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Combination therapy with histone deacetylase inhibitors and lithium chloride:

a novel treatment for carcinoid tumors

Joel T. Adler, BA, Daniel G. Hottinger, Muthusamy Kunnimalaiyaan, PhD, and Herbert Chen, MD, FACS

Endocrine Surgery Research Laboratories, Department of Surgery, University of Wisconsin, Madison, WI

Abstract

Background—In carcinoid cell lines, the histone deacetylase (HDAC) inhibitors valproic acid (VPA) and suberoyl bis-hydroxamic acid (SBHA) activate the Notch1 pathway, while lithium inhibits glycogen synthase kinase-3 β (GSK-3 β). These compounds limit growth and decrease hormonal secretion *in vitro*. We hypothesized that lower-dose combination therapy of HDAC inhibitors and lithium chloride could achieve similar growth inhibition to that of the drugs alone.

Methods—GI and pulmonary carcinoid cells were treated with either VPA or SBHA and lithium chloride for up to 48 hours. Western analysis was used to measure the effects on the Notch1 and GSK-3 β pathways and the neuroendocrine tumor marker chromogranin A (CgA). Growth was measured by a cellular proliferation assay.

Results—With lower-dose combination therapy, a decrease in CgA was observed. The HDAC inhibitors increased the amount of active Notch1 protein, while treatment with lithium was associated with inhibition of GSK-3 β . Moreover, growth was inhibited with lower-dose combination therapy.

Conclusions—Treatment of carcinoid cells with either VPA or SBHA and lithium chloride suppresses the neuroendocrine marker CgA while upregulating Notch1 and inhibiting GSK-3 β . This combination effectively reduces growth. Thus, lower-dose combination therapy may be a viable therapeutic approach for carcinoid tumors.

Synopsis—In carcinoid cell lines, activate of the Notch1 pathway and inhibition of the glycogen synthase kinase-3 β limit growth and decrease hormonal secretion *in vitro*. Lower-dose combination therapy to simultaneously target these pathways effectively reduced growth and limited hormonal secretion. Thus, lower-dose combination therapy may be a viable therapeutic approach for carcinoid tumors.

Keywords

carcinoid; Notch1; GSK-3 β ; histone deacetylase inhibitor; combination therapy

Introduction

Carcinoid tumors are slow growing malignancies that arise from neuroendocrine cells, most commonly of the gastrointestinal system or lungs. With an incidence of 1.5 cases per 100,000, they are the most common neuroendocrine (NE) cancer.[1] Patients suffering from carcinoid

tumors frequently present with metastases and experience debilitating symptoms from the secretion of hormones and peptides. Current chemotherapy regimens are unsatisfactory[2], and surgical resection remains the only therapeutic option but is often impossible because of widespread metastasis. Moreover, many patients develop recurrent disease.[3] As such, there is need for new treatment modalities.

The Notch1/hairy enhancer of split 1 (HES-1)/achaete-scute complex-like 1 (ASCL1) pathway can act as a tumor suppressor or oncogene depending on its cellular context.[4,5] It has been identified as a tumor suppressor for targeted molecular based therapy of neuroendocrine tumors.[6] Notch1 is a transmembrane receptor that undergoes two proteolytic cleavages upon binding its ligand. After cleavage, Notch1 translocates to the nucleus, where it binds to CBF-1 complex and regulates transcription of its target genes. Activation of the Notch1 pathway has been shown to inhibit growth and suppress the neuroendocrine phenotype in medullary thyroid cancer and gastrointestinal (GI) carcinoid cell lines.[7,8]

Histone deacetylase (HDAC) inhibitors are a class of drug that has recently been identified as an activator of the Notch1 pathway. One such drug, valproic acid (VPA), is a branched chain fatty acid commonly used for the treatment of epilepsy. Pharmacological activation of Notch1 by VPA has been shown to suppress tumor growth and secretion of hormonal markers in both GI and pulmonary carcinoid cell lines.[9] Another promising HDAC inhibitor is suberoyl bis-hydroxamic acid (SBHA), a derivative of the well-known suberoylanilide hydroxamic acid (SAHA). SBHA has also been shown to activate Notch1, controlling both growth and phenotype in GI and pulmonary carcinoid cell lines.[10]

