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Thiocoraline activates the Notch pathway in carcinoids and reduces tumor progression *in vivo*

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Abstract

Carcinoids are slow-growing neuroendocrine tumors (NETs) that are characterized by hormone overproduction; surgery is currently the only option for treatment. Activation of the Notch pathway has previously been shown to have a role in tumor suppression in NETs. The marine-derived thiodepsipeptide thiocoraline was investigated *in vitro* in two carcinoid cell lines (BON and H727). Carcinoid cells treated with nanomolar concentrations of thiocoraline resulted in a decrease in cell proliferation and an alteration of malignant phenotype evidenced by decrease of NET markers, ASCL-1, CgA, and NSE. Western blot analysis demonstrated the activation of Notch1 on the protein level in BON cells. Additionally, thiocoraline activated downstream Notch targets HES1, HES5, and HEY2. Thiocoraline effectively suppressed carcinoid cell growth by promoting cell cycle arrest in BON and H727 cells. An *in vivo* study demonstrated that thiocoraline, formulated with polymeric micelles, slowed carcinoid tumor progression. Thus, the therapeutic potential of thiocoraline, which induced activation of the Notch pathway, in carcinoid tumors was demonstrated.

Keywords

thiocoraline; carcinoid; Notch 1 pathway; polymeric micelle; in vivo

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Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author Contributions

R Jaskula-Sztul designed the experiments. H Cho designed the thiocoraline formulation. T Wyche, A Dammalapati, H Cho, A Harrison, and R Jaskula-Sztul performed the experiments. T Wyche wrote the manuscript with contributions from R Jaskula-Sztul and reviewed by T Bugni, G Kwon, and H Chen.

Introduction

Carcinoids are slow-growing neuroendocrine tumors (NETs) that are characterized by hormone overproduction.[1–4] Carcinoids, which make up about 0.5% of all malignant tumors,[5–6] are most commonly found in the small intestine but can also be present in the lungs, rectum, appendix, and stomach, among other locations. Symptoms from carcinoids are often absent but metastases can lead to carcinoid syndrome, which includes effects such as diarrhea, bronchospasm, and right-sided valvular heart lesions.[1–4] Treatment of carcinoids remains an ever-present issue as they are resistant to current therapeutics: the single agent chemotherapy response rate is only 20%.[4] Responses to chemotherapy are typically short-lived and not correlated with prolonged survival. Consequently, surgery remains the only curative option for treatment. Therefore, further studies are necessary to find more viable options for treatment of carcinoids.

Activation of Notch1 signaling in carcinoids has been shown to have a role in tumor suppression.[7] Notch isoforms (1–4) are transmembrane receptors that are activated by proteolytic cleavage following ligand binding.[8–10] The Notch intracellular domain (NICD) translocates into the nucleus where it forms a complex with centromere binding factor 1 (CBF1) and other proteins to activate gene transcription (HES and HEY genes). The Notch1 signaling pathway is not active in carcinoid tumors.[7] As a result of studies implicating Notch as a tumor suppressor in carcinoid tumors, therapeutic leads that activate this signaling pathway represent promising strategies for treatment of carcinoids.

Thiocoraline was originally isolated from a marine *Micromonospora* sp.[11–12] and has demonstrated potent cytotoxicity against lung, breast, colon, renal, and melanoma cancer cells and *in vivo* efficacy against human carcinoma xenografts.[12–14] Thiocoraline is a bisintercalator and does not damage DNA or inhibit topoisomerase II; however, it does inhibit DNA elongation by DNA polymerase α.[13] More recently, we isolated thiocoraline and new analogs from a marine ascidian-derived *Verrucosispora* sp..[15] We also demonstrated that thiocoraline altered the neuroendocrine phenotype and activated the Notch pathway in medullary thyroid cancer (MTC).[16] Due to the need for additional therapeutic options for neuroendocrine cancers we aimedto investigate the effect of thiocoraline on carcinoids.

In this study, we investigated thiocoraline's effect on cell proliferation in human pancreatic carcinoid tumor cells (BON) and human bronchopulmonary carcinoid cells (H727). Additionally, we determined thiocoraline's ability to alter the neuroendocrine phenotype of carcinoids. Moreover, we have shown that thiocoraline transcriptionally activates the Notch pathway. To better understand the mechanism of action of thiocoraline's antiproliferative effects, we performed cell cycle analysis by flow cytometry and validated protein expression of cell cycle markers by western blot. Finally, a formulation for thiocoraline was developed to overcome solubility problems using nanoparticle polymeric micelles in order to improve the solubility for *in vivo* studies. Thiocoraline slowed the progression of carcinoid tumor growth in mice. Altogether, the results from this study provided evidence for the therapeutic potential of thiocoraline against carcinoid tumors.

Materials and Methods

Cell Culture

BON human pancreatic carcinoid tumor cells,[17] and H727 human bronchopulmonary carcinoid cells (ATCC #CRL-5815)(ATCC, Manassas, VA, USA) were maintained as previously described.[18–19] The BON cell line was authenticated in May 2012 at Genetica DNA laboratories.[20] For the purpose of *in vivo* study, BON cells were stably transfected with luciferase–expressing plasmid*luc2* (pGL4.50[*luc2*/CMV/Hygro], Promega, Madison, WI, USA) using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Twenty-four hours after transfection, cells were cultured in the BON cells medium additionally containing selective antibiotic – 100μg/mL of hygromycin B (Invitrogen) – for 3 weeks to obtain hygromycin-resistant clones. Selected clones were then tested for luciferase expression using a luciferase assay system kit (Promega), and the clone with the highest activity was chosen for the *in vivo* experiments.

Thiocoraline

Chondrilla caribensis f caribensis sponge specimens were collected in the Florida Keys on February 10, 2010 as was previously described.[15] Thiocoralinewas isolated and purified from the marine bacterium *Verrucosispora* sp., as was previously described.[15] Thiocoraline was dissolved in dimethyl sulfoxide (DMSO) and diluted in standard media to achieve desired concentrations.

Cell Proliferation Assay and IC_{50} determination

Cell proliferation was measured via 3-(4,5-dimethylthiazol-2yl)-2,5 diphenyltetrazolium bromide (MTT) assay as previously described.[19,21] Cells were plated in quadruplicate in 24-well plates under standard conditions and allowed to attach overnight. The following day, cells were treated with thiocoraline (0–40 nM) and incubated for up to 8 days. Control cells (0 nM) received DMSO at 0.5% final concentration. Cell proliferation was assessed after 2, 4, 6, and 8 days. Following two days of thiocoraline treatment, the dose effect curve was plotted to determine the IC_{50} value. The MTT assay was performed by replacing the standard media with 250 μL of serum-free RMPI 1640 containing 0.5mg/mL MTT and incubated for 3.5 hours at 37 °C. After incubation, 750 μL of DMSO was added per well. Plates were shaken for 5 minutes to enhance dissolution. Absorbance at 540 nM was measured via a spectrophotometer (μQuant; Bio-Tek Instruments, Winooski, VT).

Flow Cytometry

To analyze the cell cycle progression of BON and H727 cells, the DNA content was quantified via flow cytometry. BON and H727 cells were treated for two days with thiocoraline (0–40 nM). After treatment, cells were washed with cold 1X PBS pH 7.2 (Life Technologies, Carlsbad, CA, USA) and harvested with trypsin (Life Technologies) to enhance dissociation. Cells were then centrifuged at 1200 rpm at 4 °C and washed twice with cold 1X PBS before fixed with cold 70% ethanol and kept at −20 °C before staining. Prior to staining, cells were again washed twice with cold 1X PBS with centrifugation after each wash. The pellet was suspended in a propidium iodide (PI) staining solution containing

20 mg/mL RNAse-A (Sigma-Aldrich, St. Louis, MO, USA) and 330 μg/mL propidium iodide dissolved in 1X PBS. Cells were stained in the dark overnight at 4 °C. Samples were filtered prior to analysis. FACS analysis was performed on a flow cytometer at 488 nM (FACS Calibur flow cytometer; BD Biosciences), and results were analyzed with ModFit LT 3.2 software (Verity, Topsham, ME, USA).

