Valproic acid induces NET cell growth arrest and enhances tumor suppression of the receptor-targeted peptide–drug conjugate via activating somatostatin receptor type II

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Abstract
Background: Human pancreatic carcinoids, a type of neuroendocrine tumors, are asymptomatic and difficult to diagnose, with the effects of traditional anti-cancer therapies being limited. The histone deacetylase (HDAC) inhibitor valproic acid (VPA) was evaluated for its effects alone and in combination with receptor-targeting peptide–drug conjugate via increasing drug internalization.

Materials and methods: The in vitro and in vivo assays were used to evaluate the effects of VPA and somatostatin receptor-targeting camptothecin–somatostatin conjugate (CPT-SST).

Results: VPA induced proliferation suppression, cell apoptosis and cell cycle arrest. VPA acts as a HDAC inhibitor to induce a decrease of HDAC4 and an increase of acetylated histone 4 (AcH4). Meanwhile, most importantly, besides activating Notch signaling, VPA was observed to stimulate the expression of somatostatin receptor type 2 (SSTR2) that has been applied for receptor-targeting therapies. This characteristic was used for a combination therapy of VPA and CPT-SST. The combination displayed much more potent anti-tumor effects on carcinoid tumor growth by increasing SSTR2 density and drug internalization in target tumor cells.

Conclusion: The combination of VPA and a SSTR2-targeting agent provides us a promising approach in treatment of carcinoid tumors.

Keywords
Carcinoid, HDAC inhibitor, peptide–drug conjugate, receptor-targeting, tumor suppression, valproic acid

Introduction
The anti-convulsant drug valproic acid (VPA), a branched short-chain fatty acid, has been found to affect cancer progression in many types of cancers [1–4] such as colon rectal cancer [1], prostate cancer [2] and cervical cancer [3]. VPA’s anti-cancer effects are associated with multiple molecular signaling pathways such as the Wnt signaling pathway [5–8], p21 pathway [7,9], GSK3 α/β [10,11], histone acetylation/deacetylation signaling [8,10,11] and Notch signaling [2]. Histone acetylation via histone acetyltransferases (HATs) and histone deacetylation via histone deacetylase (HDAC) could activate or inactivate gene transcription and regulate cancer progression [7,12,13]. Thus, the strategies to inhibit HDAC activity via HDAC inhibitors could be applied towards cancer treatment [13]. In many cancer cells, VPA was believed to act mainly as a HDAC inhibitor inducing tumor suppression via blocking histone deacetylation and enhancing histone acetylation [10–12]. Conversely, VPA seems to act as a HDAC activator in certain cancer cells. For example, VPA reportedly reduces the expression of histone H3 and its acetylation in renal cell cancer (RCC) Caki-1 cells [14]. Thus, the precise VPA-mediated anti-cancer mechanisms may be up to different types of cancers and need to be carefully evaluated.

Valproic acid (VPA) as a HDAC inhibitor potentially displays its anti-tumor therapeutic effects in neuroendocrine tumors (NETs) [15–19]. Carcinoids, a type of neuroendocrine tumor (NET), are rare and grow slowly [20]. Most carcinoid tumors are asymptomatic and difficult to diagnose, with only a small number secreting the excessive hormones that cause carcinoid syndrome [21,22]. Patients diagnosed with carcinoids most likely have malignant metastasis and lose surgery as a treatment option. Traditional chemotherapy and radiotherapy also have very limited effects on the metastasized carcinoid tumor [17,22,23]. The HDAC inhibitor VPA was
found to induce anti-tumor inhibitory effects in carcinoids via modulating histone deacetylation and/or Notch signaling [15–19,23]. In this study, VPA was investigated for its effects on pancreatic carcinoid tumor cells.

Valproic acid (VPA) alone has limited effects and so is more frequently used as a safe adjuvant in combination with other cytotoxic agents such as Capecitabine, Decitabine and Irrerotecan [24–28]. These combination treatments display the enhanced anti-tumor effects. Multiple combination treatments are currently under investigation and evaluation for many types of cancers [24–28]. However, most of these combination therapies could not selectively deliver drugs to target tumor sites. Moreover, certain treatments show non-synergic effects. For instance, VPA in combination with Decitabine has been administered to patients carrying acute myelogenous leukemia with no observed improvement compared to when Decitabine was used alone [26]. Thus, a new strategy may help to improve treatments.

