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Inhibition of growth in medullary thyroid cancer cells with histone deacetylase inhibitors and lithium chloride

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Abstract

Background—While representing only 3% of thyroid malignancies, medullary thyroid cancer (MTC) accounts for 14% of thyroid cancer deaths. MTC has a high rate of recurrence and lacks effective treatments. The histone deacetylase (HDAC) inhibitors valproic acid (VPA) and suberoyl bis-hydroxamic acid (SBHA) activate the Notch1 signaling pathway, while lithium chloride inhibits the glycogen synthase kinase-3 β (GSK-3 β) pathway. These compounds have been shown to limit growth and suppress hormonal secretion; thus, targeting different signaling pathways may be an effective treatment.

Methods—MTC cells were treated with varying combinations of up to 20 mM lithium chloride with either 3 mM VPA or 20 μ M SBHA for 48 hours. Western analysis was used to measure the effects on Notch1, GSK-3 β , and neuroendocrine (NE) markers. Growth was assessed by a methylthiazolyldiphenyl-tetrazolium (MTT) bromide cellular proliferation assay. Western analysis was used to determine the mechanism of growth regulation

Results—Combination therapy increased active Notch1, inhibited the GSK-3 β pathway, and decreased NE markers. Additive inhibition of growth was observed with combination therapy. Lower-dose combination therapy achieved greater decreases on NE markers and growth than treatment with any of the drugs alone. Moreover, an increase in the cleavage of the apoptotic markers caspase-3 and PARP was observed.

Conclusions—Combination therapy with lithium chloride and HDAC inhibitors suppresses NE markers and decreases growth via apoptosis of MTC cells *in vitro*. With the possibility of increased efficacy and decreased toxicity, combination therapy may represent a new strategy to treat MTC.

Keywords

medullary thyroid cancer; Notch1; GSK-3 β ; histone deacetylase inhibitor; combination therapy; lithium chloride; valproic acid; suberoyl bis-hydroxamic acid

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Introduction

Medullary thyroid cancer (MTC) is a neuroendocrine (NE) tumor that arises from the calcitonin-secreting parafollicular cells of the thyroid gland. MTC is a relatively rare malignancy, representing only 3% of all thyroid malignancies; nevertheless, it accounts for 14% of all thyroid cancer-related deaths.(1,2) Moreover, MTC metastasizes in more than 50% of cases, precluding surgical resection and causing significant morbidity.(3,4) Currently, surgery is the only curative therapy for MTC, and chemotherapeutic regimens are palliative at best. Thus, there is an urgent need for new and different treatment modalities.

Glycogen synthase kinase-3 β pathway (GSK-3 β) is a serine/threonine protein kinase that regulates multiple cellular processes including differentiation, metabolism, and proliferation, and survival.(5,6) GSK-3 β is highly active and non-phosphorylated in MTC, and its activity is inhibited by phosphorylation of a single serine residue (Ser9). Inhibition of GSK-3 β with lithium chloride decreased hormonal secretion and suppressed growth of MTC *in vitro* and *in vivo*.(7) Another important signaling pathway in NE tumors is the Notch1 signaling pathway. The Notch1 signaling pathway plays an important role in cellular differentiation and proliferation. Upon binding its ligand, the transmembrane receptor Notch1 is cleaved, and its intracellular domain translocates to the nucleus and activates transcription of target genes. (8) In NE tumors, Notch1 functions as a tumor suppressor, and its overexpression in MTC cells is associated with growth inhibition and a decrease in NE markers.(9-11) Moreover, pharmacologic activation of the Notch1 pathway by valproic acid (VPA) and suberoyl bis-hydroxamic acid (SBHA) has been shown to decrease NE markers and suppress growth in MTC cells *in vitro* (12,13) and *in vivo*.(14)

While both the Notch1 and GSK-3 β pathways have been identified as potential therapeutic targets for treatment of MTC, the effects of targeting both pathways simultaneously are not known. Because various pathways may be dysregulated in cancer, targeting multiple pathways simultaneously may prove an effective treatment modality. The combination of HDAC inhibitors with lithium chloride could lead to an equally effective treatment with potentially lower toxicity for patients with MTC. We were interested to determine if simultaneous targeting of the Notch1 and GSK-3 β pathways could be an effective treatment for MTC.

