA activates Notch1 signaling and alters the neuroendocrine phenotype in MTC cells, we next examined its effects on cell proliferation with the MTT assay. VPA treatment inhibited the growth of MTC cells in a dose-dependent manner (Figure 4). At a concentration as low as 1 mM, a statistically significant suppression of cancer cell growth was observed. Cells treated with VPA in concentrations of 2 mM and greater actually exhibited a progressive decrease in cell viability as measured by the MTT assay.

We then assessed the ability of another HDAC inhibitor, TSA, to suppress growth of MTC cells. Similar to VPA, TSA inhibited MTC cell proliferation in a dose-dependent fashion (Figure 5), suggesting that MTC growth suppression may be a general property of HDAC inhibitors.

VPA induces apoptosis in MTC cells

Having established that VPA inhibits cell growth in MTC cells, we were interested in determining the mechanism of action for this effect. Previous studies have shown that VPA induces apoptosis or programmed cell death in a variety of cancers, including leukemia^{16, 17}, multiple myeloma¹⁸, prostate cancer^{19, 20}, cervical cancer²¹, thoracic cancer²², medulloblastoma²³, melanoma²⁴, and well-differentiated thyroid cancer²⁵. We performed Western blot analysis to look for evidence of apoptosis in VPA-treated MTC cells.

The caspases are a family of proteases that mediate apoptosis²⁶. Caspase-9 is the initiator caspase of the mitochondrial or intrinsic pathway, and caspase-3 is the common effector caspase of both the intrinsic and extrinsic apoptosis pathways. The DNA repair enzyme poly (ADP-ribose) polymerase (PARP) is a target of active caspase-3, and PARP cleavage is a marker of apoptosis. Western blot analysis demonstrated an increase in active caspase-9 and caspase-3, as well as PARP cleavage, after VPA treatment of MTC cells (Figure 6A). We then used a cell death ELISA kit to confirm induction of apoptosis with VPA treatment. Compared to untreated control cells, MTC cells exposed to VPA for 2 days had a 14-fold increase in cytoplasmic histone-associated DNA fragments (Figure 6B), indicating apoptosis. We also performed immunoblot analysis to look for changes in mediators of cell cycle arrest, including p21, p27, and cyclin D1, but did not observe any significant changes in the expression of these proteins with VPA treatment (data not shown).

Discussion

Notch1 signaling is minimal or absent in neuroendocrine tumors such as small cell lung cancer^{15, 27}, carcinoid tumors^{8, 14, 28, 29}, and medullary thyroid cancer⁵, suggesting that in these cancers Notch1 may act as a tumor suppressor³⁰. Ectopic Notch1 expression in MTC cells, carcinoid tumor cells, and small cell lung cancer cells resulted in suppression of hormones and neuroendocrine tumor markers, and inhibition of cancer cell growth^{5, 27, 28}. These findings led us to consider Notch1 activation an attractive target for the treatment of neuroendocrine tumors, including MTC. However, until recently, no small molecule Notch1 activators have been described.

Stockhausen and colleagues reported an increase in Notch1 protein after VPA treatment of neuroblastoma cells⁷. Based on this observation, we treated human gastrointestinal and pulmonary carcinoid cancer cell lines with VPA, and noted an increase in Notch1 signaling and inhibition of tumor cell growth *in vitro*. VPA also suppressed tumor growth *in vivo*, in a mouse xenograft model of carcinoid cancer. Importantly, the median peak serum VPA level in treated animals was well within the therapeutic range of human patients treated with VPA for epilepsy, indicating that it is possible to activate Notch1 and suppress neuroendocrine tumor growth with VPA at non-toxic concentrations. *Notch1* gene knockdown with small interfering RNA (siRNA) blocked the inhibitory effects of VPA on carcinoid cancer cell growth and neuroendocrine tumor marker expression, providing further evidence to support the hypothesis that Notch1 is a crucial pathway for neuroendocrine tumorigenesis⁸.

