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Key Words. Valproic acid • Carcinoid tumors • Neuroendocrine tumors • Notch-1 Achaete-scute complex-like 1 (ASCL-1)

ABSTRACT

Carcinoid tumors are neuroendocrine malignancies that frequently metastasize and secrete hormones that cause debilitating symptoms in patients. In this study we report the effects of valproic acid (VPA), a drug long used for the treatment of epilepsy, on the growth and neuroendocrine phenotype of human carcinoid cancer cells. VPA treatment of gastrointestinal and pulmonary carcinoid cells resulted in a dose-dependent inhibition of cancer cell growth. Western blot analysis revealed degradation of cyclin D1 and an increase in cyclin-dependent kinases p21 and p27 with VPA treatment. Flow cytometry confirmed that the mechanism of VPA-induced growth inhibition is G_1 phase cell cycle arrest. Furthermore, VPA suppressed expression of the neuroendocrine tumor marker chromogranin A. In addition to these effects, VPA also increased levels of full-length Notch-1 and the active Notch-1 intracellular domain. Luciferase reporter assays incorporating the centromere-binding factor 1 (CBF-1) binding site and the achaete-scute complex-like 1 (ASCL-1) promoter confirmed the functional activity of VPA-induced Notch-1. Transfection of Notch-1 small-interfering RNA into carcinoid tumor cells blocked the effects of VPA on Notch-1 activation, ASCL-1 suppression, p21 induction, and cell growth inhibition. VPA also suppressed growth of carcinoid tumors in vivo in a mouse tumor xenograft experiment. These findings confirm the important role of Notch-1 in regulating the growth and neuroendocrine phenotype of carcinoid tumor cells. On the basis of this study, a clinical trial of VPA for patients with advanced carcinoid cancer will be conducted. ***The Oncologist*** 2007;12:942–951

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Carcinoid tumors are neoplasms that arise from the disseminated neuroendocrine cell system of the gastrointestinal (GI) tract, lungs, and other organs. The incidence of carcinoid tumors in the U.S. is estimated at 1.5 cases per 100,000 people, or approximately 2,500 new cases of carcinoid tumors per year [1, 2]. While they are slow growing compared to adenocarcinomas, carcinoids frequently metastasize to the liver, and can cause debilitating symptoms in patients as a result of tumor secretion of various hormones and peptides. Surgery is the only potentially curative treatment, but complete resection is often impossible because of widespread disease [3]. Other conventional cancer treatments such as chemotherapy and external beam radiation are

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largely ineffective against carcinoids [4]. Therefore, there is a pressing need for the development of new therapeutic approaches to advanced carcinoid tumor disease.

We have previously reported that ectopic expression of Notch-1 in carcinoid and other neuroendocrine tumor cells resulted in decreased production of neuroendocrine tumor markers and suppression of cancer cell growth [5–8]. We concluded that activation of Notch-1 signaling was an attractive target for the development of new treatments for carcinoids and other neuroendocrine tumors. Until now, however, no small-molecule activators of Notch-1 signaling in carcinoid tumors have been described.

Valproic acid (VPA) is a branched-chain fatty acid that has long been used for the treatment of patients with epilepsy and other neuropsychiatric disorders [9]. In addition to other properties, VPA is a well-established histone deacetylase (HDAC) inhibitor and is currently in clinical trials for various cancers. Stockhausen and colleagues recently described the ability of VPA to increase Notch-1 protein levels in neuroblastoma cells [10]. We hypothesized that VPA may also be able to activate Notch-1 signaling in carcinoid cancer cells, with antitumor effects.

In this study we describe the effects of VPA on human GI and pulmonary carcinoid tumor cell lines. VPA treatment resulted in a dose-dependent inhibition of carcinoid cell growth in vitro. Western blot analysis and flow cytometry demonstrated that this growth inhibition is mediated by cell cycle arrest at the G_1 phase. Besides inhibiting cancer cell growth, VPA also suppressed production of the neuroendocrine tumor marker chromogranin A (CgA). These effects of VPA on carcinoid cell growth and CgA production were associated with Notch-1 signaling activation by an increase in protein levels of both full-length Notch-1 and the active Notch-1 intracellular domain (NICD). Luciferase reporter assays confirmed that the Notch-1 induced by VPA binds with centromere-binding factor 1 (CBF-1) and the achaete-scute complex like 1 (ASCL-1) promoter repressor, indicating that it is functionally active. Small-interfering RNA (siRNA) against Notch-1 countered the effects of VPA on carcinoid cell growth and neuroendocrine marker production, providing further evidence that VPA acts through Notch-1. Finally, VPA inhibited carcinoid tumor growth in vivo in a xenograft model. These findings demonstrate that VPA has antitumor effects in carcinoid cells in vitro and in vivo, and suggest that these effects are mediated by activation of Notch-1 signaling. These data form the basis for a clinical trial of VPA in the treatment of patients with advanced carcinoid cancer, a disease for which few effective therapies currently exist.

Materials and Methods

Cell Culture

BON human GI carcinoid tumor cells, kindly provided by Drs. B. Mark Evers and Courtney M. Townsend, Jr. (University of Texas Medical Branch, Galveston, TX), and NCI-H727 human pulmonary carcinoid tumor cells (American Type Culture Collection, Manassas, VA) were maintained as previously described [11, 12].

Cell Proliferation Assay

Carcinoid tumor cell proliferation was measured by the methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO) rapid colorimetric assay as previously described [12]. Briefly, cells were seeded in quadruplicate on 24-well plates and incubated for 24 hours under standard conditions to allow cell attachment. The cells were then treated with VPA (2-propylpentanoic acid; Sigma-Aldrich) in concentrations of 0–4 mM and incubated for up to 6 days. The MTT assay was performed by replacing the standard medium with 250 μl of serum-free medium containing MTT (0.5 mg/ml) and incubating at 37°C for 3 hours. After incubation, 750 μl of dimethyl sulfoxide (Sigma-Aldrich) was added to each well and mixed thoroughly. The plates were then measured at 540 nm using a spectrophotometer (μQuant; Bio-Tek Instruments, Winooski, VT).

