A Thyroid Hormone Receptor Beta Specific Agonist Suppresses Anaplastic Thyroid Cancer Cell Phenotype and Increases Efficacy of Therapeutic Agents

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Short title: TRβ agonist inhibits ATC progression and increases therapeutic efficacy

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Abstract

Background: Anaplastic thyroid cancer (ATC) is one of the most lethal endocrine cancers, with an average survival time of six months after diagnosis. These aggressive tumors have very limited treatment options highlighting a need for a deeper understanding of its mechanisms for development of more effective therapies. We have previously shown that the liganded thyroid hormone receptor beta (TRβ) can function as a tumor suppressor and induce re-differentiation in ATC cells. We therefore tested the hypothesis that selective activation of TRβ with sobetirome (GC-1) could reduce the tumorigenic phenotypes of ATC cell lines and improve the efficacy of clinically relevant therapeutics.

Methods: We used a panel of four ATC cell lines with variable genetic backgrounds to assess the ability of GC-1 to reduce the aggressive phenotype. The effects of GC-1 alone or in combination with buparlisib, alpelisib, sorafenib, and palbociclib on cell growth, viability, and migration were determined and compared with the gene expression levels of selected markers. The impact of these treatments on the cancer stem cell population was assessed by tumorsphere assay. Thyroid differentiation markers were measured by gene analysis, and sodium iodide symporter (NIS) protein level and function were determined.

Results: Our results show that GC-1 alone can decrease cell viability, growth, and slow cell migration in all four ATC cell lines. In addition, GC-1 is able to further block each of these phenotypes when combined with buparlisib, alpelisib, sorafenib, or palbociclib. GC-1 alone blocks thyrosphere outgrowth in all cell lines and increases the efficacy of each of the therapeutic agents tested. GC-1 increased NIS transcript and protein levels to allow for increased iodide uptake in ATC cells.

Conclusion: Activation of TRβ with selective agonist sobetirome (GC-1) reduces the aggressive phenotype and induced re-differentiation in ATC cells and increases the efficacy of therapeutic

agents that are currently used in the treatment of ATC. These results indicate that selective activation of TRβ not only induces a tumor suppression program de novo but enhances the effectiveness of anti-cancer agents.

Introduction

The global incidence of thyroid cancer, the most common endocrine malignancy, is increasing faster than any other solid tumor almost tripling over the past few decades (1,2). While the prognoses for patients with differentiated tumors receiving standard therapies are generally very good, outcomes for patients with resistant or recurrent disease and poorly differentiated thyroid cancers (PDTC) are extremely poor (3). Due to a lack of effective therapies, patients with advanced or metastatic anaplastic thyroid cancer (ATC) have a higher mortality rate than all other endocrine cancers combined with a mean survival time of 6 months (4). The pathogenesis of thyroid cancer is characterized by dysregulation of intracellular signaling pathways, abnormalities in expression of tumor suppressors, cell cycle regulators, and apoptotic signaling. ATC tumors are refractory to radioiodine therapy due to loss of sodium-iodide symporter (NIS), and traditional chemotherapy is of limited benefit. Activation of phosphatidylinositol 3-kinase (PI3K)–Akt–mammalian target of rapamycin (mTOR) pathway (PI3K-Akt) and mitogen-activated protein kinase (MAPK) signaling are recognized drivers of thyroid oncogenesis and blocking this activity is a therapeutic focus. Yet inhibitors of the major components of these signaling cascades rarely provide a durable response due to the development of resistance.

The aggressive behavior of ATC and poor outcome with conventional therapies has led to the continued evaluation of new drugs with variable levels of toxicity which limit their clinical use (5-7). Molecular diagnostics, notably identification of $BRAF^{V600E}$ mutations, provide rationale for intervention with combination therapies (8). Thus far, the use of combination treatments has not yielded significantly improved responses due to the development of resistance, and there are still no curative options. Therefore, there remains an urgent need for innovative and less-toxic therapeutic options for ATC.

There is compelling evidence that the ligand-activated thyroid hormone receptor beta (TRβ) functions as a suppressor of tumor progression, invasion, and metastases in a number of endocrine cancers, including differentiated thyroid tumors. Our recent studies revealed that TRβ in the presence of triiodothyronine (T_3) induced a tumor-suppressive transcriptomic program in anaplastic thyroid cancer (ATC) cells (9,10). We identified key signaling pathways by which activation of TRβ could reduce tumor cell progression, by altered gene expression, implicating thyroid hormone signaling as a potential therapeutic pathway in ATC. However, activation of TRβ by non-isoform selective ligands such as T_3 is contraindicated as a therapeutic approach due to potential for adverse side-effects. Isoform-selective T_3 analogs that preferentially bind to and activate TRβ mitigate these deleterious effects and have been clinically successful for the treatment of metabolic disorders (11-14). The analogue sobetirome, or GC-1, induces similar transcriptomic changes to T_3 (15,16).