In the current study we report the results of VPA treatment of another type of neuroendocrine tumor, MTC. At baseline, Notch1 protein was essentially undetectable in MTC cells. VPA treatment resulted in an increase in both the full-length and cleaved, active form of Notch1. Furthermore, VPA suppressed levels of *ASCL1*, an established target of Notch signaling, as well as the neuroendocrine tumor marker chromogranin A, and the hormone calcitonin. These results are consistent with our previous findings in an inducible Notch1 model of MTC, where Notch1-mediated silencing of *ASCL1* gene transcription down-regulated protein expression of chromogranin A and calcitonin⁵. The phenotypic change induced by VPA may have implications for the treatment and palliation of patients with advanced MTC disease, as these patients often suffer from endocrinopathies secondary to tumor secretion of hormones such as calcitonin.

The mechanism by which VPA activates Notch1 signaling is yet to be determined. VPA is known to inhibit histone deacetylase (HDAC) enzymes. HDAC inhibitors increase histone acetylation, alter chromatin structure, and modulate gene transcription³¹. Several HDAC inhibitors including VPA and suberoylanilide hydroxamic acid (SAHA) have shown antineoplastic effects in a variety of cancers, and are currently being evaluated in clinical trials³². The effects of HDAC inhibitors such as VPA in MTC and other neuroendocrine tumors may be mediated in part by activation of the Notch1 signaling pathway^{8, 33}.

VPA treatment of MTC cells resulted in dose-dependent inhibition of cancer cell proliferation. This effect was also seen after treatment of MTC cells with another HDAC inhibitor, trichostatin A. Western blot analysis revealed an increase in active caspase-9 and caspase-3, as well as PARP cleavage, indicating the induction of apoptosis after VPA treatment. A cell death ELISA confirmed that the mechanism of VPA-induced growth inhibition in MTC cells is apoptosis. Notch1 is known to mediate apoptosis of neural progenitor cells during embryonic development³⁴. The ability of VPA to activate apoptotic pathways in MTC cells may form the basis of a novel targeted therapy for this disease. As the drug has a proven safety profile in humans, a clinical trial of VPA in patients with advanced MTC could be initiated relatively quickly.

Mini-Abstract

The authors treated human medullary thyroid cancer (MTC) cells with valproic acid (VPA), a histone deacetylase inhibitor long used for the treatment of epilepsy. VPA activated Notch1 signaling, down-regulated neuroendocrine tumor markers, suppressed calcitonin, inhibited MTC cell growth, and induced apoptosis. Based on these findings, the authors propose a clinical trial of VPA in patients with advanced MTC.

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References

1. National Cancer Institute. SEER. Incidence: Thyroid Cancer. 2004.
2. Greenblatt DY, Elson D, Mack E, et al. Initial lymph node dissection increases cure rates in patients with medullary thyroid cancer. *Asian J Surg* 2007;30:108–112. [PubMed: 17475579]
3. Chen H, Roberts JR, Ball DW, et al. Effective long-term palliation of symptomatic, incurable metastatic medullary thyroid cancer by operative resection. *Ann Surg* 1998;227:887–895. [PubMed: 9637552]
4. Greenblatt DY, Chen H. Palliation of Advanced Thyroid Malignancies. *Surgical Oncology* 2007;16:237–247. [PubMed: 17855077]
5. Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, et al. Overexpression of the NOTCH1 intracellular domain inhibits cell proliferation and alters the neuroendocrine phenotype of medullary thyroid cancer cells. *J Biol Chem* 2006;281:39819–39830. [PubMed: 17090547]
6. Henry TR. The history of valproate in clinical neuroscience. *Psychopharmacol Bull* 2003;37(Suppl 2): 5–16. [PubMed: 14624229]
7. Stockhausen MT, Sjolund J, Manetopoulos C, et al. Effects of the histone deacetylase inhibitor valproic acid on Notch signalling in human neuroblastoma cells. *Br J Cancer* 2005;92:751–759. [PubMed: 15685243]
8. Greenblatt DY, Vaccaro A, Jaskula-Sztul R, et al. Valproic acid activates Notch1 signaling and regulates the neuroendocrine phenotype in carcinoid cancer cells. *The Oncologist* 2007;12:942–951. [PubMed: 17766653]
9. Chen H, Carson-Walter EB, Baylin SB, et al. Differentiation of medullary thyroid cancer by C-Raf-1 silences expression of the neural transcription factor human achaete-scute homolog-1. *Surgery* 1996;120:168–72. discussion 173. [PubMed: 8751579]
10. Van Gompel JJ, Kunnimalaiyaan M, Holen K, et al. ZM336372, a Raf-1 activator, suppresses growth and neuroendocrine hormone levels in carcinoid tumor cells. *Mol Cancer Ther* 2005;4:910–917. [PubMed: 15956248]
11. Sippel RS, Carpenter JE, Kunnimalaiyaan M, et al. Raf-1 activation suppresses neuroendocrine marker and hormone levels in human gastrointestinal carcinoid cells. *Am J Physiol Gastrointest Liver Physiol* 2003;285:G245–54. [PubMed: 12851216]
12. Sippel RS, Carpenter JE, Kunnimalaiyaan M, et al. The role of human achaete-scute homolog-1 in medullary thyroid cancer cells. *Surgery* 2003;134:866–71. discussion 871–3. [PubMed: 14668716]
13. Chen H, Kunnimalaiyaan M, Van Gompel JJ. Medullary thyroid cancer: the functions of raf-1 and human achaete-scute homolog-1. *Thyroid* 2005;15:511–521. [PubMed: 16029117]
14. Nakakura EK, Sriuranpong VR, Kunnimalaiyaan M, et al. Regulation of neuroendocrine differentiation in gastrointestinal carcinoid tumor cells by notch signaling. *J Clin Endocrinol Metab* 2005;90:4350–4356. [PubMed: 15870121]