A second potential target for molecular based treatment of NE cancers is glycogen synthase kinase (GSK). GSK is a serine/threonine protein kinase regulating multiple cellular processes involving differentiation, metabolism, proliferation, and survival.[11] Commonly used to treat bipolar disorder, lithium chloride is a known pharmacologic inhibitor of the beta isoform, glycogen synthase kinase-3 β (GSK-3 β). Inactivation of GSK-3 β via phosphorylation with lithium is associated with decreased growth and hormonal secretion in medullary thyroid cancer[11] and pheochromocytoma[12] tumor cells.

The aforementioned studies have shown that activation of the Notch1 pathway by the HDAC inhibitors VPA and SBHA, as well as inactivation of GSK-3 β through treatment with lithium chloride, can effectively inhibit growth and decrease hormonal secretion in neuroendocrine cell lines. However, whether there is therapeutic benefit in simultaneous targeting of both pathways is not known. We wanted to study whether combination therapy with lithium and either VPA or SBHA could more effectively inhibit growth and regulate phenotypic expression in carcinoids, specifically in GI (BON) and pulmonary (H727) cell lines. We hypothesized that we could achieve equal efficacy with lower dose combinations of the drugs, compared to higher dose treatment with any single drug alone.

Methods

Cell culture

BON human GI carcinoid tumor cells, courtesy of 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.[13,14]

Western blot analysis

BON GI carcinoid cells and H727 pulmonary carcinoid cells were treated with varying combinations of VPA (Sigma-Aldrich, St. Louis, MO), SBHA (Biomol, Plymouth Meeting,

PA), and lithium chloride (Sigma-Aldrich). Whole cell lysates were prepared as previously described.[13] An equal volume of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was used as a control. 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 Notch1 (Santa Cruz Biotechnology, Santa Cruz, CA), pGSK-3 β (Cell Signaling Technology, Beverly, MA), and chromogranin A (CgA; Zymed Laboratories, San Francisco, CA); 1:2,000 for p21 and p27 (Cell Signaling Technology); and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Trevigen, Gaithersburg, MD).

Horse radish peroxidase conjugated goat anti-rabbit IgG (1:2000, Cell Signaling Technology) secondary antibody was used for pGSK-3 β , CgA, GAPDH, Notch1, and p21, while goat anti-mouse IgG (1:200, Pierce Biotechnology) secondary antibody was used for p27. For visualization of the protein signal, Immunstain (Bio-Rad Laboratories, Hercules, CA) was used for pGSK-3 β , CgA, and GAPDH. SuperSignal West Femto (Pierce Biotechnology) was used for Notch1, p21, and p27 per the manufacturer's instructions. Relative amount of CgA expression was quantified using QuantityOne (Version 4.6.3, BioRad Laboratories, Hercules, CA) and standardized to the amount of protein loaded in each lane.

Luciferase reporter assay

To determine the functional activity of Notch1, BON cells were transfected with CBF-1 luciferase constructs as previously described.[9] Cells were plated onto 6-well plates in triplicate and allowed to adhere overnight. Cells were treated with one drug or a combination of lithium and either of the HDAC inhibitors for 2 days. All cells were then harvested, lysed, and a luciferase assay (Promega, Madison, WI) was performed in accordance with the manufacturer's instructions. Luciferase levels were measured using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA) and were normalized to the amount of protein as measured by the bicinchoninic assay described above.