Methods

Cell culture

Human MTC cells (TT) were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 18% fetal bovine serum (Sigma-Aldrich, St Louis, MO), and 100 IU/ml penicillin and 100 μ g/ml streptomycin (Life Technologies) in a humidified atmosphere of 5% CO₂ in air at 37°C as previously described.(7,8)

Western blot analysis

TT cells were treated with varying combinations of VPA (Sigma-Aldrich, St. Louis, MO), SBHA (Biomol, Plymouth Meeting, PA), and lithium chloride (Sigma-Aldrich), and an equal volume of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was used as a control. After two days of treatment, whole cell lysates were prepared as previously described.(15) 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 as follows: 1:500 for chromogranin A (CgA; Zymed Laboratories, San Francisco, CA); 1:1,000 for Notch1 (Santa Cruz Biotechnology, Santa Cruz, CA), achaete scute complex-like 1 (ASCL1; BD Biosciences, San Diego, CA), pGSK-3 β , poly-ADP ribose phosphate (PARP), and cleaved caspase-3 (Cell Signaling Technology, Beverly, MA); and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Trevigen, Gaithersburg, MD).

Horseradish peroxidase conjugated goat anti-rabbit IgG (1:2000, Cell Signaling Technology) secondary antibody was used for CgA, Notch1, pGSK-3 β , PARP, cleaved caspase-3, and GAPDH, while goat anti-mouse IgG (1:200, Pierce Biotechnology) secondary antibody was used for ASCL1. For visualization of the protein signal, SuperSignal West Femto (Pierce Biotechnology) was used for Notch1 and ASCL1. Immunstar (Bio-Rad Laboratories, Hercules, CA) was used for CgA, pGSK-3 β , PARP, cleaved caspase-3, and GAPDH per the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was measured by a methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) rapid colorimetric assay. To perform this assay, 100,000 cells were seeded in quadruplicate on 24-well plates and incubated overnight. After incubation, MTC cells were treated with DMSO as control, lithium chloride, VPA, or SBHA, or the combination of lithium chloride with VPA or SBHA. Cells were incubated for up to 6 days. Every 2 days, treatment media was changed, and the MTT assay was performed by replacing the treatment medium with 250 μ L of serum-free RPMI 1640 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, Winooski, VT).

Statistical analysis

One-way analysis of variance (ANOVA) was performed using SPSS (Version 11; SPSS, Inc., Chicago, IL). A *P* value of ≤ 0.05 was considered to be significant. All points represent the average of four readings plus/minus standard error.

Results

Combination therapy upregulates Notch1 and inhibits GSK-3 β

After observing that combination therapy with HDAC inhibitors and lithium chloride inhibited growth, we wanted to determine how these compounds affected target pathways. After two days of treatment, Western analysis was used to determine the effects of VPA, SBHA, and lithium chloride upon signaling pathways in MTC cells. At baseline, MTC cell lines express relatively little cleaved, active Notch1 (NICD; Figure 1, lane 1).⁽⁸⁾ Treatment with the HDAC inhibitors VPA (lane 3) and SBHA (lane 5) upregulated Notch, as evidenced by an increase in NICD. This is consistent with our earlier reports that these drugs inhibit histone deacetylation and upregulate Notch1 in MTC cells.^(14,16) We observed no effect on the GSK-3 β pathway with treatment by HDAC inhibitors (Figure 1).

GSK-3 β , in contrast to other kinases, becomes inactivated by phosphorylation in response to signaling cascades. Lithium chloride is a known inhibitor of GSK-3 β in MTC cells.⁽¹⁷⁾ Treatment with lithium chloride was associated with an increase in the phosphorylation of GSK-3 β , indicating inhibition of the pathway (pGSK-3 β , Figure 1: lane 2). Moreover, when combined with HDAC inhibitors, lithium chloride did not affect the amount of active Notch1 in MTC cells (lanes 4 and 6). Thus, treatment with HDAC inhibitors upregulated Notch1, and treatment with lithium chloride induced phosphorylation of GSK-3 β .