Our previous study has shown that VPA could not only induce growth suppression, but also significantly up-regulate certain cell surface receptors in cervical cancer cells [29]. These receptors can be used in receptor-targeted therapy, such as receptor-targeting peptide-/antibody-drug conjugates. These conjugates are the new generation of receptor-specific chemistry, having their effects via coupling cytotoxic agents to the peptide or antibody vehicles and further delivering these non-selective anti-tumor agents to the receptor-specific target tumor sites while enhancing anti-tumor efficacy and reducing toxic side effects [30,31]. The up-regulation of receptor density could more quickly internalize the cytotoxic agents and improve their anti-tumor efficacy [29,30,32]. Therefore, besides exploring its anti-tumor efficacy, we also investigate the effects of VPA on certain G protein-coupled receptors (GPCRs) in pancreatic carcinoid tumor cells and, more importantly, evaluate the novel application of VPA in combination with the receptor-targeting peptide-drug conjugate against SSTR-abundant NETs [33–35]. In this study, we found that, besides suppressing cancer cell growth, VPA also induced somatostatin receptor type II (SSTR2) up-regulation and for carcinoid treatment this suggested the possibility of a combination therapy of VPA with SSTR2-specific camptothecin–somatostatin conjugate (CPT-SST).

Materials and methods

Materials

Valproic acid sodium salt (VPA), Dibenzazepine (DBZ) and Trichostatin A (TSA) were purchased from Sigma (St. Louis, MO). Antibodies against SSTR2 (Cat. No.: sc-11609), p21 (sc-756), p63 (sc-8343), COX2 (sc-7951), Chromogranin A (CgA, sc-13090), Histone 4 (H4, sc-10810), Histone deacetetylase 4 (HDAC4, sc-11418), Acetylated histone 4 (AcH4, sc-8660-R) and β-actin (sc-1616-HRP) were purchased from Cell Signaling Technology (Santa Cruz, CA), with Notch1 (Cat. No.: 4380) from Cell Signaling Technology (Danvers, MA). The cytotoxic peptide–drug conjugate CPT-SST was synthesized in our laboratories and dissolved in 0.01% acetic acid [36].

Cell culture and transfection

The human pancreatic carcinoid BON cell line was a gift of Dr. Courtney Townsend (University of Texas–Galveston) and was grown and maintained in DMEM/F12 (1:1) medium from Life Technologies (Grand Island, NY), supplemented with 1% Pen Strep (Life Technologies) and 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO80524). For cell transfection, 500 μl of cells (2 × 10^5 cells/ml) was plated in each well of 24-well plates. Two milliliters of Lipofectamine™ 2000 (Lipo-2000) and 0.8 μg of DNA were added separately in each vial with 50 μl Opti-MEM transfection medium, and combined together after a 5–10 min incubation. The DNA-Lipo-2000 complexes were incubated for 20–30 min and then added to each well. Growth medium was replaced 4–5 h later and cells were incubated for 72 h.

RT-PCR and real-time PCR

All primers for SSTR2, gastrin-releasing peptide receptor (GRPR) and Notch receptors were obtained from previously published reports [29,32,37] except for the MMP2 primers (F: 5′ AAG GAC CGC AAC CGC ATC CTG 3′, R: 5′ AAA GTG GGC AAC GCC CGT GTG 3′), p53 primers (F: 5′ CAG CAT CTT ATC CGA GTG AAG AA GGA 3′, R: 5′ CAC AAA CAC GCA CCC AAA GC 3′), p21 primers (F: 5′ TGA TGC GCT AAT GGC GGG CT 3′, R: 5′ TGC TGG TCT GCC GCT GTT TT 3′) and CgA primers (F: 5′ TGA ATA AAG GGG ATA CCC AGG TG 3′, R: 5′ CGC TGT TCT TCT GCT CTT GTG 3′). RT-PCR assays were performed as described [29]. Real-time PCR assays were set up using iScript™ cDNA Synthesis Kit and iQTM SYBR Green Supermix (Bio-Rad) and performed on a Bio-Rad iCycler (Hercules, CA) as previously described [32]. Beta-actin was used as the internal control and results were calculated with comparative 2^(-ΔΔCT) methods. The experiments were repeated three times.