Cell proliferation assay

Cell proliferation was measured by the methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) rapid colorimetric assay. Cells were seeded in equal amounts (30,000 and 50,000 cells per well for BON and H727, respectively) in quadruplicate on 24-well plates and incubated overnight. Carcinoid tumor cells were treated with only lithium, VPA, or SBHA or the combination of lithium with VPA or SBHA for a total of five different treatments plus control. Cells were incubated for up to 6 days. Every 2 days, treatment media was changed, and the MTT assay was performed by replacing the standard medium with 250 μ L of serum-free medium containing MTT (0.5 mg/mL) and incubating at 37°C for 4 hours. After incubation, 750 μ L DMSO was added to each well and mixed thoroughly. The plates were then measured at 540 nm using a spectrophotometer (μ Quant; Bio-Tek Instruments).

Statistical analysis

As appropriate to presented data, one-way analysis of variance (ANOVA) and the independent samples *T* test were performed using SPSS (Version 11; SPSS, Inc., Chicago, IL). A *P* value of ≤ 0.05 was considered to be significant.

Results

Combination therapy upregulates Notch1 and inhibits GSK-3 β

Western analysis was used to determine the effects of combination therapy with lithium chloride and either VPA or SBHA on carcinoid cells. At baseline, both GI carcinoid and pulmonary carcinoid cell lines express relatively little of the cleaved, active Notch1 (NICD, Figure 1). Treatment with either VPA (lane 3) or SBHA (lane 5) upregulated Notch1, which is consistent with our previous observations.[9,10,15-18] Treatment with lithium chloride (lane 2) did not induce active Notch1 protein. Treatment with HDAC inhibitors (lanes 2 and 4) had no effect on the GSK-3 β pathway.

In contrast to other kinases, GSK-3 β is highly active and non-phosphorylated in unstimulated cells, and it becomes inactivated by phosphorylation in response to signaling cascades. Lithium chloride is a known inhibitor of this pathway in neuroendocrine cells.[12] Lithium chloride increases phosphorylated GSK-3 β , indicating inhibition of the pathway (pGSK-3 β , Figure 1: lane 2). Moreover, when combined with the HDAC inhibitors, lithium did not affect the amount of active Notch1 in either GI or pulmonary carcinoid cell lines (lanes 4 and 6).

We confirmed the results of our Western analyses by utilizing BON cells stably transfected with a luciferase reporter construct incorporating the CBF-1 binding site (Figure 2). In agreement with the results from Western analysis, Notch1 binding activity to CBF-1 was upregulated by treatment with both VPA and SBHA, and lithium chloride did not impact Notch1 levels.

Lower-dose combination therapy lowers hormonal secretion in carcinoid cells

After measuring the effect on the Notch1 and GSK-3 β pathways, we looked to see how combination therapy affected hormonal secretion by measuring CgA levels. CgA is an acidic glycoprotein cosecreted with hormones by NE tumors whose reduction is correlated with decreases in hormonal secretion measured in extracellular media.[6,9] In GI carcinoid cells, our combination therapy consisted of 2 mM VPA or 15 μ M SBHA with 15 mM lithium. In pulmonary carcinoid cells, the combination of 2 mM VPA or 40 μ M SBHA with 15 mM lithium was used. Our intent was to see if lower-dose combination therapy could effectively limit CgA as much as treatment with single drugs at higher doses. As shown in Figure 3, combination treatment with lower doses limited hormonal secretion with approximately the same effectiveness as treatment with the drugs alone. In fact, lower-dose combination therapy was more effective than either drug alone in pulmonary carcinoid cells. This suggests that targeting different pathways is an effective method for controlling hormonal secretion and can be achieved with lower doses.

Combination therapy inhibits growth of carcinoid cells

After observing that lower-dose combination therapy effectively limited hormonal secretion, we wanted to see if this approach was associated with similar effects on growth inhibition. The MTT growth assay was used to determine the impact of combination therapy with either VPA or SBHA and lithium on carcinoid cell growth. In addition to the full doses used above, we utilized the combination of 2 mM VPA or 15 μ M SBHA with 15 mM lithium in GI carcinoid cells. In pulmonary carcinoid cells, we used the combination of 2 mM VPA or 40 μ M SBHA with 15 mM lithium. Growth was inhibited by lower-dose combination therapy as well as or better than the drugs used alone (Figure 4). Importantly, growth was suppressed significantly ($P < 0.01$, one-way ANOVA) after only 2 days by the lowest doses used. This became more significant ($P < 0.001$) after four days. In comparison to treatment with the drugs alone, lower-dose combination therapy was as effective at growth inhibition of carcinoid cells.