Western Blot Analysis

Carcinoid cancer cells were treated with VPA and whole cell lysates were prepared as previously described [11]. Total protein concentrations were quantified with a bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL). Denatured cellular extracts were resolved by SDS-PAGE, transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH), blocked in milk, and incubated with appropriate antibodies. The antibody dilutions were: 1:1,000 for Notch-1 (Santa Cruz Biotechnology, Santa Cruz, CA), mammalian ASCL-1 (BD Biosciences, San Diego, CA), CgA (Zymed Laboratories, San Francisco, CA), and cyclin D1 (Cell Signaling Technology, Danvers, MA); 1:2,000 for p21 (Cell Signaling Technology) and p27 (Santa Cruz Biotechnology); and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (G3PDH; 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 per the manufacturer's instructions.

Flow Cytometry

GI carcinoid cells treated with or without VPA were labeled for 1 hour with 10 μM bromodeoxyuridine (Sigma-Aldrich), counterstained with 50 μg/ml propidium iodide, and analyzed by two-dimensional flow cytometry (FACSCalibur flow cytometer; BD Biosciences) to detect both fluorescein and propidium iodide as previously described [13]. Results were analyzed with FlowJo software (Tree Star, Ashland, OR).

Luciferase Reporter Assays

GI carcinoid tumor cells were transiently transfected with luciferase constructs as previously described [6]. Wild-type (4xwtCBF1Luc; 2 μg) or mutant (4xmutCBF1Luc; 2 μg) CBF-1–luciferase reporter plasmids were cotransfected with cytomegalovirus β-galactosidase (CMV-β-gal; 0.5 μg) [14]. In experiments with the γ-secretase inhibitor, *N*-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; EMD Biosciences, Darmstadt, Germany), to inhibit Notch-1 cleavage and activation, cells were pretreated for 45 minutes with DAPT (50 μM) then treated with 4 mM VPA. In the second study, a plasmid containing the ASCL-1 promoter construct p-3600/+37 (2 μg) was cotransfected with CMV-β-gal (0.5 μg), as previously described [15]. After transfection, cells were treated with or without VPA for 48 hours. Cells were harvested and lysed, and luciferase and β-galactosidase assays (Promega, Madison, WI) were performed in accordance with the manufacturer's instructions. Luciferase levels were measured using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA). Luciferase activity was expressed relative to β-galactosidase activity.

Notch-1 RNA Interference Assays

siRNA against Notch-1 and nonspecific siRNA (Santa Cruz Biotechnology, sc-44226 and sc-37007) were transfected into BON GI carcinoid cells using Lipofectamine 2000 (Invitrogen, San Diego, CA) per the manufacturer's instructions. The next day, the medium containing the transfection complexes was replaced with fresh medium with or without VPA (4 mM), and the cells were incubated for another 48 hours. The cells were then harvested and cell lysates were prepared for immunoblotting as described above. To determine the effect of Notch-1 on carcinoid cell proliferation, BON cells were transfected with Notch-1 siRNA or nonspecific siRNA and incubated overnight. The next day, the cells were trypsinized, counted, and plated in equal amounts (10,000 cells per well) onto 24-well plates. On the following day, the cells were treated with control or VPA (4 mM) and the MTT assay was performed every 2 days, as described above.

Xenograft Studies

BON cells (1×10^6) were suspended in Hanks' balanced salt solution (Invitrogen) and injected s.c. into the right flank of 12 male nude athymic NU/NU mice (Charles River Laboratories, Wilmington, MA) under anesthesia. After palpable tumors developed, the mice were divided into two groups of six animals. The control group received daily i.p. injections of saline while the treatment group received daily i.p. injections of VPA (366 mg/kg) for 20 days. The length and width of tumors were measured with a vernier caliper every 4 days and tumor volumes were calculated using the formula volume = width^2 $\times$ length $\times$ 0.52. At the end of the experiment the mice were sacrificed and the tumors were resected and frozen in liquid nitrogen. Whole tumor cell protein lysates were prepared for immunoblotting as previously described [16]. This experiment was performed in accordance with the protocols of the University of Wisconsin Medical School Animal Care and Use Committee.

Statistical Analysis

Analysis of variance with Bonferroni post hoc testing was performed using a statistical analysis software package (SPSS version 10.0, SPSS, Chicago, IL). A p-value of $<.05$ was considered significant.

RESULTS

VPA Suppresses Growth of Carcinoid Tumor Cells In Vitro

VPA has been shown to inhibit the growth of a variety of human cancer cells, including multiple myeloma [17], lymphoid cancers [18], malignant glioma [19], medulloblastoma [20, 21], neuroblastoma [10, 22, 23], endometrial cancer [24], cervical cancer [25], ovarian cancer [26], breast adenocarcinoma [25], colon adenocarcinoma [25], sarcoma [25], thyroid cancer [27, 28], and melanoma [29]. However, the effects of VPA on carcinoid tumor cell growth have not been characterized to date. We utilized the MTT assay to measure cell viability after VPA treatment of BON GI and H727 pulmonary human carcinoid tumor cells. GI carcinoid cells treated with VPA had a profound dose-dependent inhibition of growth (Fig. 1A). After 6 days of treatment, growth of cells exposed to 1 mM of VPA was inhibited by 50% relative to untreated cells. Statistically significant growth inhibition was also seen in pulmonary carcinoid cells treated with VPA (Fig. 1B).