Lower-dose combination therapy inhibits growth of MTC cells

After measuring the effect on the Notch1 and GSK-3 β pathways, we wanted to see if a lower-dose combination therapy could achieve similar growth inhibition to that of the higher doses of drugs used alone. An MTT growth assay was used to determine the impact of combination therapy with either VPA or SBHA and lithium chloride on MTC cell growth. In addition to the full doses used above, we utilized the combination of 15 mM lithium chloride with either 2 mM VPA or 15 μ M SBHA in MTC cells. Growth was more inhibited by lower-dose combination therapy than higher doses of the drugs used alone (Figure 2). Importantly, growth was suppressed significantly ($P < 0.01$, one-way ANOVA) after only 2 days with any treatment. This became more significant ($P < 0.001$) after four days. In comparison to higher-dose treatment with the drugs alone, lower-dose combination therapy was more effective at growth inhibition.

Combination therapy causes growth inhibition through apoptosis

After observing that growth was significantly limited with lower-dose combination therapy, we wanted to explore the mechanism of growth inhibition. We utilized a Western blot for PARP and caspase-3. Both of these proteins are well-known markers of the apoptotic pathway, and their cleavage is indicative of apoptosis. After 2 days of treatment with combination therapy, increased cleavage was noted in both PARP and caspase-3 (Figure 3). This suggests that combination therapy with HDAC inhibitors and lithium chloride causes growth inhibition via apoptosis.

Lower-dose combination therapy reduces NE tumors markers in MTC cells

After observing that lower-dose combination therapy effectively limited growth via apoptosis, we sought to understand how combination therapy affected the NE phenotype by measuring ASCL1. Notch1 negatively regulates ASCL1, a basic helix-loop-helix transcription factor that promotes neuronal differentiation and is a marker for NE hormone production.(18-20) Treatment with lithium chloride (Figure 4, lane 2) and HDAC inhibitors (lanes 3 and 5) decreased the amount of ASCL1, suggesting a suppression of the NE phenotype. CgA is an acidic glycoprotein cosecreted with hormones by MTC, and the reduction of CgA is correlated with decreases in hormonal secretion.(21) A decrease was also seen in CgA, demonstrating that these treatments reduce NE markers in MTC cells.

After observing these decreases, we treated MTC cells with a lower-dose combination therapy of 15 mM lithium chloride with either 2 mM VPA or 15 μ M SBHA. Our intent was to see if lower-dose combination therapy could effectively lower ASCL1 and CgA as much as treatment with single drugs at higher doses. Not only was this treatment approach effective, it was actually more effective than any higher dose single drug treatment (lanes 4 and 6). This suggests that targeting different pathways is an effective method for controlling the NE phenotype and hormonal secretion, and it can be achieved with lower doses.

Discussion

MTC is a relatively rare cancer, accounting for only 3% of thyroid malignancies but approximately 14% of deaths.(1,2,4) Derived from the parafollicular calcitonin-secreting cells of the thyroid gland, these NE tumors frequently metastasize and cause a poor quality of life. Unfortunately, there are limited therapeutic options for patients with these tumors, and surgical resection is the only potentially curative treatment. Thus, there is an urgent need for new treatment modalities. This study is an attempt to combine differing treatment modalities to assess their efficacy in MTC.

In this study, we address two pathways known to play an important role in MTC and other NE tumors: the Notch1 and the GSK-3 β pathways. While oncogenic in many cancers, Notch1 appears to function as a tumor suppressor in NE tumors such as MTC.(21) Overexpression of the Notch1 intracellular domain inhibits growth and affects the NE phenotype(8). The HDAC inhibitors VPA and SBHA are able to pharmacologically activate the pathway in MTC(12,13), carcinoid(18,19), small cell lung cancer(20), and pheochromocytoma(22) cells. We have also recently shown that the GSK-3 β pathway is important in NE tumors, and lithium chloride inhibits the GSK-3 β pathway, limits hormonal secretion, decreases NE markers, and inhibits growth in MTC(7) and pheochromocytoma(17) cells.

Whether the combination of the two drugs could be therapeutically useful is not known, so we asked if such combination was a more efficient method to limit growth and hormonal secretion in MTC. We accomplished this by first demonstrating the effects of the compounds on the pathways alone, and then to see if similar effects could be achieved with lower-dose combinations of the different compounds. As expected based upon previous results, treatment with the HDAC inhibitors VPA and SBHA upregulated Notch1, and lithium chloride inhibited GSK-3 β . Alteration of both of these pathways led to a suppression of ASCL1 and CgA. After this, we utilized lower doses of HDAC inhibitors paired with lithium chloride. Not only were the NE markers decreased by lower-dose combination therapy, but they were actually significantly lower than results achieved with any single drug. A similar effect was shown for growth: with the same therapeutic approach, lower-dose combination therapy limited growth more effectively than higher doses of the drugs. Up to the concentrations tested, this growth inhibition was shown to occur through apoptosis. These results suggest that lower-dose combination therapy is a viable therapeutic option for MTC.