Western Blot Analysis

Cell lysates—BON and H727 cells were treated for two days with thiocoraline (0–40 nM) and protein extracts were harvested and quantified as previously described.[18,22] Denatured cellular extracts (30–40 μg) were subjected to gel electrophoresis on 7.5% or 10% SDS-PAGE (Invitrogen), transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA), and blocked in milk solution.[23] Membranes were incubated overnight at 4°C with the appropriate primary antibody. The following primary antibodies were used at the following concentrations: anti-NOTCH1 (1:2000); anti-MASH1 (mammalian ASH1) to detect ASCL1 (1:2000; Pharmingen, San Diego, CA, USA); anti-CgA (1:1000; Zymed Laboratories Inc., San Francisco, CA, USA); anti-p21 (1:2000); anti-p27 (1:2000); anti-cyclin B1 (1:1000), anti-cyclin D1 (1:1000), anti-NSE (1:2000), anti-glyceraldehyde-3 phosphate (GAPDH) (1:10,000; Trevigen Inc., Gaithersburg, MD, USA) and vinculin (1:2000; Cell Signaling, Danvers, MA, USA). Following primary antibody incubation, membranes were washed as previously described and incubated with secondary antibody.[23] The following secondary antibodies at the indicated dilutions were used: goat anti-rabbit (Notch1 1:4000, Cyclin D1 1:2000, p27 1:6000, CgA 1:4000, GAPDH 1:3000); goat anti-mouse (Cyclin B1 1:3000, p21 1:6000, ASCL1 1:5000, NSE 1:6000, and vinculin 1:2000). Following secondary antibody incubation, the membranes were washed as previously described.[22,23] Proteins were visualized using SuperSignal West Femto, West Dura, West Pico (Pierce, Rockford, IL, USA), or Immunstar (Bio-Rad Laboratories) chemiluminescent substrate according to manufacturers' directions. The detection of GAPDH was used as a loading control.

Tumor Extracts—Tumor tissue (2 mm^3) was pulverized in the Cryoprep tissue homogenizer (Covaris, Woburn, MA, USA) and the tissue powder was used for protein lysates preparation as described previously.[24] Briefly, the tissue powder was dissolved in 500μl of lysis buffer containing 50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Igepal CA-630, 0.1% sodium dodecyl sulfate, 0.1 μmol/L phenylmethylsulfonyl fluoride, 5 mmol/L ethylene diaminetetraacetic acid, 12 μL/mL protease inhibitor cocktail (Sigma-Aldrich); incubated on ice for 45 minutes; and centrifuged at 13,000 RPM for 30 minutes at 4°C. The supernatants were collected, and protein concentration was determined by the bicinchoninic acid protein as say kit (Pierce). Western blot analysis for Notch1 expression was performed as described above.

Quantitative Real-time PCR (qRT-PCR)

Following two-day thiocoraline treatment, RNA was isolated using RNeasy Mini-kit (Qiagen, Valencia, CA, USA) and reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed by the iCycler IQ detection system (Bio-Rad). A 25 μL volume reaction containing 2 μL cDNA sample (200 ng/μL), 200 nM

forward and reverse primers, and 12.5 μL SYBR Green Supermix (Bio-Rad) was used. The following PCR forward and reverse primer pairs were used: Notch1 (5′-GTCAACGCCGTAGATGACCT-3′ and 5′-TTGTTAGCCCCGTTCTTCAG-3′), HES1 (5′-TTGGAGGCTTCCAGGTGGTA-3′ and 5′-GGCCCCGTTGGGAATG-3′), HES2 (5′-CTCATTTCGGACCTCGGTT-3′ and 5′-TTCGAGCAGTTGGAGTTCT-3′), HES5 (5′-ACCGCATCAACAGCAGCATT-3′ and 5′-AGGCTTTGCTGTGCTTCAGGT-3′) and s27 (5′-TCTTTAGCCATGCACAAACG-3′ and 5′-TTTCAGTGCTGCTTCCTCCT-3′), as a loading control. The RT-PCR reactions were performed in duplicate under previously described conditions.[23] Results were normalized to s27 mRNA levels, and expression was plotted as average ± standard error of the mean (SEM).

Luciferase reporter assay

Notch1 functional activity was measured, by the degree of CBF1-binding, utilizing a luciferase construct containing four CBF1-binding sites (4xCBF1-Luc). BON cells were transiently transfected with CBF1-luciferase reporter construct and then treated with 0 or 30 nM of thiocoraline for 48 hours. To normalize for transfection efficiency, 0.5 μg of cytomegalovirus β-galactosidase (CMV-β-gal) was cotransfected as previously described.[22]

Preparation of thiocoraline-incorporated polymeric micelle

Poly (ethylene glycol)-*block*-poly (D, L-lactide) (PEG-*b*-PLA; 7.4k-*b*-2.3k) was purchased from Advanced Polymer Materials Inc. (Montreal, Canada). The solvent evaporation method was used for the preparation of thiocoraline-loaded PEG-*b*-PLA micelles.[25] Briefly, 1, 3, or 5mg of thiocoraline and 10, 30, or 50mg of the polymer were completely dissolved in acetone and transferred into round bottom flasks. Acetone was evaporated under low pressure by rotary evaporation in a 60 °C water bath until a clear thin-layered film was formed. This film was rehydrated by adding 1 mL of 0.9% sodium chloride solutionin 60 °C water bath. The rehydrated thiocoraline-incorporated PEG-*b*-PLA solution was centrifuged for 5 min at 10,000 g and filtered with 0.22 μm regenerated cellulose filter to remove unloaded thiocoraline and obtain sterilized a polymeric micelle solution. Thiocoraline acquired aqueous solubility of 4.23 ± 1.15 mg/mL in water by forming polymeric micelles with PEG-*b*-PLA. Thiocoraline encapsulated in polymeric micelle was detected and quantified by UV Cary 100 Bio UV-visible spectrophotometer (Varian, Palo Alto, CA, USA) at 360nm. Particle size distributions of PEG-*b*-PLA micelles containing thiocoraline were determined by dynamic light scattering measurement, using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK).

Maximum tolerate dose (MTD) study

Female 6–8week-old balb/c mice were purchased from National Cancer Institute (Rockville, MD, USA) and randomized into 6 groups. Empty vehicle, 5, 7, 10, 20, 30, and 40mg/kg thiocoraline-incorporated PEG-*b*-PLA micelles were intravenously injected to mice and animal body weight changes were monitored for 8 consecutive days. Institutional guidelines were followed for maintenance of animals and end point of animal studies.

Xenograft study

Four-week-old male athymic nude mice were obtained from Charles Rivers (Wilmington, MD, USA). Before beginning the experiment, the mice were allowed to acclimate one week in the animal facility to reduce stress after arrival. Mice were maintained under specific pathogen-free conditions. BON cells stably transfected with the vector encoding the luciferase reporter gene *luc2*were subcutaneously inoculated into the left flank of mouse (5×10^6 cells/animal) in 100 μL of Hanks Balanced Salt Solution (Mediatech, Inc., Manassas, VA, USA). Fifteen days after inoculation, mice with palpable tumors were randomized into two groups (n=6), and intravenously (i.v.) injected with 5 mg/kgBW thiocoraline-incorporated micelles and vehicle (empty PEG-*b*-PLA micelles), respectively. The treatment was repeated at day 22and 29. Tumor volumes were measured from day 15 by external caliper every four days and then were calculated by the modified ellipsoidal formula: $\text{Tumor volume} = \frac{1}{2}(\text{length} \times \text{width}^2)$. Additionally, mice were imaged weekly using a cooled CCD camera (Xenogen IVIS) to validate the dynamics of tumor growth. In brief, 250 μL (3.75mg) of substrate D-Luciferin (Caliper Life Science, Hopkinton, MA, USA) in PBS was injected i.p. in each mouse 12 minutes prior to the whole-body imaging. The image acquisition and tumor size analysis based on the total photon counts of bioluminescence were done by using Live Imaging software (Caliper Life Science, Hopkinton, MA, USA). At the end of the experiment, mice were sacrificed and the tumors were dissected from the surrounding tissues and flash frozen in liquid nitrogen for storage in −80°C. Postmortem examination of the lungs, liver, kidneys, and spleen were performed to confirm that there was no evidence of metastases or tumor growth outside of the inoculation site. All experimental procedures were performed in compliance with our animal care protocol approved by the University of Wisconsin-Madison Research Animal Resources Committee in accordance with the NIH Guideline for the Care and Use of Laboratory Mice.

Statistical Analysis

Statistical analyses were performed using a one-way analysis of variance (ANOVA) and the Kruskal-Wallis rank sum test or by repeated measures ANOVA. A value of $p \leq 0.05$ was considered statistically significant.