The Mechanism of VPA-Induced Carcinoid Growth Suppression Is Cell Cycle Arrest

After establishing that VPA inhibits cell proliferation in carcinoids, we were interested in determining the mecha-

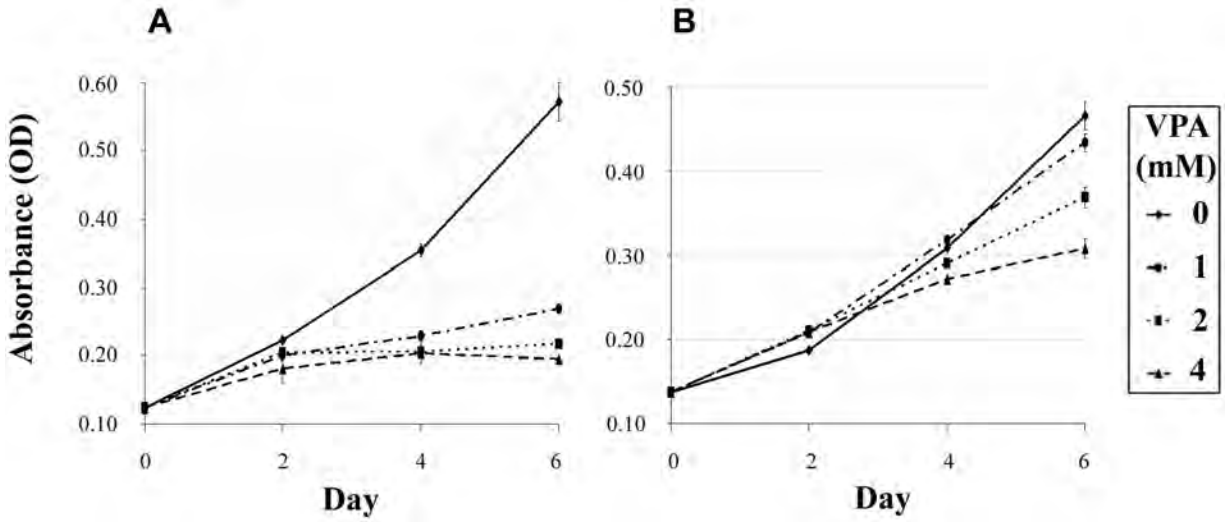

Figure 1. VPA suppresses growth of carcinoid tumor cells in vitro. Gastrointestinal **(A)** and pulmonary **(B)** carcinoid cancer cells were treated with VPA (0–4 mM) for up to 6 days, and cell viability was measured with the MTT assay.

Abbreviations: MTT, methylthiazolyldiphenyl-tetrazolium bromide; OD, optical density; VPA, valproic acid.

nism of action for this effect. Previously, VPA has been shown to induce cell cycle arrest in a variety of cancer cell lines by modulating expression of p21 and other regulatory proteins [17, 20, 23, 28]. We performed Western blot analysis using BON cell lysates after 2-day and 4-day treatment with VPA to measure the effect of the drug on cell cycle regulators. Treatment of BON cells with 1 mM of VPA resulted in an increase in protein levels of the cyclin-dependent kinase inhibitors p21 and p27 (Fig. 2A). The cell cycle promoter cyclin D1 was suppressed by VPA, with levels almost undetectable after 4 days of treatment with 4 mM of VPA. Similar results on expression of p21, p27, and cyclin D1 were seen in H727 cells treated with VPA (data not shown). To confirm the induction of cell cycle arrest with VPA treatment, we performed flow cytometry. VPA treatment of BON cells resulted in a dose-dependent, statistically significant increase in the percentage of cells in the G_1 phase of the cell cycle, accompanied by a decrease in cells in the S phase (Fig. 2B). Taken together, these immunoblot and flow cytometry data indicate that VPA inhibits carcinoid cell growth by inducing G_1 phase cell cycle arrest.

VPA Decreases Levels of CgA

Neuroendocrine tumors such as carcinoids frequently cause debilitating symptoms in patients as a result of excess tumor secretion of various bioactive amines and peptides. CgA is an acidic glycoprotein that is cosecreted with hormones such as serotonin by carcinoid tumors. All neuroendocrine tumors produce CgA, and the protein is thus a useful marker for this class of malignancies. We were interested in whether VPA had the ability to alter the neuroendocrine

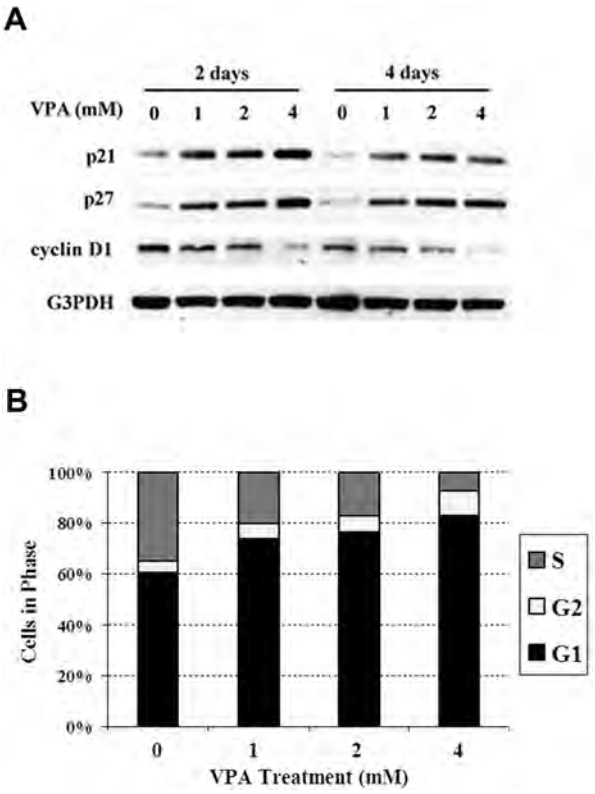

Figure 2. The mechanism of VPA-induced growth inhibition is cell cycle arrest. **(A):** Western blot analysis was performed on VPA-treated GI carcinoid cancer cells to measure protein expression of the cell cycle regulators p21, p27, and cyclin D1. G3PDH was used as a loading control. **(B):** Flow cytometry was performed on GI carcinoid cells to measure the proportion of cells in each cell cycle phase.