Western blot analysis

This assay was employed as described in the protocol (Santa Cruz). Briefly, cells were harvested, re-suspended in RIPA buffer with cocktail inhibitors, homogenized with a 25-gauge needle, spun down at 4°C for 10 min at 12 000 rpm, the supernatants were mixed with loading buffer and heated for 7 min at 95°C. Samples were loaded to run on an 8–16% Tris-glycine gel. Protein was transferred to a nitrocellulose membrane and blocked with 5% fat-free milk, washed and incubated with primary antibody. The membrane was washed again and incubated with secondary antibody (Santa Cruz). Films were developed according to the ECL system protocol (Amersham Biosciences, Buckinghamshire, England). The experiments were repeated three times.

Cell proliferation assay (MTT)

The cell proliferation assay (Promega, Madison, WI) was performed as described [29,36]. Fifty microliters of medium containing different concentrations of compounds were added to 96-well plates, with 50 μl of cells (0.5–1 × 10^5 cells/ml) dispensed into each well. The plates were incubated at 37°C for 3 days, before 15 μl of the dye solution was added and continuously incubated at 37°C for 4 h, followed by the
addition of 100 \( \mu l \) per well of the solubilization solution. The plates were measured at 570 nm once the contents became a uniform-color. Data were analyzed using GraphPad Prism 4.0 (San Diego, CA). The experiments were repeated three times.

**Cell apoptosis and cell cycle assays**

Cells were plated on six-well plates and incubated overnight. The test compounds were added. Cells were incubated overnight and then harvested for the assay. For the apoptosis assay, the Annexin V-FITC apoptosis detection kit (Cat. No.: APOAF) was purchased from Sigma (Saint Louis, MO). Into 500 \( \mu l \) of cell suspension was added 5 \( \mu l \) of Annexin V-FITC conjugate and 10 \( \mu l \) of propidium iodide solution. The mixture was incubated for 10 min and assayed to determine the fluorescence using a Beckman-Coulter Gallios analyzer. Data were analyzed with Gallios software. For the cell cycle assay, the Coulter DNA Prep reagents kit (Cat. No.: PN 6607055) from Beckman Coulter (Fullerton, CA) was used. Analysis was done on a Beckman-Coulter Epics FC500 analyzer using CXP software for acquisition and the ModFit LT version 3.1 (Verity Software House, Topsham, ME) for Cell Cycle Modeling. Both experiments were repeated three times.

**In vivo tumor growth and treatment**

The protocol was approved by the Tulane Institutional Animal Care and Use Committee (IACUC). Pancreatic carcinoid BON cells were harvested, washed and re-suspended in ice-cold PBS. 100 \( \mu l \) of the cell suspension (4 \( \times \) 10⁶ cells) was placed in the flanks of 5–7 weeks old nude mice (NCI, Frederik, MD). Tumor-carrying mice were separated into four groups (\( n = 8–10 \)) with one control group and three test groups. The tested compounds were s.c. injected into the flank opposite of the tumors. One group received 1 mg/kg of cytotoxic SST conjugate CPT-SST (100 \( \mu l \) per mouse) and one group received 200 mg/kg of VPA (100 \( \mu l \)). The last group was treated with 50 mg/kg of VPA in combination with 0.5 mg/kg of CPT-SST (total 100 \( \mu l \)), with the control group being given 100 \( \mu l \) of 0.01% acetic acid. All mice were injected once a day, five times a week. Tumor volumes were measured and bodyweights taken once a week. Data were analyzed with one-way ANOVA among the groups.