Combination therapy causes growth inhibition through cell cycle arrest

After noting that growth was significantly limited with lower-dose combination therapy, we wanted to explore the mechanism of growth inhibition. We carried out a Western blot for p21 and 27, two well-characterized cell cycle inhibitors. An increase in these Cip/Kip family CDK-inhibitors is associated with G1-phase cell cycle arrest.[19] Treatment with lower-dose combination therapy increased both p21 and p27 proteins, suggesting that the mechanism of growth inhibition is cell cycle arrest at the G1 phase (Figure 5). The highest doses tested are those reported, and a dose-dependent effect was observed when lower doses were utilized (data not shown).

Discussion

Carcinoid tumors are rare cancers with an incidence of 1.5 cases per 100,000 people in the United States.[1,20,21] These tumors typically arise from neuroendocrine tissue in the digestive tract or lungs. While slow growing, carcinoids frequently metastasize and secrete hormones that often cause debilitating symptoms and a poor quality of life. Unfortunately, there are limited therapeutic options for patients with these tumors, and surgical resection is the only viable cure for patients with benign disease. For patients with metastatic disease, treatments are even less effective. Thus, new treatment modalities must be discovered.

We have previously shown that Notch1 plays an important role in growth inhibition and hormonal suppression in neuroendocrine tumors.[7,8,22] Until recently, there were no known pharmacologic activators of the Notch1 pathway in neuroendocrine tumors. We have demonstrated that both VPA and SBHA are potent activators of Notch1 and associated with growth inhibition and hormonal suppression in GI and pulmonary carcinoid[9,10], medullary thyroid cancer[16,17], small cell lung cancer[18], and pheochromocytoma cell lines.[15] Clearly, this is a viable therapeutic target for neuroendocrine tumors. We have also recently shown that the GSK-3 β pathway is important in neuroendocrine tumors: lithium inhibits the GSK-3 β pathway, limits hormonal secretion, decreases neuroendocrine markers, and inhibits growth.[11,12] Whether the combination of the two drugs could be therapeutically useful is not known. The combination of multiple drugs acting on different pathways or mechanisms is common in the treatment of several cancers. Serving as the basis for our study, we asked if there was a more efficient method to limit growth and hormonal secretion in carcinoid tumors.

Consistent with our previous results, treatment with the HDAC inhibitors VPA and SBHA upregulated Notch1 in both GI and pulmonary carcinoid cells, suggesting that the observed effects on hormonal secretion and growth inhibition are due, in part, to Notch1 activity. We also successfully inhibited the GSK-3 β pathway with lithium; likewise, GSK-3 β must also contribute to limiting hormonal secretion and growth inhibition. We observed that HDAC inhibitors could upregulate Notch1, lithium could inhibit GSK-3 β , and the combination of the drugs does not affect the other pathway. Therefore, we set out to see if we could utilize lower doses of these drugs to achieve similar effects to that of either lithium or HDAC inhibitors alone.

We were successful in limiting hormonal secretion with lower doses of both drugs in combination. In GI carcinoid cells, we utilized either lower-dose VPA or SBHA with less lithium to achieve a similar decrease in CgA to that of the drugs alone at higher doses. In pulmonary carcinoid cells, the lower-dose combination was able to decrease CgA levels more than that of any drug alone. These data suggest that hormonal secretion can be limited effectively by lower-dose combination therapy, which serves as an important proof of concept. A similar effect was shown on growth: with the same doses in both GI and pulmonary carcinoid cell lines, lower-dose combination therapy limited growth as well as higher doses of the drugs. Moreover, this growth inhibition was shown to occur through cell cycle arrest. In aggregate,

these results show that lower-dose combination therapy is a viable therapeutic option for neuroendocrine tumors.