Abbreviations: G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GI, gastrointestinal; VPA, valproic acid.

phenotype in carcinoid tumor cells, so we performed Western blot analysis for expression of CgA. After 2 days of treatment of GI (Fig. 3A) and pulmonary (Fig. 3B) carcinoid cancer cells with VPA, protein levels of CgA decreased, indicating a change in the neuroendocrine phenotype of these tumor cells.

VPA Activates Notch-1 Signaling in Carcinoid Cells

After establishing that VPA inhibits carcinoid cell growth and suppresses production of CgA, we wanted to identify the mechanism of action for these effects. We have previously shown that Notch-1 signaling is absent at baseline in neuroendocrine tumors, and that Notch-1 overexpression with an inducible Notch-1 construct causes inhibition of cell growth and hormone production [5–8]. Based on a recent report describing the ability of VPA to increase Notch-1 protein levels in neuroblastoma cells [10], and our previous research on negative regulation of ASCL-1 by Notch-1, we hypothesized that VPA might also activate Notch-1 signaling in carcinoid cells, with antitumor effects.

To assess the effect of VPA on Notch-1 protein expression, we performed Western blot analysis. There was a lack of Notch-1 at baseline in the two carcinoid cell lines. Interestingly, VPA treatment of GI (Fig. 4A) and pulmonary (Fig. 4B) carcinoid cells led to an increase in both full-length Notch-1 and NICD, the active form of the protein. We next asked, is the Notch-1 induced by VPA functionally active? Notch-1 is activated by a series of proteolytic cleavage events, mediated by γ-secretase and other enzymes, resulting in the liberation of NICD. NICD then translocates into the nucleus, where it binds with CBF-1 and other proteins to form a DNA-binding complex. This complex activates transcription of target genes such as hairy enhancer of split 1 [30–33]. We used a luciferase reporter assay incorporating the CBF-1 binding site to measure the functional activity of Notch-1 induced by VPA [14]. VPA treatment of GI carcinoid cells transfected with this construct resulted in a fivefold greater luciferase activity (Fig. 5A). Importantly, the γ-secretase inhibitor DAPT blocked the effect of VPA on CBF-1 binding, indicating that the increase in CBF-1 binding was a result of induction of NICD.

We have previously shown that activation of Notch-1 signaling in carcinoid cells results in suppression of ASCL-1, a basic helix-loop-helix transcription factor that regulates the neuroendocrine phenotype [6, 7]. If VPA is able to activate Notch-1 signaling in carcinoids, therefore, we would expect to see a decrease in expression of ASCL-1. Western blot analysis of VPA-treated GI (Fig. 5B) and pulmonary (Fig. 5C) carcinoid cancer cells showed a dose-dependent decrease in ASCL-1 protein.

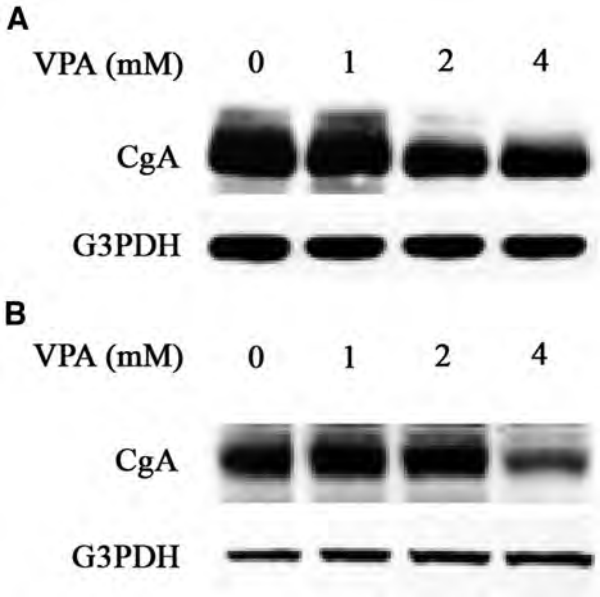

Figure 3. VPA decreases levels of CgA in carcinoid cells. Western blot analysis of GI **(A)** and pulmonary **(B)** carcinoid cells for protein expression of the neuroendocrine tumor marker CgA after 2 days of treatment with VPA. G3PDH was used to confirm equal protein loading.

Abbreviations: CgA, chromogranin A; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GI, gastrointestinal; VPA, valproic acid.

Exposure of H727 cells to 4 mM of VPA for 2 days suppressed ASCL-1 to undetectable levels. To determine the mechanism of VPA-mediated suppression of ASCL-1, we used an ASCL-1 promoter construct [15]. As expected, at baseline, GI carcinoid cells bearing this construct had a high level of luciferase activity. However, VPA treatment caused an almost fourfold lower luciferase activity, indicating transcriptional repression of ASCL-1 (Fig. 5D). Thus, VPA treatment led to a significant reduction in ASCL-1 transcription. Since Notch-1 is known to silence ASCL-1 transcription, this provides further evidence that VPA-mediated ASCL-1 reduction is through Notch-1 signaling.