**Results**

The human pancreatic carcinoid BON cell is a classical NET cell model and has been applied in the study of anti-NET drug development. In our previous studies, multiple GPCRs such as SSTRs and GRPR were found aberrantly expressed in BON cells [36]. The HDAC inhibitor VPA was found to suppress tumor cell growth and also to up-regulate the expression of certain receptors in many cancer cells [29]. Herein, we investigated the anti-tumor effects of VPA on NET BON cell growth and on the GPCR member SSTR2, and also for its novel potential application in combination with the SSTR2-targeting cytotoxic conjugate CPT-SST for treating pancreatic carcinoid tumors.

**VPA-induced cell proliferation suppression**

Histone acetylation/deacetylation and Notch signaling are reportedly involved in cell proliferation and differentiation in NETs [15,19]. VPA is well-known as a HDAC inhibitor and has also been reported to be able to induce Notch activation [15,29,38]. Thus, we assessed the effects of VPA on BON cell proliferation. Another HDAC inhibitor, TSA and a Notch signaling inhibitor, DBZ, were used to compare their effects. As shown in Figure 1, VPA obviously induced cell proliferation suppression in a dose-dependent manner, with a VPA-induced decrease of cell proliferation marker PCNA (Supplementary Figure S1). Further in vivo assays also demonstrated that VPA could partly suppress BON tumor growth in xenograft mice (discussion below). The HDAC inhibitor TSA at low concentration showed its more potent effects on suppression of BON cell growth, but DBZ did much less (Figure 1). DBZ’s lesser effects possibly may be associated with inactivation of Notch signaling in BON cells [39].

**VPA-induced cell apoptosis**

We further compared the effects of the three agents VPA, TSA and DBZ on BON cell apoptosis. We found that VPA, at 1 mM and an 18-h incubation, resulted in a slight induction of cell apoptosis, with the apoptotic rate being 7% (Supplementary Figure S2B) compared to the rate of the control (3.8%) (Supplementary Figure S2A). VPA at a higher concentration (4 mM) with a long-time incubation (72 h) resulted in enhanced cell apoptosis, with the apoptotic rate over 60% (Figure 2B). The RT-PCR and real-time PCR analysis showed that the anti-apoptotic marker BCL-2 was also down-regulated by VPA in BON cells (Supplementary Figures S1 and S3A). Apoptosis was also observed in the treatment using TSA at 1 \( \mu M \) and an 18-h incubation, with an apoptotic rate of 8% (Supplementary Figure S2C). However, DBZ at 20 \( \mu M \) did not, with the apoptotic rate (3.6%) (Supplementary Figure S2D) being similar to that of the control (3.8%) (Supplementary Figure S2A).

**VPA-induced cell cycle arrest**

We also compared the effects of these anti-cancer agents, VPA, TSA and DBZ, on cell cycle progression. After an 18-h incubation, VPA at 4 mM and TSA at 1 \( \mu M \) could arrest cell...
growth at the same G2 phase, with the increased rates being 25% for VPA and 33% for TSA, respectively, compared to 12% for control. However, the Notch inhibitor DBZ at 20 and 50 μM did not (Table 1). Interestingly, DBZ was found to weaken the effects of VPA on BON cell cycle arrest at phase G2, with VPA-induced rate at phase G2 dropping down from 24.5% to 16.7% (Supplementary Table S1). We also found that VPA displayed different effects with different incubation times. Compared to the control, VPA at 4 mM induced cell cycle arrest at phase S, G2 and G1 with incubation times of 3, 18 and 72 h, respectively (Supplementary Table S2 and Figure 3). This suggested that VPA might play different roles in acute and chronic treatments.

Meanwhile, we found that VPA induced a significant increase in cell cycle-associated tumor repressors p21 and p63 (Figure 4, Supplementary Figures S1 and S3). VPA increased both p21 and p63 at the mRNA level as seen by RT-PCR (Supplementary Figure S1) and at the protein level by Western blot (Figure 4A and B and Supplementary Figure S3B). As illustrated by the time course assay, VPA-induced increase of both p21 and p63 was different, with p21 quickly increasing in a short incubation (3 and 6 h) and then dropping down with incubation being extended (36 and 72 h; but still above control levels; (Figure 4A) and with p63 not increasing until after longer incubation times (18 h or more).