Results

Thiocoraline inhibits BON and H727 cell proliferation in vitro

Thiocoraline has previously been shown to decrease cell proliferation in medullary thyroid cancer-TT cells at nanomolar concentrations,[16] and consequently, the survival of BON and H727 cells treated with thiocoraline was investigated. Thiocoraline exhibited low nanomolar potency against BON and H727 cells (Fig. 1a, 1b). Additionally, cell survival over the course of eight days was monitored after treatment with thiocoraline (0–40 nM; Fig. 1c, 1d). For both BON and H727 cells, treatment of thiocoraline at concentrations between 20–40 nM resulted in a steady decrease in cell proliferation over the course of eight days. Treatment of thiocoraline at 5 and 10 nM resulted in a decrease in cell proliferation through six days of treatment in H727 cells.

Thiocoraline induces cell cycle arrest in BON and H727 cells

After determining the ability of thiocoraline to reduce cell proliferation in BON and H727 cells, the mechanism of action of thiocoraline was investigated by western blot and flow cytometry (Fig. 2). Cell cycle marker proteins (p21, p27, cyclin B1, and cyclin D1) were monitored by western blot and suggested arrest in the G2/M phase induced by thiocoraline in BON and H727 cells. An increase in expression of p21, a cyclin dependent kinase (Cdk) inhibitor which promotes cell cycle suppression, was demonstrated by western blot after treatment of BON and H727 cells with thiocoraline. Additionally, flow cytometry analyses of BON and H727 cells treated with thiocoraline (0–40 nM) suggested that thiocoraline causes cell cycle arrest in the G2/M phase. An increase in cell population in the G2/M phase with concomitant decrease in cell population in the G1 phase was displayed with increasing concentrations of thiocoraline in the BON and H727 cell lines. The percentage of cells in the S phase showed variable change in both cell lines.

Thiocoraline decreases neuroendocrine tumor markers in vitro

Achaete-scute complex like-1 (ASCL-1), chromogranin A (CgA), and neurospecific enolase (NSE) have been characterized as markers of NETs.[26–27] Treatment of thiocoraline (0–40 nM) in BON and H727 cells effectively changed their NE phenotype and resulted in a dose-dependent decrease in expression of ASCL-1, CgA, and NSE as demonstrated by western blot analysis (Fig. 3).

Thiocoraline induces Notch isoforms expression in BON cells

After demonstrating the ability of thiocoraline to cause cell cycle arrest in BON and H727 cells, western blot analysis and qRT-PCR were used to further investigate thiocoraline's mechanism of action. Clinical studies have shown that clinical efficacy was correlated with activation of the Notch pathway in NETs[28]; therefore, Notch activation was investigated. The expression of Notch1 was monitored by western blot and qRT-PCR in BON cells treated with thiocoraline (0–40 nM). A dose-dependent increase of Notch1 expression in BON cells was apparent by western blot analysis and qRT-PCR (Fig. 4). qRT-PCR showed a four-fold increase in expression of Notch1 between the control (0 nM) and treatment with 40 nM thiocoraline. H727 cells treated with thiocoraline did not demonstrate a statistically significant increase in expression of Notch1 at the protein or mRNA level. To further determine whether thiocoraline functionally activated the Notch pathway, a luciferase reporter assay incorporating four CBF1-binding sites was used. Thiocoraline treatment of BON cells resulted in a nearly 3-fold induction of luciferase activity (Fig. 4c) indicating that this increase was caused by Notch activation followed by Notch-CBF1 binding. Additionally, activation of downstream targets of Notch was apparent by qRT-PCR. A dose dependent increase in the mRNA levels of HES1, HES5, and HEY2 was detected in BON cells treated with thiocoraline. These results suggest that thiocoraline functionally activates Notch signaling in carcinoids.

Thiocoraline reduced tumor progression *in vivo*

After investigating thiocoraline's *in vitro* effect against carcinoid cell lines, an *in vivo* study was pursued. Thiocoraline has poor solubility in DMSO and aqueous solutions, which

initially hindered *in vivo* experiments, but a method for formulation of thiocoraline with polymeric micelles (PEG-*b*-PLA) increased the aqueous solubility to 4.23 ± 1.15 mg/mL. This formulation enabled a maximum tolerated dose (MTD) study in mice; the MTD was determined to be 7 mg/kg. There was 100% survival of mice and no loss in body weight at this dose.

After determining the MTD, an *in vivo* study investigated the therapeutic potential of thiocoraline. Mice were inoculated with BON cells stably transfected with luciferase–expressing plasmid *luc2*, and tumors were allowed to grow for 14 days. Thiocoraline formulated in polymeric micelles was dosed to 6 mice on days 15, 22, and 29 at 5 mg/kg. Likewise, empty micelles were dosed to 6 additional mice at the same time points. Thiocoraline slowed the progression of tumor growth compared to the vehicle control (Fig. 5). There was a 62.6% reduction in tumor volume between mice treated with thiocoraline and vehicle. Moreover, validation of tumor growth by bioluminescence imaging (73.4% reduction in tumor growth) confirmed that thiocoraline formulated in polymeric micelles has significant antitumor activity. Western blot analysis of proteins from the tumor tissue and qRT-PCR demonstrated increased expression of Notch1 in only mice treated with thiocoraline.

Discussion

Activation of the Notch signaling pathway has been demonstrated to play a role in NET suppression.[7] The Notch signaling pathway is not present in carcinoid tumors; therefore, small molecules that can activate this signaling pathway represent promising strategies for treatment of carcinoids. Several of these Notch activators, such as valproic acid, have made it to clinical trials.[28] Despite the potential for these therapeutic leads, the only effective treatment for carcinoid cancer is surgery, and therefore, there is a need for additional therapeutic options. This study demonstrated that thiocoraline causes a decrease in cell proliferation in BON and H727 cells, acting by cell cycle arrest. Additionally, a decrease in NET markers and an increase in Notch1 protein and mRNA levels suggested that thiocoraline functionally activates the Notch pathway in BON cells. In general, these results parallel recent work investigating the potential for thiocoraline as a treatment for MTC.[16]

While this study demonstrated that thiocoraline activates the Notch pathway in carcinoids, these results do not examine thiocoraline's effects on other pathways that may also contribute to the anti-proliferative effects. The phosphatidylinositol 3′ kinase (PI3)/Akt pathway and mitogen-activated protein kinase (MAPK) pathway have demonstrated a role in the suppression of NETs.[2,19] Future work could entail the use of siRNA to block Notch to determine if thiocoraline's anti-proliferative effects are a result of activation of the Notch pathway.

The ability of thiocoraline to activate Notch signaling and cause a decrease in cell proliferation in carcinoids is a promising step towards finding alternative forms of treatment for NETs. Importantly, the *in vivo* study presented here demonstrated that thiocoraline can slow progression of carcinoid tumors. While Faircloth *et al* demonstrated that thiocoraline had *in vivo* anti-tumor activity,[29] limited *in vivo* studies have been reported for thiocoraline.

Therefore, the *in vivo* study presented here is significant, but additional work is necessary in order to develop thiocoraline into a viable drug lead.

This study complements other recent work on thiocoraline as a potential therapeutic. Considerable progress has been made for the total synthesis of thiocoraline, which could make it more amenable to pharmaceutical development. Thiocoraline was originally synthesized by Boger and co-workers,[30–31] and work has been done more recently by Albericio and co-workers to complete anefficient solid-phase synthesis of thiocoraline using enzyme-labile protecting groups.[32] Additionally, the biosynthesis of thiocoraline has been studied,[33–37] which could help provide more potent analogs. Another issue for thiocoraline is its poor aqueous solubility, which hinders its ability to be delivered *in vivo*. Our formulation using polymeric micelles helps alleviate this problem. Additionally, recent work has been done with liposomes to improve the ability to deliver thiocoraline.[38–39] Overall, considerable work is still necessary, but many of the pieces are in place for thiocoraline as a potential therapeutic.

Thus, thiocoraline activates the Notch signaling pathway and reduces cell proliferation in carcinoids. Treatment of BON and H727 cells with thiocoraline at nanomolar concentrations resulted in a decrease in NET markers (ASCL-1, CgA, and NSE). Cell cycle analyses by flow cytometry and western blot demonstrated arrest in the G2/M phase induced by thiocoraline in BON and H727 cells. A dose dependent increase of Notch1 on the protein and mRNA levels in BON cells treated with thiocoraline, as well as an increase of downstream targets of Notch, points towards the tumor suppression role of thiocoraline in carcinoids. Importantly, thiocoraline slowed carcinoid tumor progression *invivo*. While further work is necessary to better understand thiocoraline's mechanism of action, these results suggest that thiocoraline could be a potential treatment for NETs.