Notch-1 RNA Interference Blocks the Effects of VPA in Carcinoid Cells

VPA has diverse biological effects. Besides Notch-1 signaling, VPA is known to influence Akt [34], glycogen synthase kinase-3β [35], inositol [36–38], angiogenesis factors [39], and HDAC enzymes [40–42]. In order to confirm that the effects of VPA on carcinoid cells—i.e., inhibition of growth, induction of cell cycle arrest, and suppression of neuroendocrine tumor markers—are a result of activation of Notch-1 signaling, we used RNA interference. BON GI carcinoid cells were transiently transfected with siRNA

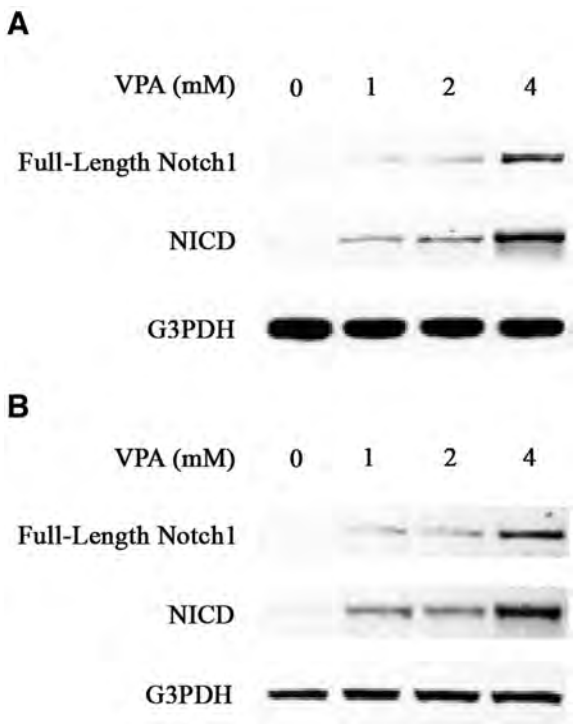

Figure 4. VPA activates Notch-1 in carcinoid cells. GI **(A)** and pulmonary **(B)** carcinoid cancer cells were treated with VPA (0–4 mM) for 2 days, and cell lysates were immunoblotted for full-length Notch-1 and the active NICD. Equal loading was confirmed with G3PDH.

Abbreviations: G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GI, gastrointestinal; NICD, Notch-1 intracellular domain; VPA, valproic acid.

against Notch-1, nonspecific siRNA, or vehicle (Lipofectamine) alone, and cell lysates were analyzed by immunoblotting. In the absence of siRNA, VPA treatment led to greater Notch-1 and p21 levels, and a lower level of ASCL-1 (Fig. 6A, lane 2). Similar results were obtained in the presence of nonspecific siRNA (Fig. 6A, lane 4). Blockade of VPA-mediated Notch-1 induction was achieved with Notch-1 siRNA. Importantly, the abrogation of Notch-1 induction with siRNA reversed the VPA-mediated changes in ASCL-1 and p21 expression (Fig. 6A, lane 6).

We next assessed the impact of Notch-1 siRNA on carcinoid cell growth in combination with VPA treatment. BON cells were again transfected with anti–Notch-1 siRNA or controls, treated with or without VPA, and the MTT cell proliferation assay was performed. Cells transfected with siRNA against Notch-1 were partially protected from the antiproliferative effect of VPA (Fig. 6B). The difference in relative cell viability between cells transfected with Notch-1 siRNA and controls was statistically significant. This suggests that the growth inhibition seen with VPA treatment is mediated in part by Notch-1 signaling.

VPA Inhibits the Growth of Carcinoid Tumors In Vivo

After confirming that VPA activates Notch-1 signaling and suppresses carcinoid cell growth in vitro, we were interested in testing its efficacy in vivo. s.c. human GI carcinoid tumors were developed in immunocompromised mice, which were then given daily i.p. injections of VPA, and the tumors were measured every 4 days for 20 days. The tumors of the saline-treated control mice grew at a higher rate than did those of the VPA-treated animals (Fig. 7A). Indeed, the growth of the tumors in the treatment group was almost static. On the last day of the experiment, blood was drawn from each animal in the treatment group 2 hours after i.p. injection of VPA. Significantly, the median serum VPA level was 46 μg/ml, which is below the therapeutic range of 50–125 μg/ml commonly used for human patients treated with VPA for epilepsy.

After the animals were sacrificed, the tumors were resected and frozen, and protein was isolated for Western blot analysis. As shown in Figure 7B, tumors from the VPA-treated mice had a lower expression of ASCL-1 than in controls. As ASCL-1 is an established target of Notch-1 signaling [6], the downregulation of ASCL-1 seen in the VPA-treated tumors provides evidence that systemically administered VPA is able to activate Notch-1 signaling in carcinoid tumors in vivo.

Discussion

Notch-1 signaling is minimal or absent in neuroendocrine tumors such as small cell lung cancer [43, 44], medullary thyroid cancer [7], and carcinoid tumors [5, 6, 45]. Furthermore, Notch-1 overexpression in medullary thyroid cancer and carcinoid tumor cell lines results in inhibition of cell growth and suppression of neuroendocrine tumor markers and hormones [5, 7, 8], indicating that in neuroendocrine cancers Notch-1 acts as a tumor suppressor. However, pharmacologic methods to activate Notch-1 signaling in carcinoid tumors in vivo have, until now, not been described.

In the current study we report that VPA, a branched-chain fatty acid that has been used for decades in the treatment of patients with epilepsy and other neuropsychiatric disorders, activated Notch-1 signaling in human GI and pulmonary carcinoid cancer cells. At baseline, no detectable Notch-1 protein was present in these cells. However, with VPA treatment, a dose-dependent induction of both full-length Notch-1 protein and the active, cleaved NICD was seen. Luciferase reporter assays using the CBF-1 binding site and the ASCL-1 promoter confirmed that the Notch-1 protein induced by VPA is functionally active and signals to established downstream Notch-1 targets.