How these seemingly independent pathways may interact is not entirely clear. Clearly, these drugs all have an effect on neuroendocrine hormone secretion and cellular proliferation. These mechanistic details represent a possible future avenue of investigation. Additionally, the cause of different effects of the HDAC inhibitors is not clear: different levels of Notch1 activation seem to result in similar growth inhibition, which could be explained by other properties of the compounds. Also, pulmonary carcinoid cells appear to be less sensitive to the HDAC inhibitors for unclear reasons. This study lays the groundwork for eventual exploration of targeting multiple pathways in an *in vivo* model of neuroendocrine tumors.

In conclusion, combination therapy in carcinoid cells with either VPA or SBHA and lithium chloride effectively up-regulates Notch1, inhibits GSK-3 β , suppresses hormonal secretion, and reduces growth through cell cycle arrest. Significantly, these effects were achieved with lower doses when used in combination than when any of the three drugs were used alone. Thus, targeting both the Notch1 and GSK-3 β signaling pathways simultaneously may be an effective treatment modality with the potential benefit of equal efficacy in the treatment for carcinoid tumors.

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References

1. Modlin IM, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. *Cancer* 2003;97:934–959. [PubMed: 12569593]
2. Lal A, Chen H. Treatment of advanced carcinoid tumors. *Current opinion in oncology* 2006;18:9–15. [PubMed: 16357558]
3. Modlin IM, Oberg K, Chung DC, et al. Gastroenteropancreatic neuroendocrine tumours. *Lancet Oncol* 2008;9:61–72. [PubMed: 18177818]
4. Kadesch T. Notch signaling: the demise of elegant simplicity. *Curr Opin Genet Dev* 2004;14:506–512. [PubMed: 15380241]
5. Maillard I, Pear WS. Notch and cancer: best to avoid the ups and downs. *Cancer cell* 2003;3:203–205. [PubMed: 12676578]
6. Kunnimalaiyaan M, Chen H. Tumor suppressor role of Notch-1 signaling in neuroendocrine tumors. *Oncologist* 2007;12:535–542. [PubMed: 17522241]
7. Kunnimalaiyaan M, Traeger K, Chen H. Conservation of the Notch1 signaling pathway in gastrointestinal carcinoid cells. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G636–642. [PubMed: 16160079]
8. Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, Chen H. 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]
9. Greenblatt DY, Vaccaro AM, Jaskula-Sztul R, et al. Valproic acid activates notch-1 signaling and regulates the neuroendocrine phenotype in carcinoid cancer cells. *Oncologist* 2007;12:942–951. [PubMed: 17766653]
10. 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;11:1515–1520. [PubMed: 17874277]discussion 1520

11. Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, Chen H. Inactivation of glycogen synthase kinase-3 β , a downstream target of the raf-1 pathway, is associated with growth suppression in medullary thyroid cancer cells. *Mol Cancer Ther* 2007;6:1151–1158. [PubMed: 17363508]
12. Kappes A, Vaccaro A, Kunnimalaiyaan M, Chen H. Lithium ions: a novel treatment for pheochromocytomas and paragangliomas. *Surgery* 2007;141:161–165. [PubMed: 17263970] discussion 165
13. 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:G245–254. [PubMed: 12851216]
14. Van Gompel JJ, Kunnimalaiyaan M, Holen K, Chen H. ZM336372, a Raf-1 activator, suppresses growth and neuroendocrine hormone levels in carcinoid tumor cells. *Mol Cancer Ther* 2005;4:910–917. [PubMed: 15956248]
15. Adler JT, Hottinger DG, Kunnimalaiyaan M, Chen H. Histone deacetylase inhibitors upregulate notch1 and inhibit growth in pheochromocytoma cells. *Surgery*. 2008In press
16. Ning L, Greenblatt DY, Kunnimalaiyaan M, Chen H. Suberoyl bis-hydroxamic acid activates notch-1 signaling and induces apoptosis in medullary thyroid carcinoma cells. *Oncologist* 2008;13:98–104. [PubMed: 18305053]
17. Greenblatt DY, Cayo MA, Adler JT, et al. Valproic acid activates Notch1 signaling and induces apoptosis in medullary thyroid cancer cells. *Ann Surg* 2008;247:1036–1040. [PubMed: 18520232]
18. Platta CS, Greenblatt DY, Kunnimalaiyaan M, Chen H. Valproic acid induces Notch1 signaling in small cell lung cancer cells. *J Surg Res* 2008;148:31–37. [PubMed: 18570928]
19. Morgan DO. Principles of CDK regulation. *Nature* 1995;374:131–134. [PubMed: 7877684]
20. Van Gompel JJ, Sippel RS, Warner TF, Chen H. Gastrointestinal carcinoid tumors: factors that predict outcome. *World J Surg* 2004;28:387–392. [PubMed: 14994141]
21. Sippel RS, Chen H. Carcinoid tumors. *Surg Oncol Clin N Am* 2006;15:463–478. [PubMed: 16882492]
22. 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]