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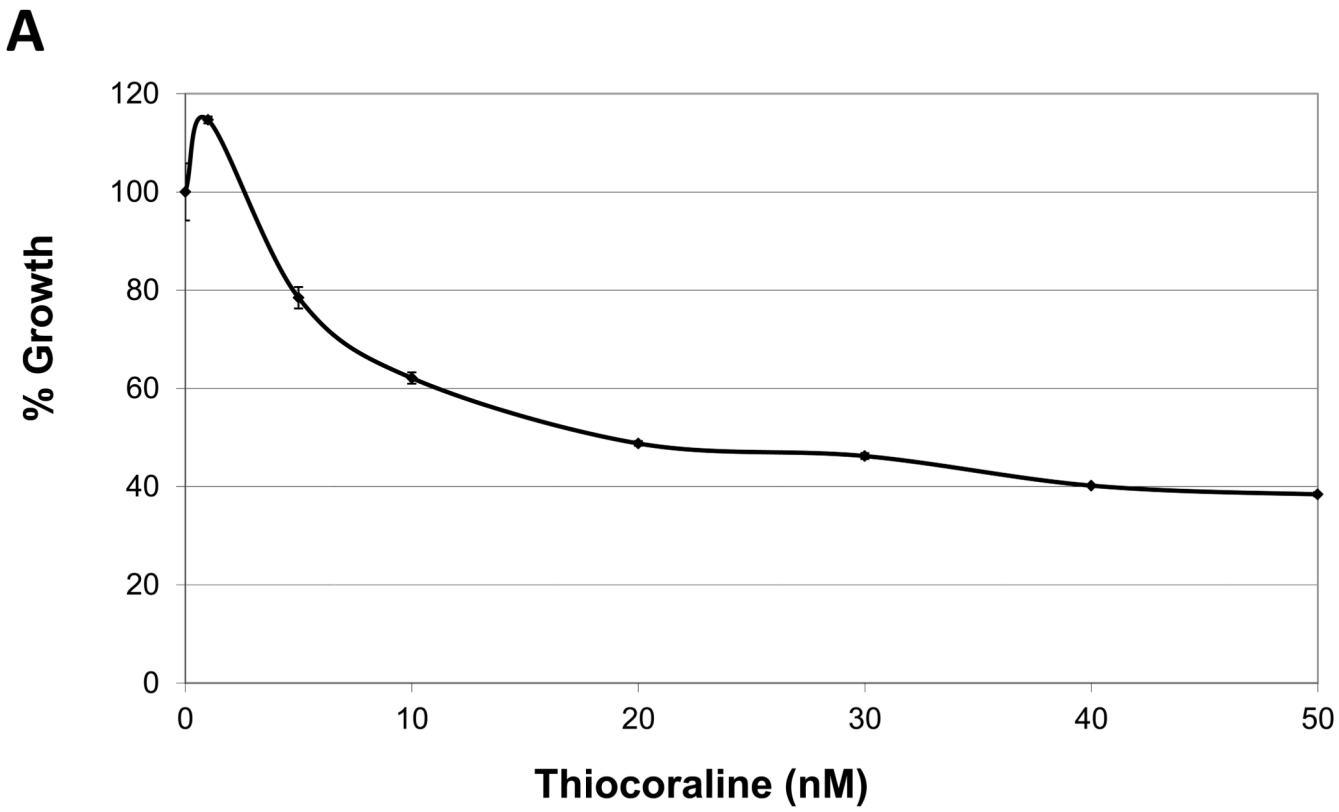
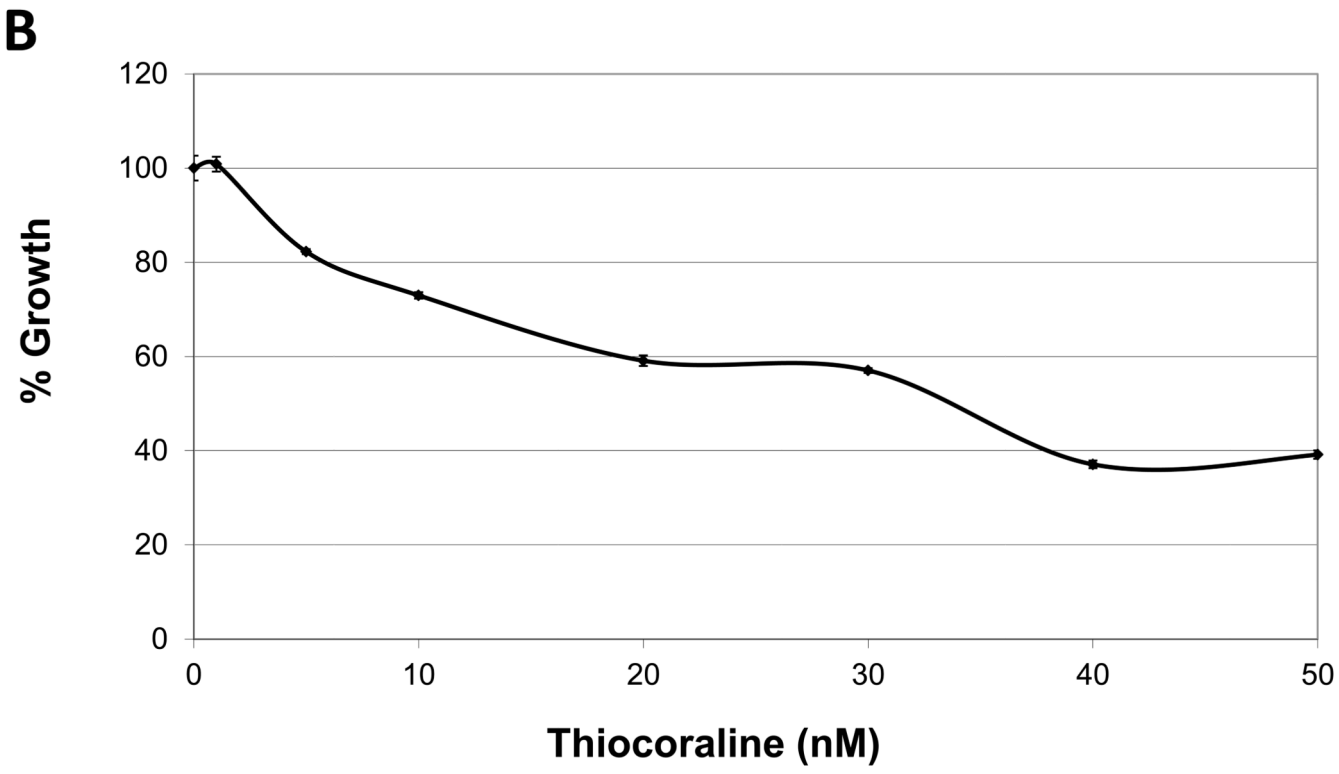