15. Sriuranpong V, Borges MW, Strock CL, et al. Notch signaling induces rapid degradation of achaete-scute homolog 1. *Mol Cell Biol* 2002;22:3129–3139. [PubMed: 11940670]
16. Tsapis M, Lieb M, Manzo F, et al. HDAC inhibitors induce apoptosis in glucocorticoid-resistant acute lymphatic leukemia cells despite a switch from the extrinsic to the intrinsic death pathway. *Int J Biochem Cell Biol*. 2007
17. Xue HM, Li WY, Guo HX, et al. Experimental study on the mechanism of the apoptosis of leukemic cells induced by valproic acid. *Zhonghua Er Ke Za Zhi* 2005;43:894–898. [PubMed: 16412349]
18. Kaiser M, Zavrski I, Sterz J, et al. The effects of the histone deacetylase inhibitor valproic acid on cell cycle, growth suppression and apoptosis in multiple myeloma. *Haematologica* 2006;91:248–251. [PubMed: 16461312]
19. Angelucci A, Valentini A, Millimaggi D, et al. Valproic acid induces apoptosis in prostate carcinoma cell lines by activation of multiple death pathways. *Anticancer Drugs* 2006;17:1141–1150. [PubMed: 17075313]
20. Shabbeer S, S Q Kortenhorst M, Kachhap S, et al. Multiple Molecular pathways explain the anti-proliferative effect of valproic acid on prostate cancer cells in vitro and in vivo. *Prostate* 2007;67:1099–1110. [PubMed: 17477369]
21. Chen J, Ghazawi FM, Bakkar W, et al. Valproic acid and butyrate induce apoptosis in human cancer cells through inhibition of gene expression of Akt/protein kinase B. *Mol Cancer* 2006;5:71. [PubMed: 17156483]
22. Ziauddin MF, Yeow WS, Maxhimer JB, et al. Valproic acid, an antiepileptic drug with histone deacetylase inhibitory activity, potentiates the cytotoxic effect of Apo2L/TRAIL on cultured thoracic cancer cells through mitochondria-dependent caspase activation. *Neoplasia* 2006;8:446–457. [PubMed: 16820090]
23. Li XN, Shu Q, Su JM, et al. Valproic acid induces growth arrest, apoptosis, and senescence in medulloblastomas by increasing histone hyperacetylation and regulating expression of p21Cip1, CDK4, and CMYC. *Mol Cancer Ther* 2005;4:1912–1922. [PubMed: 16373706]
24. Valentini A, Gravina P, Federici G, et al. Valproic Acid Induces Apoptosis, p(16INK4A) Upregulation and Sensitization to Chemotherapy in Human Melanoma Cells. *Cancer Biol Ther* 2007;6:185–191. [PubMed: 17218782]
25. Shen WT, Wong TS, Chung WY, et al. Valproic acid inhibits growth, induces apoptosis, and modulates apoptosis-regulatory and differentiation gene expression in human thyroid cancer cells. *Surgery* 2005;138:979–84. discussion 984–5. [PubMed: 16360381]
26. Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* 1999;68:383–424. [PubMed: 10872455]
27. Sriuranpong V, Borges MW, Ravi RK, et al. Notch signaling induces cell cycle arrest in small cell lung cancer cells. *Cancer Res* 2001;61:3200–3205. [PubMed: 11306509]
28. Kunnimalaiyaan M, Traeger K, Chen H. Conservation of the Notch1 signaling pathway in gastrointestinal carcinoid cells. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G636–42. [PubMed: 16160079]
29. Kunnimalaiyaan M, Yan S, Wong F, et al. Hairy Enhancer of Split-1 (HES-1), a Notch1 effector, inhibits the growth of carcinoid tumor cells. *Surgery* 2005;138:1137–42. discussion 1142. [PubMed: 16360401]
30. Kunnimalaiyaan M, Chen H. Tumor suppressor role of Notch1 signaling in neuroendocrine tumors. *Oncologist* 2007;12:535–542. [PubMed: 17522241]
31. Cress WD, Seto E. Histone deacetylases, transcriptional control, and cancer. *J Cell Physiol* 2000;184:1–16. [PubMed: 10825229]
32. Gallinari P, Di Marco S, Jones P, et al. HDACs, histone deacetylation and gene transcription: from molecular biology to cancer therapeutics. *Cell Res* 2007;17:195–211. [PubMed: 17325692]
33. Greenblatt DY, Cayo M, Ning L, et al. Suberoyl bishydroxamic acid inhibits cellular proliferation by inducing cell cycle arrest in carcinoid cancer cells. *J Gastrointest Surg* 2007;11:1515–20. discussion 1520. [PubMed: 17874277]
34. Yang X, Klein R, Tian X, et al. Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. *Dev Biol* 2004;269:81–94. [PubMed: 15081359]