How VPA is able to induce protein expression of Notch-1

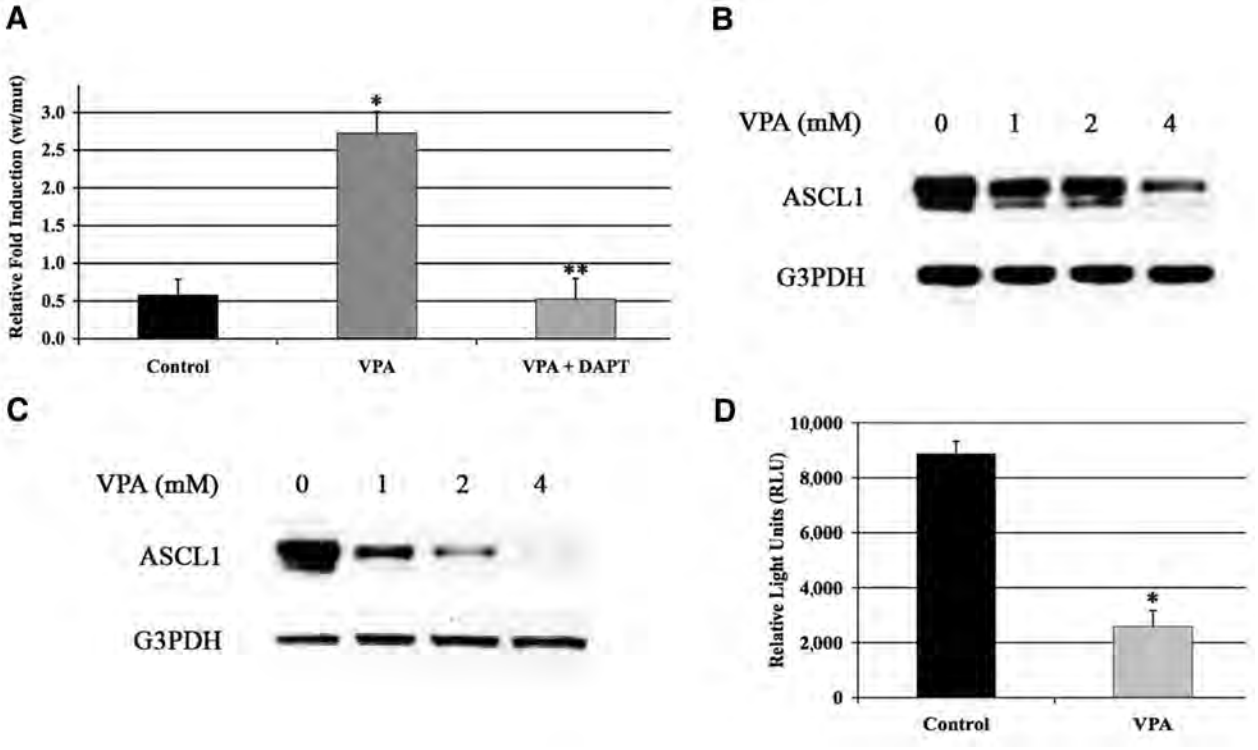

Figure 5. VPA-induced Notch-1 is functionally active. **(A):** GI carcinoid cells were transiently transfected with a luciferase reporter containing the CBF-1 binding site (wild-type or mutant) as well as a CMV-β-galactosidase plasmid and then treated with VPA with or without the γ-secretase inhibitor DAPT. Relative luciferase activity (wild-type/mutant) relative to β-galactosidase expression is shown. Immunoblot analysis of GI **(B)** and pulmonary **(C)** carcinoid cells treated with VPA for ASCL-1, a neuroendocrine transcription factor well known to be negatively regulated by Notch-1, was performed. **(D):** GI carcinoid cells were transfected with an ASCL-1–luciferase reporter plasmid and a CMV-β-galactosidase control plasmid and treated with or without VPA. Luciferase activity relative to β-galactosidase activity is shown.

Abbreviations: ASCL-1, achaete-scute complex-like 1; CBF-1, centromere-binding factor 1; CMV, cytomegalovirus; DAPT, *N*-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; GI, gastrointestinal; VPA, valproic acid.

in carcinoid tumor cells is a subject of ongoing investigation. We know from previous studies that Notch-1 is present in neuroendocrine tumors at the mRNA level, but that at baseline no protein is detectable [5, 6]. Several ligase proteins, including Sel-10, Itch, c-Cbl, and Deltex, are responsible for Notch-1 ubiquitinization and degradation [46]. VPA may interfere with this degradation machinery, which would result in an increase in cellular Notch-1 protein.

VPA is also known to inhibit HDAC enzymes, a property we have confirmed in carcinoid tumor cells (data not shown). HDAC inhibitors modulate gene transcription by increasing histone acetylation, thereby altering chromatin structure [47]. Numerous HDAC inhibitors have shown promising antineoplastic effects in preclinical and clinical studies in a variety of cancers [48]. Recently Baradari and colleagues reported antiproliferative effects of several HDAC inhibitors, including trichostatin A and sodium butyrate, in GI carcinoid cells [49]. The antitumor effects of HDAC inhibitors such as VPA in neuroendocrine tumors may be mediated in part by activation of Notch-1 signaling.

The consequences of Notch-1 activation in carcinoid cells by VPA include suppression of the neuroendocrine tumor markers ASCL-1 and CgA, and dose-dependent cancer cell growth inhibition. We have recently shown, in medullary thyroid cancer cells, that Notch-1–mediated silencing of *ASCL-1* gene transcription results in decreased protein expression of CgA [7]. In the current study, Western blot analysis and flow cytometry provided evidence that the carcinoid cell growth inhibition caused by VPA was mediated by cell cycle arrest. This is consistent with earlier studies wherein overexpression of Notch-1 in medullary thyroid cancer cells and small cell lung cancer cells resulted in an increase in p21 and induction of cell cycle arrest [7, 43]. RNA interference experiments confirmed that these effects of VPA on expression of cell cycle regulators and carcinoid cell proliferation are mediated by Notch-1 signaling. Our current results with VPA confirm the previous findings that Notch-1 activation in neuroendocrine tumors represses ASCL-1 transcription, resulting in a decrease in CgA, and