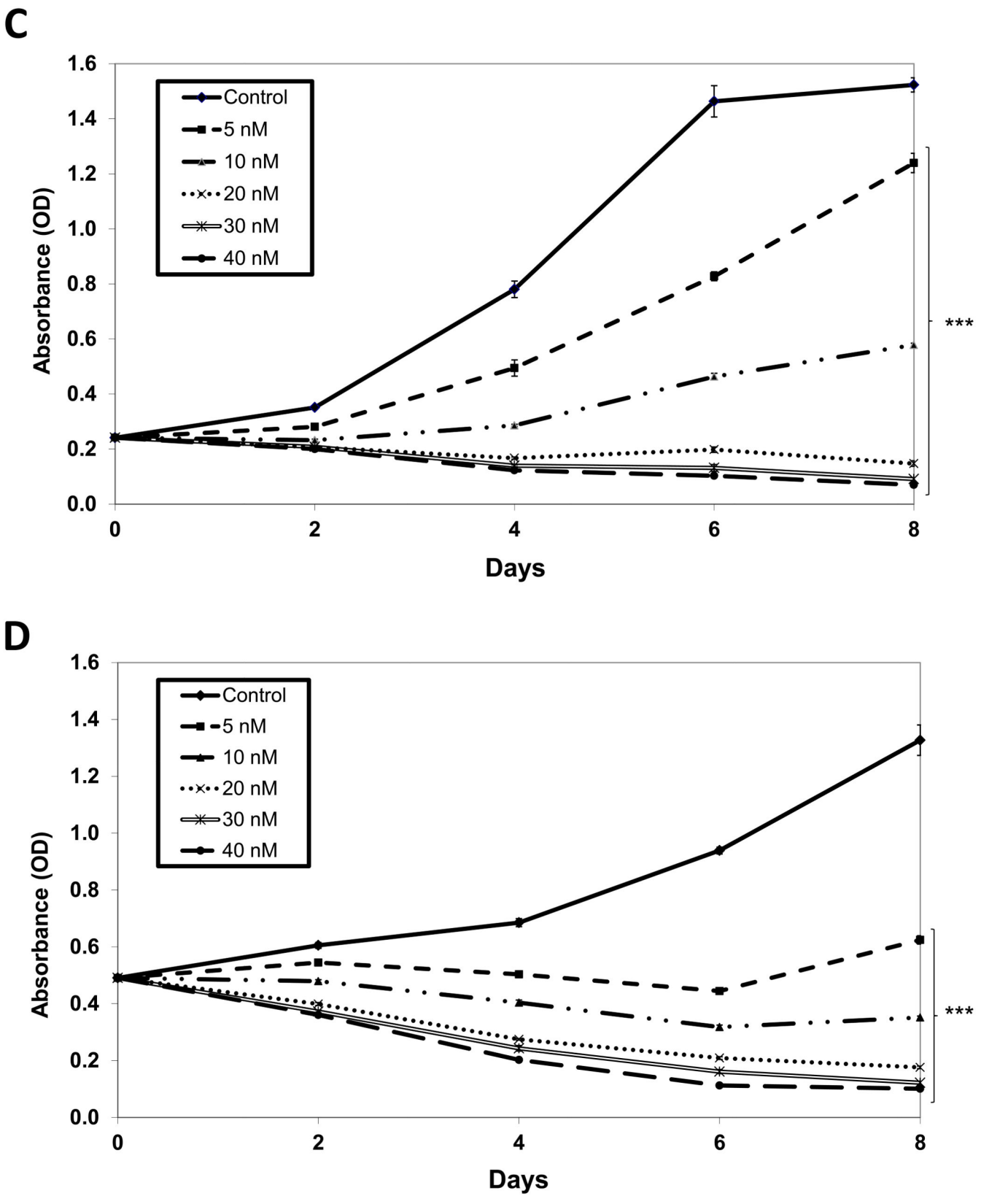
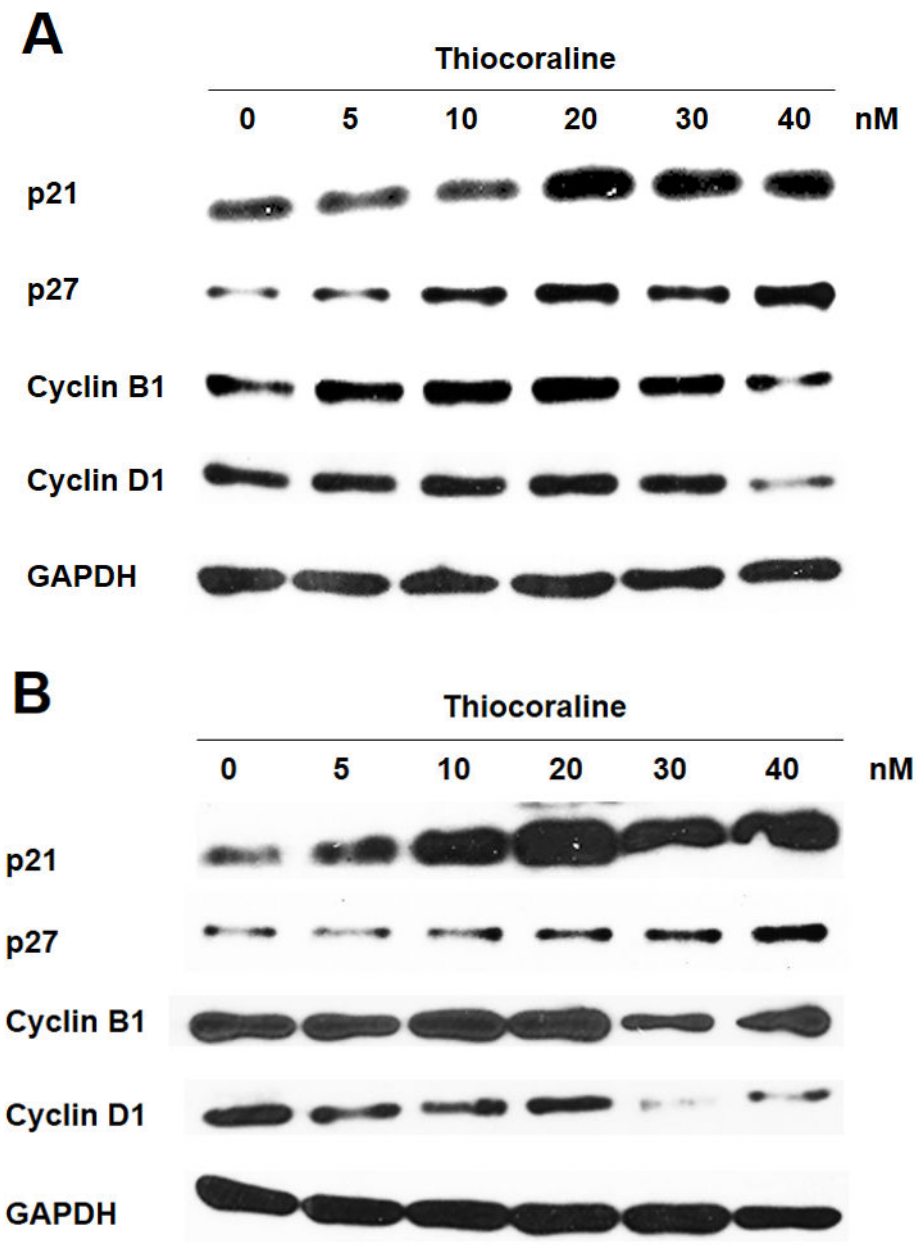
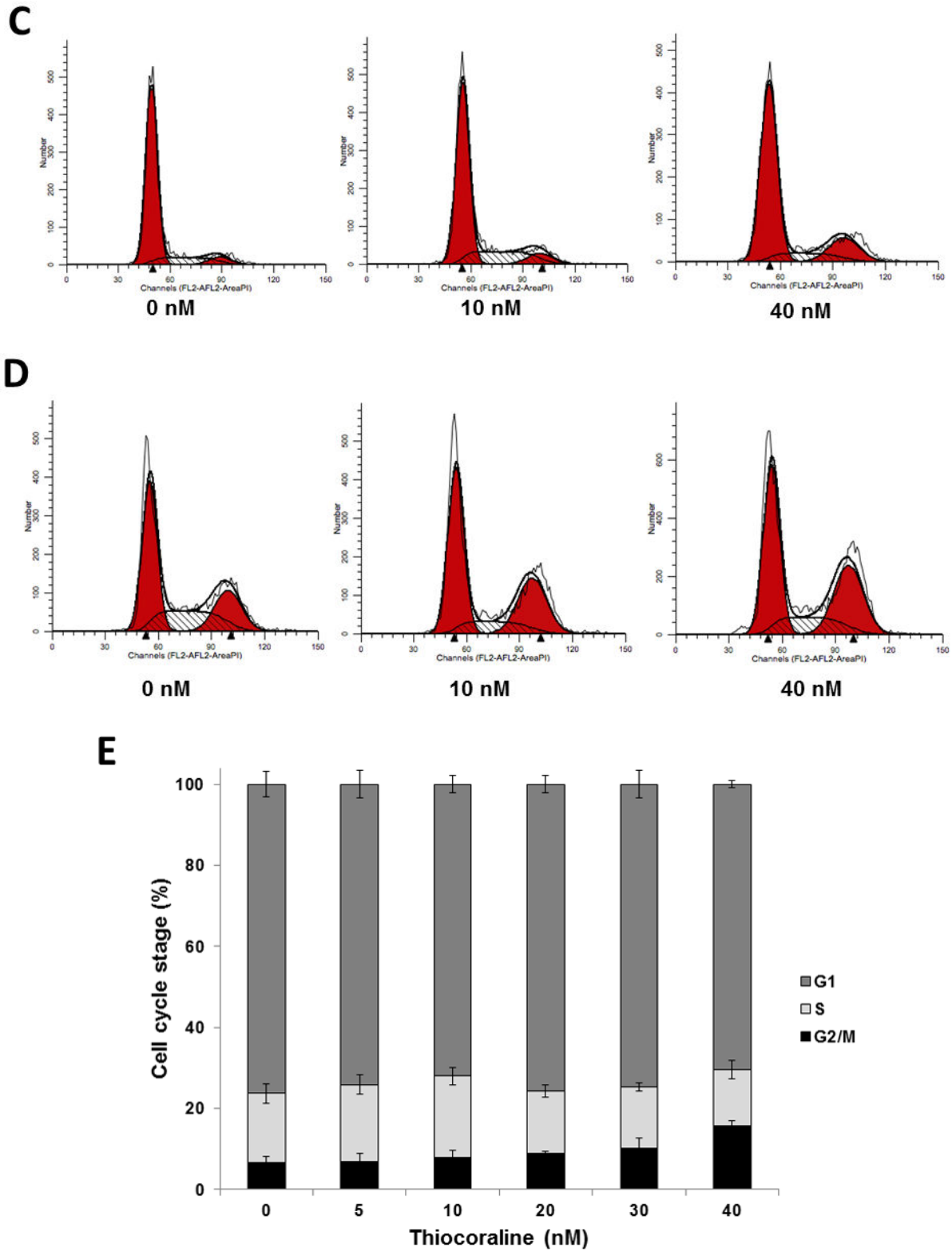