Table 1. Cell growth arrest in BON cells via cell cycle analysis (18-h incubation).

| Agents     | G1          | G2          | S           | Arrest     |
|------------|-------------|-------------|-------------|------------|
| Control    | 57.97 ± 0.35| 11.94 ± 2.00| 30.84 ± 2.96|            |
| VPA        | 59.51 ± 2.15| **24.97 ± 3.96**| 15.27 ± 3.14| G2         |
| TSA        | 51.15 ± 0.40| **32.84 ± 0.66**| 16.02 ± 1.05| G2         |
| DBZ        | 58.36 ± 0.78| 11.35 ± 1.28| 30.13 ± 0.80|            |
| DMSO       | 58.15 ± 1.71| 11.89 ± 1.25| 29.96 ± 1.60|            |

Bold values show the phase of cell cycle arrest induced by the tested agents.

VPA reduces the neuroendocrine tumor marker chromogranin A

Chromogranin A (CgA) is a NET marker and its increased level is correlated with carcinoid tumor activity and carcinoid syndrome. Thus, we investigated the effects of VPA on CgA in carcinoid BON cells. As expected, we found that CgA was readily detected in BON cells and was subsequently suppressed by VPA at the mRNA level using real-time PCR analysis (Supplementary Figure S1) and at the protein level using western blot analysis (Figure 4B and Supplementary Figure S3B). This supports that VPA can induce a phenotype change in NET cells.

VPA acts as a HDAC inhibitor and a Notch signaling activator

Valproic acid (VPA) is believed to suppress cancer cell growth mainly via acting as a HDAC inhibitor. Therefore, we first evaluated the effects of VPA on histone acetylation in carcinoid BON cells. We found that VPA suppressed HDAC and induced histone acetylation. VPA induced the down-regulation of HDAC4 and the up-regulation of histone H4 acetylation (AcH4) (Figures 3B and 4A). Interestingly, H4 was not significantly changed at an acute exposure to VPA treatment (3, 6 and 18 h), but was down-regulated at chronic exposures (36 and 72 h; Figure 4A and Supplementary Figure S3B).

Notch signaling is inactive in carcinoid BON cells [39] and Notch activation could induce BON cell suppression [15,39]. We investigated the effect of VPA on Notch signaling in BON cells. We found that this HDAC inhibitor at 4 mM and with a 72-h incubation induced an increase of Notch1 and Notch2 at the mRNA level by using RT-PCR and real-time PCR assays (Supplementary Figures S1 and S3A). We further confirmed a VPA-induced increase of Notch1 at the protein level by western blot assay. The effects of VPA on the increase of Notch1 were also dose- and time-dependent (Figure 4A and B). The assays showed that the change in Notch1
expression was not observed at acute exposures (3, 6 and 18 h; Figures 3B and 4A), but an increase was observed at chronic exposures (36 and 72 h; Figure 4A). VPA might be involved in different mechanisms during acute and chronic treatments, with the involvement of Notch signaling.

**Activation of Notch signaling induced cell growth suppression**

Notch signaling reportedly can act as a tumor suppressor. And, based on these results above, we hypothesize that the HDAC inhibitor VPA may play a tumor-suppressive role possibly via acting as a Notch signaling activator. We then investigated the effects of Notch signaling activation on carcinoid BON cells.

The over-expression of the Notch1 active form ICN1 was observed to induce cell proliferation suppression (Figure 5A), identical to VPA’s inhibitory effects, indicating that Notch signaling indeed plays the tumor-suppressive role and VPA might induce cell suppression via acting as a Notch activator (further investigation is undergoing).