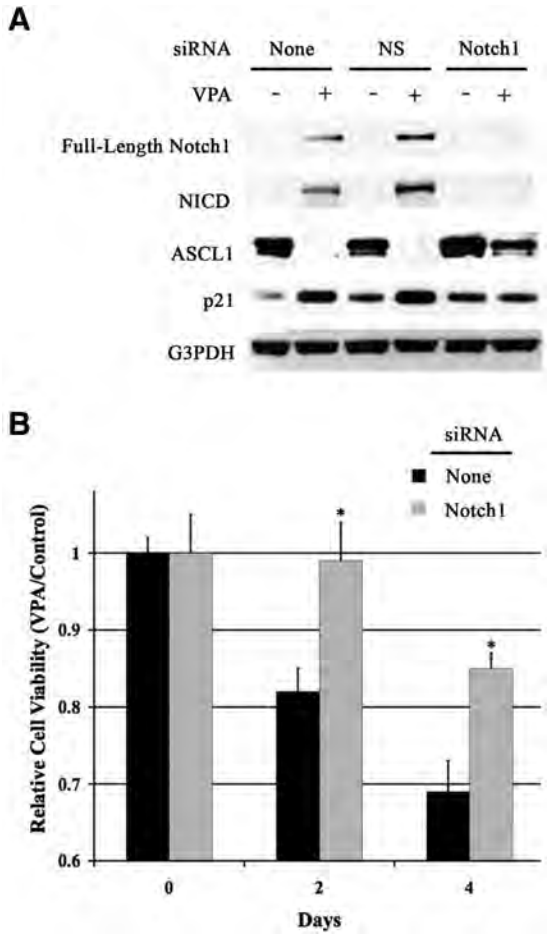

Figure 6. Notch-1 RNA interference blocks the effects of VPA in carcinoid cells. **(A):** Western blot analysis of GI carcinoid cells transfected with Lipofectamine (siRNA: None), nonspecific (NS) siRNA, or Notch-1 siRNA, and then treated with or without VPA, for expression of full-length Notch-1, NICD, ASCL-1, and p21 was performed. **(B):** MTT cellular proliferation assay of GI carcinoid cells transfected with Lipofectamine (siRNA: None) or Notch-1 siRNA and treated with or without VPA for 4 days was also performed.

Abbreviations: ASCL-1, achaete-scute complex-like 1; GI, gastrointestinal; MTT, methylthiazolyldiphenyl-tetrazolium bromide; NICD, Notch-1 intracellular domain; siRNA, small-interfering RNA; VPA, valproic acid.

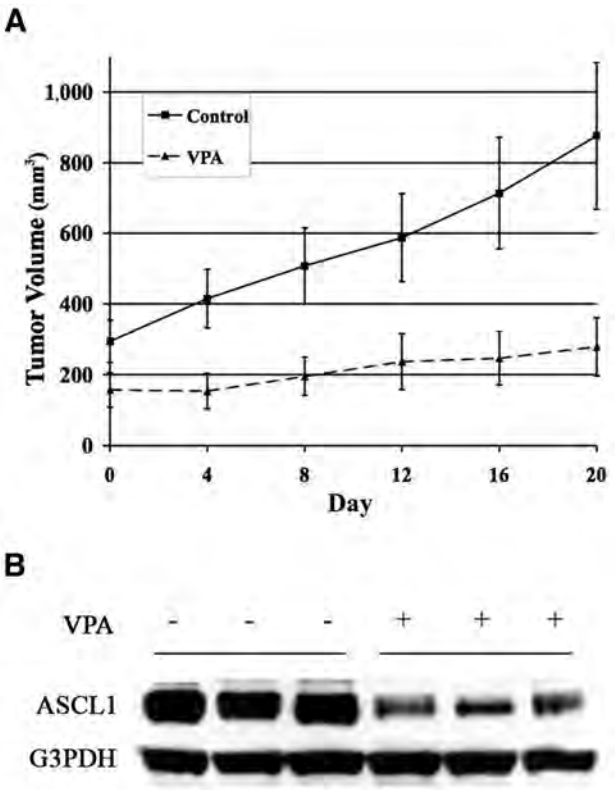

Figure 7. VPA inhibits the growth of carcinoid tumors and suppresses ASCL-1 in vivo. s.c. GI carcinoid tumors were developed in immunocompromised mice, which were then given daily i.p. injections of VPA or saline (control). **(A):** Tumor volume in VPA-treated and control animals. **(B):** Immunoblotting of resected tumor cell extracts for protein levels of the Notch-1 signaling target neuroendocrine tumor maker ASCL-1.

Abbreviations: ASCL-1, achaete-scute complex-like 1; GI, gastrointestinal; VPA, valproic acid.

induces p21 expression, resulting in cell cycle arrest and inhibition of tumor cell growth.

In this study, in addition to characterizing the effects of VPA on carcinoids in vitro, we also report the results of a mouse xenograft experiment. Carcinoid tumors in mice treated with VPA exhibited a markedly slower rate of growth than in controls. Importantly, peak VPA serum levels in the treated animals were well below the upper limit of the therapeutic range for human patients treated with VPA for epilepsy, and no signs of neurotoxicity were seen in the mice. Furthermore, Western blot analysis of the resected tumors demonstrated suppression of ASCL-1 in the VPA-treated group. As ASCL-1 is a well-established target of Notch-1 signaling, the ability of nontoxic concentrations of VPA to decrease levels of ASCL-1 provides important evidence that systemically administered VPA can activate Notch-1 in carcinoid tumors in vivo.

CONCLUSION

In summary, VPA is a small molecule that activates Notch-1 signaling in human carcinoid tumor cells in vitro and in vivo, which results in suppression of neuroendocrine tumor markers and inhibition of cell growth via induction of cell cycle arrest. As VPA is a drug approved by the U.S. Food and Drug Administration, with an established safety profile, it is an attractive candidate for clinical trials. On the basis of the data presented here, a pilot phase II clinical trial will be performed at our institution to assess the effectiveness of VPA in the treatment of patients with advanced carcinoid cancer.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

1 Modlin IM, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. Cancer 2003;97:934–959.

2 Van Gompel JJ, Sippel RS, Warner TF et al. Gastrointestinal carcinoid tumors: Factors that predict outcome. World J Surg 2004;28:387–392.