In conclusion, combination therapy in MTC cells with lithium chloride and either VPA or SBHA effectively upregulates Notch1, inhibits GSK-3 β , inhibits the NE phenotype, suppresses hormonal secretion, and reduces growth through apoptosis. Significantly, these effects were achieved with lower doses when used in combination than when any of the drugs were used alone in higher doses. 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 MTC.

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References

1. Chen H, Roberts JR, Ball DW, Eisele DW, Baylin SB, Udelsman R, et al. Effective long-term palliation of symptomatic, incurable metastatic medullary thyroid cancer by operative resection. *Ann Surg* 1998 Jun;227(6):887–95. [PubMed: 9637552]
2. Greenblatt DY, Chen H. Palliation of advanced thyroid malignancies. *Surg Oncol* 2007 Dec;16(4): 237–47. [PubMed: 17855077]
3. Udelsman R, Chen H. The current management of thyroid cancer. *Adv Surg* 1999;33:1–27. [PubMed: 10572560]
4. Sippel RS, Kunnimalaiyaan M, Chen H. Current management of medullary thyroid cancer. *Oncologist* 2008 May 1;13(5):539–47. [PubMed: 18515739]

5. Cohen P, Frame S. The renaissance of GSK3. *Nat Rev Mol Cell Biol* 2001 Oct 1;2(10):769–76. [PubMed: 11584304]
6. Cohen P, Goedert M. GSK3 inhibitors: development and therapeutic potential. *Nature reviews Drug discovery* 2004 Jun 1;3(6):479–87.
7. Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, Chen H. Inactivation of glycogen synthase kinase-3beta, a downstream target of the raf-1 pathway, is associated with growth suppression in medullary thyroid cancer cells. *Mol Cancer Ther* 2007 Mar;6(3):1151–8. [PubMed: 17363508]
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 Dec 29;281(52):39819–30. [PubMed: 17090547]
9. Ball DW. Achaete-scute homolog-1 and Notch in lung neuroendocrine development and cancer. *Cancer Lett* 2004 Feb 20;204(2):159–69. [PubMed: 15013215]
10. Nakakura EK, Sriuranpong VR, Kunnimalaiyaan M, Hsiao EC, Schuebel KE, Borges MW, et al. Regulation of neuroendocrine differentiation in gastrointestinal carcinoid tumor cells by notch signaling. *J Clin Endocrinol Metab* 2005 Jul;90(7):4350–6. [PubMed: 15870121]
11. Sriuranpong V, Borges MW, Ravi RK, Arnold DR, Nelkin BD, Baylin SB, et al. Notch signaling induces cell cycle arrest in small cell lung cancer cells. *Cancer Res* 2001 Apr 1;61(7):3200–5. [PubMed: 11306509]
12. 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 Feb;13(2):98–104. [PubMed: 18305053]
13. Greenblatt DY, Cayo MA, Adler JT, Ning L, Haymart MR, Kunnimalaiyaan M, et al. Valproic acid activates notch1 signaling and induces apoptosis in medullary thyroid cancer cells. *Ann Surg* 2008 Jun;247(6):1036–40. [PubMed: 18520232]
14. Ning L, Jaskula-Sztul R, Ndiaye MA, Kunnimalaiyaan M, Chen H. Suberoyl bishydroxamic acid activates notch1 signaling and suppresses tumor progression in an animal model of medullary thyroid carcinoma. *Ann Surg Onc*. 2008 In press.
15. Sippel RS, Carpenter JE, Kunnimalaiyaan M, Lagerholm S, Chen H. Raf-1 activation suppresses neuroendocrine marker and hormone levels in human gastrointestinal carcinoid cells. *Am J Physiol Gastrointest Liver Physiol* 2003 Aug;285(2):G245–54. [PubMed: 12851216]
16. Greenblatt DY, Cayo M, Adler JT, Ning L, Haymart M, Kunnimalaiyaan M, et al. Valproic acid activates notch1 signaling and induces apoptosis in medullary thyroid cancer cells. *Annals of Surgery*. 2008 In press.
17. Kappes A, Vaccaro A, Kunnimalaiyaan M, Chen H. Lithium ions: a novel treatment for pheochromocytomas and paragangliomas. *Surgery* 2007 Feb;142(2):161–5. discussion 5. [PubMed: 17263970]
18. Greenblatt DY, Cayo M, Ning L, Jaskula-Sztul R, Haymart M, Kunnimalaiyaan M, et al. Suberoyl bishydroxamic acid inhibits cellular proliferation by inducing cell cycle arrest in carcinoid cancer cells. *J Gastrointest Surg* 2007 Nov;11(11):1515–20. discussion 20. [PubMed: 17874277]
19. Greenblatt DY, Vaccaro AM, Jaskula-Sztul R, Ning L, Haymart M, Kunnimalaiyaan M, et al. Valproic acid activates notch-1 signaling and regulates the neuroendocrine phenotype in carcinoid cancer cells. *Oncologist* 2007 Aug;12(8):942–51. [PubMed: 17766653]
20. Platta CS, Greenblatt DY, Kunnimalaiyaan M, Chen H. Valproic acid activates the notch1 signaling pathway and inhibits growth in small cell lung cancer cells. *J Surg Res*. 2008 Oct; In press.
21. Kunnimalaiyaan M, Chen H. Tumor suppressor role of Notch-1 signaling in neuroendocrine tumors. *Oncologist* 2007 May;12(5):535–42. [PubMed: 17522241]
22. Adler JT, Hottinger DG, Kunnimalaiyaan M, Chen H. Histone deacetylase inhibitors upregulate notch1 and inhibit growth in pheochromocytoma cells. *Surgery*. 2008 In press.