**VPA enhances anti-tumor effects in combination with the receptor-targeting cytotoxic peptide–drug conjugate CPT-SST**

The effects of VPA on certain GPCR members

The characteristics of high expression of certain GPCR members such as SSTR2 and GRPR in many cancer cells
were applied in a novel receptor-targeting chemotherapy [30,31,33,34]. Furthermore, VPA frequently has been used as an ideal adjuvant for combination chemotherapy. Thus, we evaluated whether VPA could be used in combination with receptor-targeting cytotoxic peptide–drug conjugates via activating the receptors of interest. We investigated VPA-induced expression of SSTRs (SSTR1-5), bombesin receptors (GRPR, BRS3 and NMBR) and vasoactive intestinal peptide (VIP) receptors (VPAC1, VPAC2 and PAC1) at the mRNA level. We found that an increase in SSTR2, GRPR, BRS3 and VPAC2, and a decrease in SSTR1, SSTR3, SSTR4 and SSTR5, with the others being either not significantly changed or not detectable (certain results were shown in Figure 4B, Supplementary Figures S1 and S3A). Especially, we found that VPA significantly induced an increase of SSTR2 in BON cells (Figure 4B, Supplementary Figures S1 and S3A). Therefore, VPA in combination with SSTR2-targeting cytotoxic peptide–drug conjugate may be useful in treating pancreatic carcinoid tumors.

The combination therapeutics enhanced in vitro cell proliferation suppression and induced a switch at cell cycle arrest

The conjugate CPT-SST is a SSTR2-preferential cytotoxic peptide conjugate with high SSTR2-binding affinity and quick SSTR2-binding internalization [36]. We evaluated the effects of VPA, CPT-SST and their combination on cell proliferation, and found that the combination could enhance anti-cell proliferation in BON cells compared to each given alone (Figure 5B). Furthermore, cell cycle analysis showed that CPT-SST after incubation at 18 and 72 h induced cell growth arrest at phase S and phase S/G2, respectively, with VPA arresting at phase G2 and phase G1. However, VPA in combination made a switch of CPT-SST’s effects from S to
G2/S at 18-h incubation and from G2/S to G1/G2 at 72-h incubation (Supplementary Table S3).

The combination therapeutics co-regulate p21 signaling

We further investigated whether tumor repressor p21 and p63 are involved in the signaling pathways co-regulated by the effects of VPA and CPT-SST in combination. We did not observe the involvement of p63 (data not shown), but our assay showed that an enhanced increase of tumor repressor p21 is induced in BON cells by the combination of VPA and CPT-SST at mRNA level by real-time PCR (Figure 6A) and protein level by western blot (Figure 6B). Therefore, growth suppression might be through the combination co-regulating the signaling associated with tumor-suppressive p21.

VPA enhances in vivo anti-tumor efficacy of SSTR2-targeting cytotoxic conjugate

The in vivo assay further showed that the combination of VPA and CPT-SST was much more potent in suppressing carcinoid BON tumor growth than was each alone. As shown in Figure 2C, the inhibitory rates from treatments with VPA at 200 mg/kg and CPT-SST at 1 mg/kg are 17 and 41%, respectively. The suppression by the combination at much lower doses [VPA (50 mg/kg) + CPT-SST (0.5 mg/kg)] was significantly enhanced, with an inhibition rate of 66% ($p < 0.05$; Figure 5C). The results suggest that VPA-mediated SSTR2 up-regulation could increase the internalization and the anti-tumor efficacy of the SSTR2-preferential SST conjugate CPT-SST.

Discussion

The class I HDAC inhibitor VPA currently is under investigation for its new anti-cancer applications in many different types of cancers [24,29,40]. VPA also displays its potential as an effective anti-tumor therapeutic in carcinoid and other NETs [15–17,39,41]. In this study, we found effects of VPA on pancreatic carcinoid and observed that VPA-induced cell apoptosis, cell cycle arrest and cell proliferation suppression, followed with a decrease of the NET marker CgA and the endocrine cell marker somatostatin (data not shown). Also, a decrease of the anti-apoptotic marker BCL-2 and cell proliferation marker PCNA were observed in VPA-treated BON cells. These findings support that VPA induces cell growth arrest in carcinoid cells, identical to other reports about the anti-tumor effects of VPA in NETs and carcinoids with down-regulation of the NET markers [9,15–17,42]. Meanwhile, the tumor suppressors p21 and p63 were found increased, with p53 decreased. Moreover, we found that the effects of VPA on p21 and p63 were different, with an increase of p21 at acute treatments and increase in p63 at chronic treatments (18-h incubation and more). VPA may work through a different mechanism for acute and chronic treatments.