Figure 1.

Combination therapy upregulates Notch1 and inhibits GSK-3β in GI and pulmonary carcinoid cells. In both cell lines, treatment for 2 days with the HDAC inhibitors VPA (lane 3) or SBHA (lane 5) increases the amount of cleaved Notch1 protein (NICD). Additionally, treatment with lithium inhibits the GSK-3β pathway, demonstrated by phosphorylation of GSK-3β (lane 2). Combination therapy with either HDAC inhibitor and lithium (lanes 4 and 6) affects both pathways simultaneously. GAPDH is shown as a loading control. HDAC, histone deacetylase, VPA, valproic acid, SBHA, suberoyl bis-hydroxamic acid, Li, lithium chloride, GSK-3β, glycogen synthase kinase 3β, GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 2.

Combination therapy increases the amount of active Notch1-mediated CBF1 binding as measured by relative luciferase activity in gastrointestinal carcinoid cells. After 2 days of treatment with the combination of 20 mM lithium and either 3 mM VPA or 20 μM SBHA, an approximately 10-fold and 8-fold induction of Notch1 activity was seen with 3 mM and 20 μM SBHA treatment, respectively. Lithium had no effect on Notch1 activity. The increase was statistically significant ($P < 0.001$, independent samples *T* test). The experiment was performed in triplicate, VPA, valproic acid, SBHA, suberoyl bis-hydroxamic acid, Li, lithium chloride.

Figure 3.

Treatment with the combination of lithium and either VPA or SBHA reduces CgA more than treatment with full doses of the drugs alone in GI and pulmonary carcinoid cell lines. Western blot analysis showed a decrease in levels of chromogranin A (CgA), a marker of hormonal secretion. Importantly, lower-dose combination therapy was as effective (GI carcinoid) or more effective (pulmonary carcinoid) than treatment with the drugs alone. VPA, valproic acid, SBHA, suberoyl bis-hydroxamic acid, Li, lithium chloride, GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 4. Combination therapy limits growth of GI (4a) and pulmonary (4b) carcinoid cells. Carcinoid cells were treated with the indicated combinations of VPA, SBHA, and lithium for up to 6 days, and cell viability was determined by the MTT colorimetric growth assay. Points represent mean $\pm$ SE. All treatments are significantly different from control as soon as day 2 ($* = P < 0.01$, $** = P < 0.001$, one-way ANOVA). VPA, valproic acid. SBHA, suberoyl bis-hydroxamic acid. Li, lithium chloride.

Figure 5.

The mechanism of growth inhibition by combination therapy is cell cycle arrest. Carcinoid cells were treated with the indicated concentrations of VPA, SBHA, and lithium for 2 days, and total cell lysates were prepared. An increase in p21 and p27 proteins suggests that the mechanism of inhibition is cell cycle arrest. GAPDH was used as a loading control. VPA, valproic acid, SBHA, suberoyl bis-hydroxamic acid, Li, lithium chloride, GAPDH, glyceraldehyde 3-phosphate dehydrogenase.