Figure 1.

Combination therapy limits growth of MTC cells. MTC cells were treated with the indicated combinations of VPA, SBHA, and lithium chloride for up to 6 days, and cell viability was determined by an MTT colorimetric growth assay. Points represent mean $\pm$ SE. All treatments are significantly different from control after two days of treatment, and this inhibition became more significant over time (* = $P < 0.01$, ** = $P < 0.001$, one-way ANOVA). MTC, medullary thyroid cancer, VPA, valproic acid, SBHA, suberoyl bis-hydroxamic acid, Li, lithium chloride, MTT, methylthiazolyldiphenyl-tetrazolium.

Figure 2.

The mechanism of growth inhibition by combination therapy is apoptosis. MTC cells were treated with the indicated concentrations of VPA, SBHA, and lithium chloride for 2 days, and total cell lysates were prepared. An increase in the cleavage of PARP and caspase-3 suggests that the mechanism of inhibition is apoptosis. GAPDH was used as a loading control. MTC, medullary thyroid cancer, VPA, valproic acid, SBHA, suberoyl bis-hydroxamic acid, Li, lithium chloride, GAPDH, glyceraldehyde 3-phosphate dehydrogenase, PARP, poly-ADP ribose polymerase.

Figure 3.

Combination therapy upregulates Notch1 and inhibits GSK-3 β in MTC cells. At baseline, MTC cells express minimal active Notch1. 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 chloride phosphorylates GSK-3 β , demonstrating inhibition of the pathway (lane 2). Combination therapy with either VPA or SBHA and lithium chloride (lanes 4 and 6) affects both pathways simultaneously. GAPDH is shown as a loading control. MTC, medullary thyroid cancer. HDAC, histone deacetylase, VPA, valproic acid, SBHA, suberoyl bis-hydroxamic acid, Li, lithium chloride, GSK-3 β , glycogen synthase kinase-3 β , GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 4.

Treatment with the combination of lithium chloride and either VPA or SBHA reduces neuroendocrine markers more than treatment with higher doses of the drugs alone in MTC cells. Western blot analysis showed a decrease in levels of ASCL1, a pro-neuroendocrine gene, and CgA, a marker of hormonal secretion. Importantly, lower-dose combination therapy was more effective than treatment with higher doses of the drugs alone. MTC, medullary thyroid cancer, VPA, valproic acid, SBHA, suberoyl bis-hydroxamic acid, Li, lithium chloride, GAPDH, glyceraldehyde 3-phosphate dehydrogenase, CgA, chromogranin A, ASCL1, achaete scute complex-like 1.