Valproic acid (VPA) mainly was considered to regulate histone acetylation/deacetylation signaling in tumor suppression [9,43]. We observed these effects of VPA in carcinoid cells. VPA induced a decrease of HDAC4 and an increase of Ach4. However, a decrease of histone H4 was also observed at the chronic treatment times. This was identical to the report in which VPA induces a decrease of histone H3 in RCC Caki-1 cells [14]. These findings support that VPA may not only act as a HDAC inhibitor to induce tumor suppression. Notch signaling is also involved in NET cell growth suppression [9,42,44]. Notch signaling can be activated by VPA and is involved in VPA-mediated suppression [15,16]. Further, knockdown of Notch signaling could reduce a VPA-induced increase of p21 [15], indicating the effect of VPA on p21 is via activating Notch signaling. Our current study also identified that Notch1 activation via ICN1 overexpression could induce carcinoid BON cell suppression. VPA also induced cell suppression and at a chronic treatment could induce Notch activation, indicating VPA may act as a Notch signaling activator. We also observed that the Notch signaling inhibitor DBZ could lessen VPA-induced cell cycle arrest, although DBZ itself did not, possibly due to Notch signaling being naturally inactive in BON cells. Our findings support that VPA affects histone acetylation at acute and chronic treatments and Notch activation only at chronic treatments. Notch signaling may be involved in VPA-induced cell suppression.

Valproic acid (VPA) is shown to induce cell growth arrest at the G1 phase and to increase p21 and p27 [15,16]. We also found that VPA at chronic treatments induced cell growth arrest at the G1 phase and an increase of both p21 and p63. Our findings support that VPA induces cell growth suppression via cell cycle arrest and the involvement of associated cell cycle regulators. However, VPA at acute treatments displays a different effect on the cell cycle, arresting cells at phase S with 3h treatments and at phase G2 with 18h
treatments. As said, VPA also has different effects on p21 and p63 at acute or chronic treatments. Our findings support that VPA plays different roles at different incubation times, identical as described above. Further investigations are being undertaken.

Valproic acid (VPA) could induce NET tumor suppression [15–17]. Due to its limited effects, VPA is more likely to be used in combination therapeutics with other anti-cancer agents such as, with Karenitecin in treating melanoma and with Cisplatin and Topotecan (a CPT derivative) in treating cervical cancer [16,24–26,29]. However, these small molecule agents alone or in combination still are not selective or specific to tumor cells [30,31] or show non-synergistic effects [26]. In this study, VPA alone was observed having limited anti-tumor effects. However, VPA significantly up-regulates the expression of certain GPCRs, especially SSTR2, in carcinoid tumor cells. This provides a potentially novel opportunity for enhancing receptor-specific chemotherapy with VPA and a SSTR2-targeting cytotoxin peptide–drug conjugate together. This new generation of a receptor-targeted approach could enhance tumor suppression via binding with the specific receptor and increasing the internalization of the cytotoxic agents [30,32]. We indeed identified that VPA in combination with the SSTR2-targeting CPT-SST could, at lower doses, display a much more potent tumor-suppressive ability than each alone. This is due mainly to VPA having such dual functions as tumor suppression and receptor up-regulation. The combination of VPA and CPT-SST was further identified as significantly enhancing the up-regulation of tumor suppressor p21, the up-regulation of which could result in cell growth suppression [45]. Thus, the suppressive effects of VPA and CPT-SST on carcinoid tumor growth may be via co-activating p21 signaling. The VPA-mediated precise mechanism possibly with the involvement of p21, p63 and p53 is under investigation in different types of NET tumor cells.

In conclusion, VPA not only suppresses carcinoid tumor growth, but also mediates up-regulation of specific G protein-coupled receptors. This characteristic might provide us a great opportunity for the clinical applications of a combination therapeutic using VPA and receptor-targeting radio-labeled or cytotoxic conjugates in treating patients with metastasized NET and carcinoid tumors. Moreover, our findings support that VPA may modulate different signaling pathways in acute and chronic treatments.

Declaration of interest

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Supplementary materials available online
Supplementary Figures S1–S3 and Table S1–S3