Figure 1. Thiocoraline inhibits BON and H727 cell proliferation *in vitro*

The IC_{50} was determined for BON (**A**) and H727 (**B**) cells treated with thiocoraline for 48 hours using the MTT assay. Using the MTT assay, two-day treatment of thiocoraline for BON (**C**) and H727 (**D**) cells over the course of 8 days demonstrated a decrease in cell proliferation at concentrations as low as 20 nM. Experiments were done in quadruplicate, and data are plotted as mean ± SEM. ***, $P<0.001$.





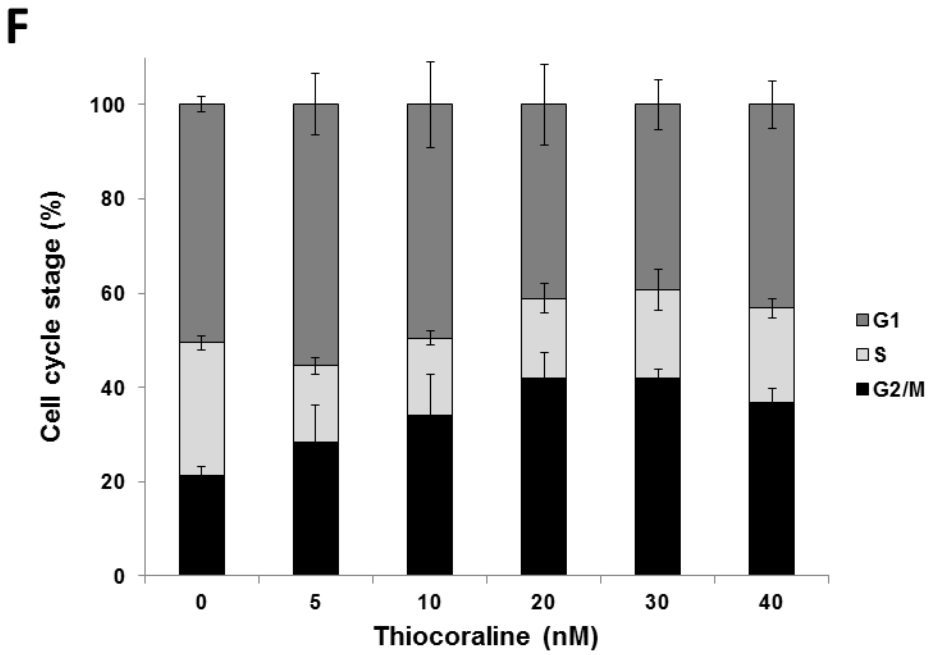


Figure 2. Thiocoraline suppresses cell proliferation through cell cycle arrest

Western blot analysis of cell cycle markers of BON (**A**) and H727 (**B**) cells treated with thiocoraline demonstrated cell cycle arrest. Equal loading was confirmed with GAPDH. Flow cytometry analysis of BON (**C**, **E**) and H727 (**D**, **F**) cells treated with thiocoraline confirmed cell cycle arrest in G2/M.

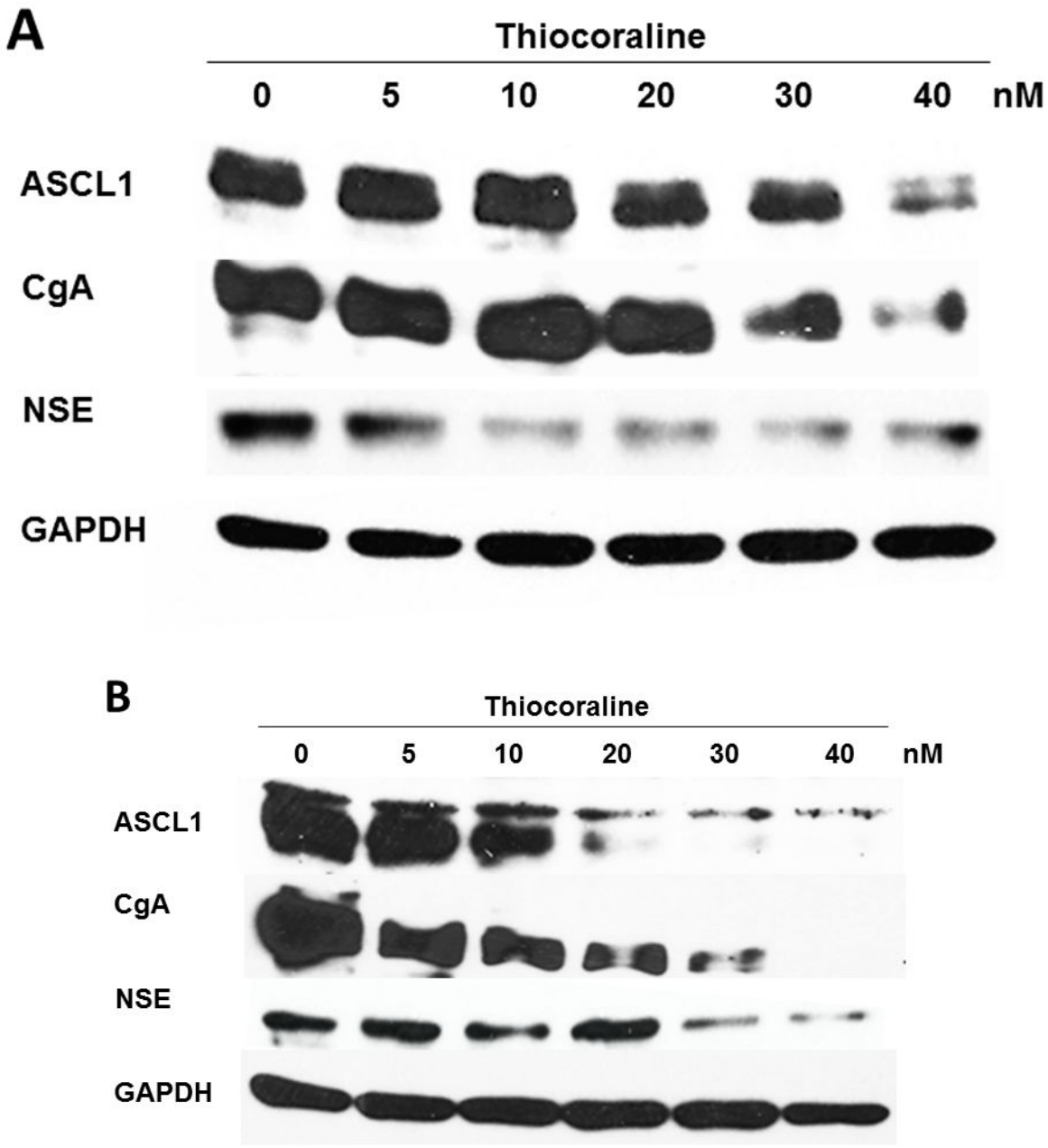
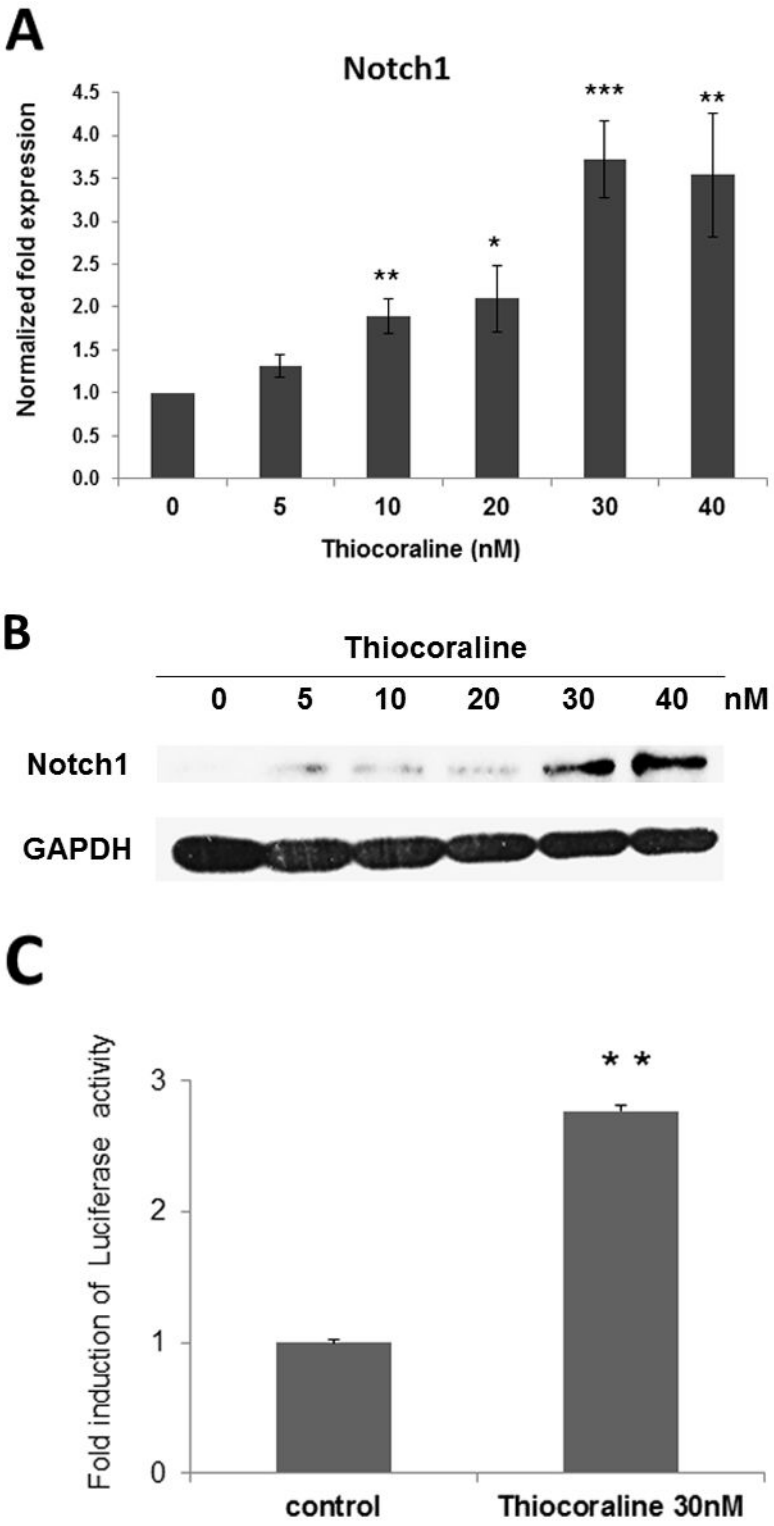