3 Sippel RS, Chen H. Carcinoid tumors. Surg Oncol Clin N Am 2006;15: 463–478.

4 Lal A, Chen H. Treatment of advanced carcinoid tumors. Curr Opin Oncol 2006;18:9–15.

5 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–G642.

6 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.

7 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.

8 Kunnimalaiyaan M, Chen H. Tumor suppressor role of Notch1 signaling in neuroendocrine tumors. ***The Oncologist*** 2007;12:535–542.

9 Henry TR. The history of valproate in clinical neuroscience. Psychopharmacol Bull 2003;37(suppl 2):5–16.

10 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.

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–G254.

12 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.

13 Hirai H, Adachi T, Tsubata T. Involvement of cell cycle progression in survival signaling through CD40 in the B-lymphocyte line WEHI-231. Cell Death Differ 2004;11:261–269.

14 Hsieh JJ, Henkel T, Salmon P et al. Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. Mol Cell Biol 1996;16:952–959.

15 Chen H, Thiagalingam A, Chopra H et al. Conservation of the *Drosophila* lateral inhibition pathway in human lung cancer: A hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. Proc Natl Acad Sci U S A 1997;94:5355–5360.

16 Vaccaro A, Chen H, Kunnimalaiyaan M. In-vivo activation of Raf-1 inhibits tumor growth and development in a xenograft model of human medullary thyroid cancer. Anticancer Drugs 2006;17:849–853.

17 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.

18 Sakajiri S, Kumagai T, Kawamata N et al. Histone deacetylase inhibitors profoundly decrease proliferation of human lymphoid cancer cell lines. Exp Hematol 2005;33:53–61.

19 Knupfer MM, Hernaiz-Driever P, Poppenborg H et al. Valproic acid inhibits proliferation and changes expression of CD44 and CD56 of malignant glioma cells in vitro. Anticancer Res 1998;18:3585–3589.

20 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.

21 Shu Q, Antalffy B, Su JM et al. Valproic acid prolongs survival time of severe combined immunodeficient mice bearing intracerebellar orthotopic medulloblastoma xenografts. Clin Cancer Res 2006;12:4687–4694.

22 Cinatl J Jr, Cinatl J, Driever PH et al. Sodium valproate inhibits in vivo growth of human neuroblastoma cells. Anticancer Drugs 1997;8:958–963.

23 Rocchi P, Tonelli R, Camerin C et al. p21Waf1/Cip1 is a common target induced by short-chain fatty acid HDAC inhibitors (valproic acid, tributyrin and sodium butyrate) in neuroblastoma cells. Oncol Rep 2005;13: 1139–1144.

24 Takai N, Desmond JC, Kumagai T et al. Histone deacetylase inhibitors have a profound antigrowth activity in endometrial cancer cells. Clin Cancer Res 2004;10:1141–1149.

25 Chavez-Blanco A, Perez-Plasencia C, Perez-Cardenas E et al. Antineoplastic effects of the DNA methylation inhibitor hydralazine and the histone deacetylase inhibitor valproic acid in cancer cell lines. Cancer Cell Int 2006;6:2–10.

26 Takai N, Kawamata N, Gui D et al. Human ovarian carcinoma cells: Histone deacetylase inhibitors exhibit antiproliferative activity and potently induce apoptosis. Cancer 2004;101:2760–2770.

27 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;138:979–984; discussion 984–985.

28 Catalano MG, Fortunati N, Pugliese M et al. Valproic acid induces apoptosis and cell cycle arrest in poorly differentiated thyroid cancer cells. J Clin Endocrinol Metab 2005;90:1383–1389.

29 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.

30 Chen H, Biel MA, Borges MW et al. Tissue-specific expression of human

achaete-scute homologue-1 in neuroendocrine tumors: Transcriptional regulation by dual inhibitory regions. Cell Growth Differ 1997;8:677–686.

31 Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: Cell fate control and signal integration in development. Science 1999;284:770–776.

32 Allenspach EJ, Maillard I, Aster JC et al. Notch signaling in cancer. Cancer Biol Ther 2002;1:466–476.

33 Bray SJ. Notch signalling: A simple pathway becomes complex. Nat Rev Mol Cell Biol 2006;7:678–689.

34 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;5:71–82.

35 Chen G, Huang LD, Jiang YM et al. The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. J Neurochem 1999;72:1327–1330.

36 O'Donnell T, Rotzinger S, Nakashima TT et al. Chronic lithium and sodium valproate both decrease the concentration of myo-inositol and increase the concentration of inositol monophosphates in rat brain. Brain Res 2000;880:84–91.

37 Vaden DL, Ding D, Peterson B et al. Lithium and valproate decrease inositol mass and increase expression of the yeast INO1 and INO2 genes for inositol biosynthesis. J Biol Chem 2001;276:15466–15471.

38 Williams RS, Cheng L, Mudge AW et al. A common mechanism of action for three mood-stabilizing drugs. Nature 2002;417:292–295.

39 Michaelis M, Michaelis UR, Fleming I et al. Valproic acid inhibits angiogenesis in vitro and in vivo. Mol Pharmacol 2004;65:520–527.

40 Gottlicher M, Minucci S, Zhu P et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 2001;20:6969–6978.

41 Phiel CJ, Zhang F, Huang EY et al. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem 2001;276:36734–36741.

42 Gurvich N, Tsygankova OM, Meinkoth JL et al. Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer Res 2004;64:1079–1086.

43 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.

44 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.

45 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;138:1137–1142; discussion 1142.

46 Miele L. Notch signaling. Clin Cancer Res 2006;12:1074–1079.

47 Cress WD, Seto E. Histone deacetylases, transcriptional control, and cancer. J Cell Physiol 2000;184:1–16.

48 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.

49 Baradari V, Huether A, Hopfner M et al. Antiproliferative and proapoptotic effects of histone deacetylase inhibitors on gastrointestinal neuroendocrine tumor cells. Endocr Relat Cancer 2006;13:1237–1250.

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