35. Kimura K, Satoh K, Kanno A, et al. Activation of Notch signaling in tumorigenesis of experimental pancreatic cancer induced by dimethylbenzanthracene in mice. *Cancer Sci* 2007;98:155–162. [PubMed: 17297654]

Figure 1.

VPA induces Notch1 in MTC cells. Human MTC cells were treated with VPA (0–5 mM) for 2 days and Western blot analysis was performed to measure protein levels of full-length Notch1 and Notch1 intracellular domain (NICD), the active form of the protein. Extracts of MiaPaCa-2 pancreatic adenocarcinoma cells, which are known to express high levels of Notch1³⁵, were used as a positive control. G3PDH expression was measured to confirm equal protein loading.

Figure 2.

VPA suppresses ASCL1 and chromogranin A. MTC cells were treated with VPA (0–5 mM) for 4 days and protein levels of ASCL1, a target of Notch1 signaling, and chromogranin A (CgA), a neuroendocrine tumor marker, were measured by immunoblotting. G3PDH was used as a loading control.

Figure 3.

VPA down-regulates calcitonin. Intracellular calcitonin levels in MTC cells treated with (VPA) or without (Control) 3 mM of VPA for 2 days were measured via ELISA.

Figure 4. VPA inhibits MTC cell proliferation *in vitro*. MTC cells were treated with VPA (0–5 mM) for up to 6 days and cell viability was measured every 2 days with the MTT assay.

Figure 5. TSA inhibits MTC cell growth. MTC cells were exposed to the HDAC inhibitor TSA (0–50 nM) for up to 6 days and cell proliferation was quantified with the MTT assay.

A **B** **Figure 6.**

VPA induces apoptosis in MTC cells. **A**, Western blot analysis was performed on MTC cells treated with VPA (0–5 mM) for 2 days. Primary antibodies against cleaved/active caspase 9 and caspase 3 as well as full-length and cleaved PARP were used. Equal loading was confirmed with G3PDH. **B**, cytosolic mono- and oligonucleosome-bound DNA fragmentation was quantified with a cell death ELISA kit. The graph shows DNA fragmentation of MTC cells treated with VPA (3 mM) for 2 days relative to untreated control cells.