Figure 3. Thiocoraline reduces neuroendocrine tumor markers in *vitro*

Western blot analysis of BON (**A**) and H727 (**B**) cells treated with thiocoraline demonstrated a dose-dependent decrease of neuroendocrine tumor markers expression: ASL1, CgA and NSE. Equal loading was confirmed with GAPDH.



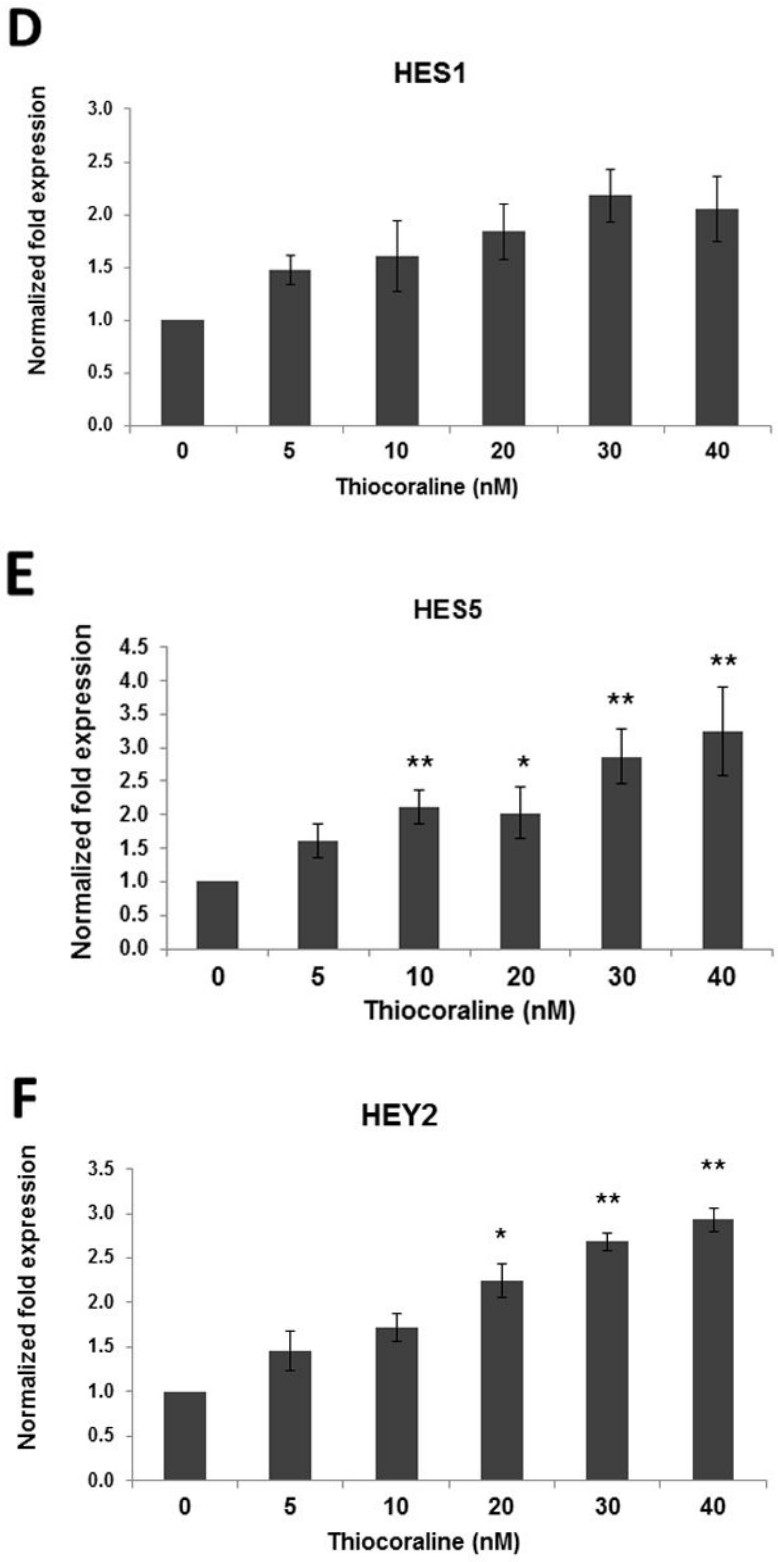
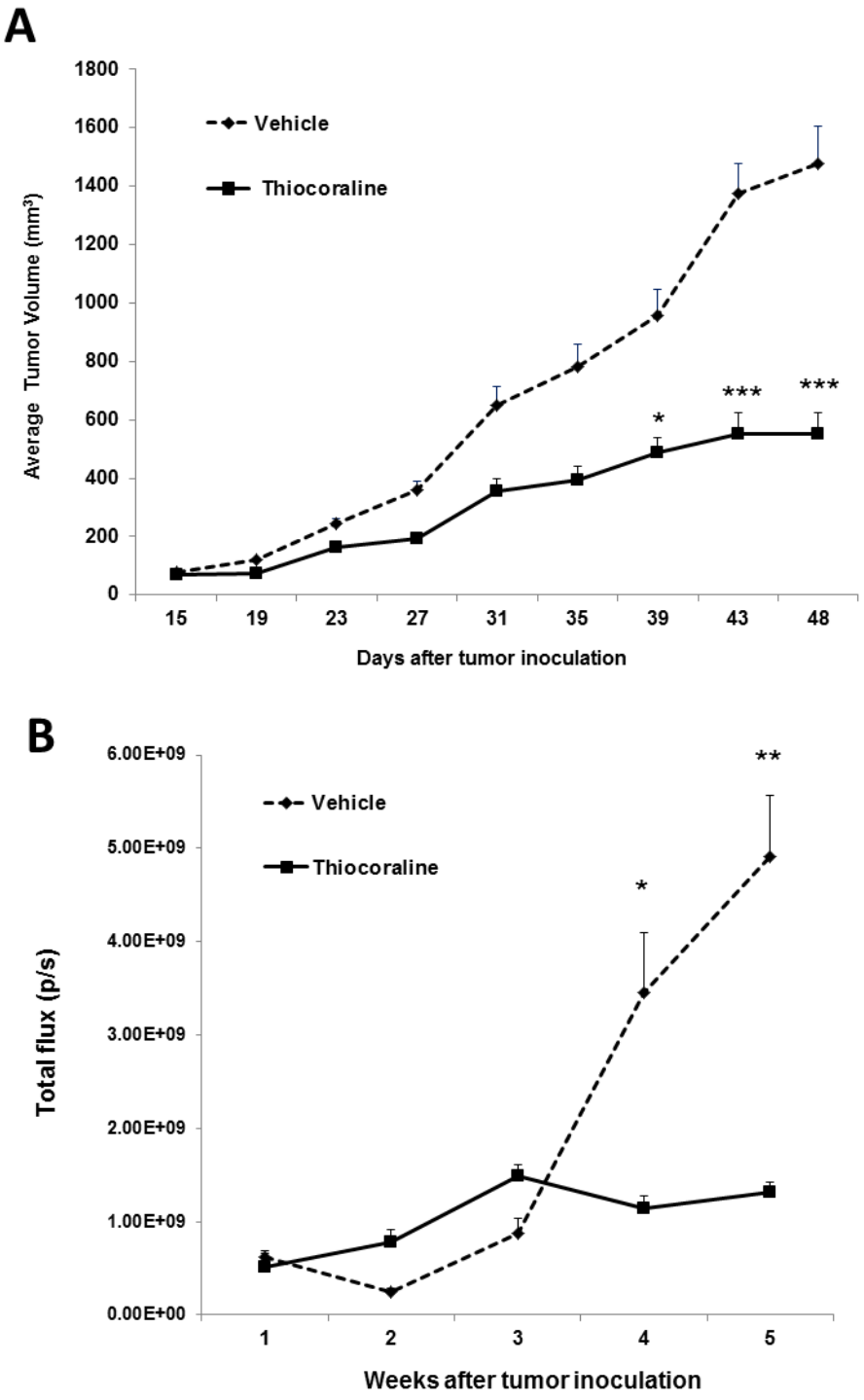


Figure 4. Thiocoraline activates Notch pathway in BON carcinoid cell line

(**A**) Quantitative RT PCR showed dose-dependent activation of Notch1 at the mRNA level in BON cells treated with thiocoraline; data were plotted relative to control cells without thiocoraline treatment. *, P<0.05, **, P<0.01,***, P<0.001. (**B**) Western blot analysis showed dose-dependent activation of Notch1 at the protein level in BON cells treated with thiocoraline. Equal loading was confirmed with GAPDH. (**C**) BON cells treated with thiocoraline induced CBF1 luciferase activity. **, P<0.01. Thiocoraline dose-dependently induced mRNA levels of downstream targets of Notch (HES1 (**D**), HES5 (**E**), and HEY2 (**F**)) monitored by qRT-PCR. *, P<0.05, **, P<0.01, ***, P<0.001.



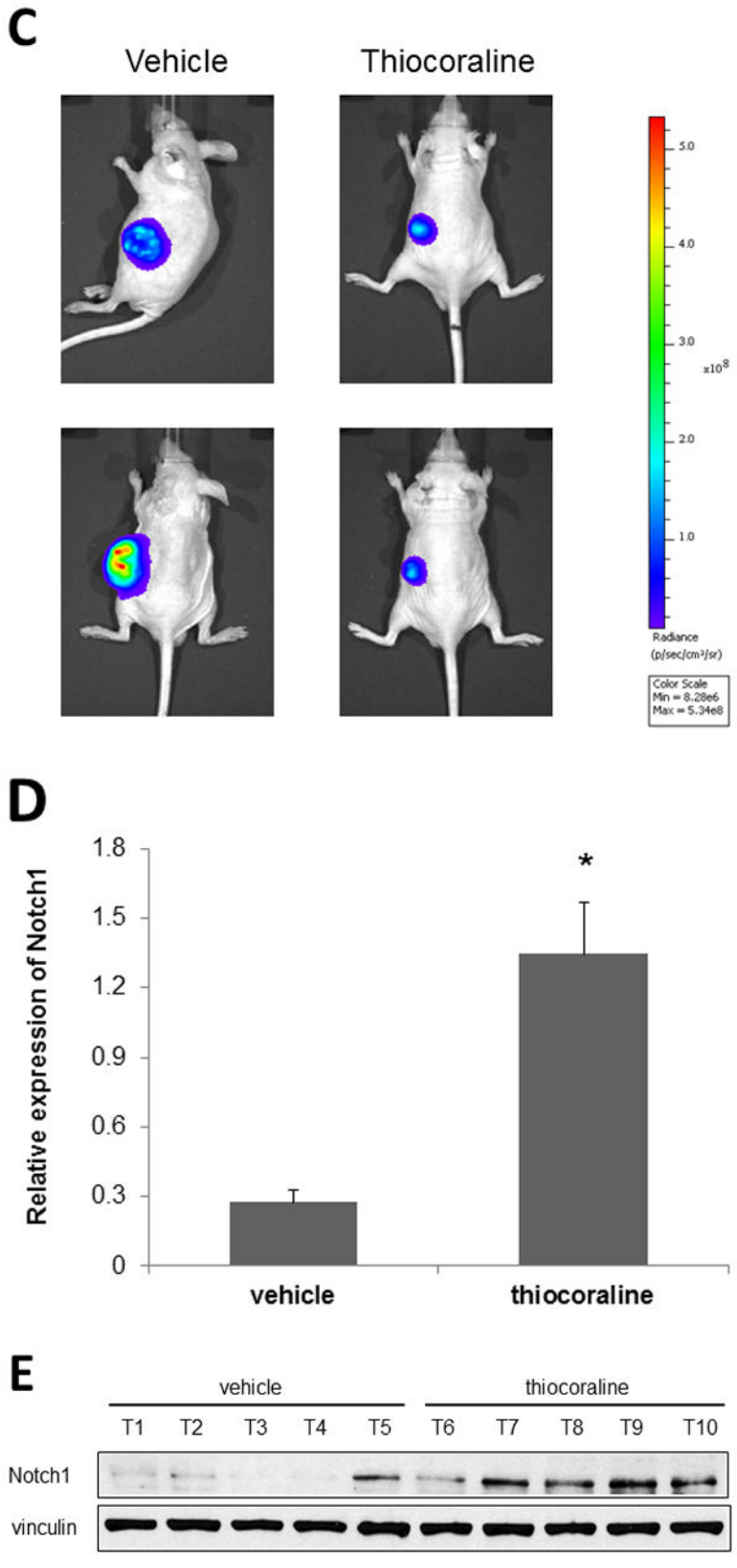


Figure 5. Thiocoraline inhibits growth of human BON xenografts

(**A**) Average tumor volume measured by caliper for mice treated with thiocoraline and vehicle. *, P<0.05, ***, P<0.001 (**B**) The bioluminescence values as a total flux (photons/sec/cm^2/steradian) of the tumor region were quantified for each group of mice and mean values ± SD are plotted over time. (**C**) Representative comparison of mice treated with vehicle and thiocoraline after four weeks of tumor growth. Expression of Notch1 in tumor tissue samples was determined by qRT-PCR (**D**) and western blot analysis (**E**) for mice treated with thiocoraline and vehicle. *, P<0.05BON tumor bearing animals are represented by five tumor lysates from each treatment group. ± SD.