In our studies presented here, we establish the effects of GC-1 alone and in combination with therapeutic agents in ATC cells with diverse genetic backgrounds (17). GC-1 alone decreases the aggressive phenotype and increases the effectiveness of MAPK, PI3K, and cell cycle inhibitors. GC-1 significantly increases expression of thyroid-specific genes, inducing re-differentiation in ATC cells. Importantly, we observe an increase in NIS protein which leads to enhanced iodide uptake. These observations indicate that selective activation with isomer-specific agonists may be an effective and practical adjuvant therapeutic strategy.

MATERIALS AND METHODS:

Culture of thyroid cell lines. Anaplastic thyroid cancer cell lines (SW1736, 8505C, OCUT-2, and KTC-2) and the normal-like thyroid cell line Nthy-ORI were cultured in RPMI 1640 growth media with L-glutamine (300 mg/L), sodium pyruvate and nonessential amino acids (1%)

(Corning Inc, Corning, NY, USA), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and penicillin-streptomycin (200 IU/L) (Corning) at 37°C, 5% CO_2, and 100% humidity. For measuring *MYC* expression via RT-qPCR, cells were maintained in media with 10% charcoal stripped fetal bovine serum (Life Technologies). SW1736 cells were modified by lentiviral transduction as recently described (10). SW1736, KTC-2, and Nthy-ORI were authenticated by the Vermont Integrative Genomics Resource at the University of Vermont using short tandem repeat profiles and Promega GenePrint10 System (SW1736, May 2019; KTC-2, October 2019; Nthy-ORI, August 2020). 8505C and OCUT2 were authenticated by the University of Colorado using short tandem repeat profiles (8505C, June 2013; OCUT-2, June 2018).

Pharmacological agents. T_3 and GC-1 (Sigma, St. Louis, MO, USA) were suspended in 1 M NaOH (T_3) and 100% ethanol (GC-1) for stock concentrations of 1 mM before further dilution in culture medium. Buparlisib, sorafenib, palbociclib, and alpelisib (MedChemExpress, Monmouth Junction, NJ, USA) were suspended in 100% DMSO for stock concentrations of 50-150 mM before further dilution in culture medium.

Cell Growth and Viability Assays. Cell growth was measured by cell counting at discrete time points. Cells were seeded in 12- well plates and growth optimized. Cells were treated with GC-1, buparlisib, alpelisib, palbociclib, or sorafenib to establish time and concentration effects of the select agents on cell growth. Cell viability was determined by a Sulforhodamine B assay (Abcam, Cambridge, UK) following the manufacturer's protocol. In brief, ATC cells were plated in 96-well clear flat-bottom plates at a density of 5,000 cells per well. Cells were fixed, stained, and then imaged using a plate reader according to manufacturer's instructions.

Migration Assay. Cell migration was determined by wound healing assay. Cells were plated and allowed to grow to 100% confluency. Four hours prior to scratching, cells were treated with

10 μg/ml mitomycin C (Sigma). A scratch was performed with a P1000 pipette tip and debris was washed away with PBS. Media was supplemented with GC-1 (10 nM) with and without 0.5 μM buparlisib, 0.5 μM alpelisib, 1 nM palbociclib, or 5 μM sorafenib. Images were obtained at 0, 16, 24, 48, and 72 hours or until 100% wound closure, depending on the cell type. The wound closure was measured using the ImageJ macro "Wound Healing Tool" (https://github.com/MontpellierRessourcesImagerie/imagej_macros_and_scripts/wiki/Wound-Healing-Tool). Percent closure was calculated relative to the area of the initial scratch.

Tumorsphere Assay. Cancer stem cell tumorspheres formed from ATC cells were used to assess self-renewal and sphere-forming efficiency. For generating tumorspheres, adherent monolayer cells were dissociated with Trypsin-EDTA, and single cells were moved to round-bottom ultra-low attachment 24-well plates at a density of 1000 cells/well (Corning). Tumorspheres were cultured in RPMI 1640 growth media supplemented with 20 ng/mL each of epidermal growth factor (EGF) and fibroblastic growth factor (FGF) (Gold Bio). Where indicated, adherent cells were treated with 0.5 μM buparlisib, 0.5 μM alpelisib, 1 nM palbociclib, 5 μM sorafenib, or vehicle for 72 hours prior to thyrosphere-forming assay. Tumorspheres were then cultured in the presence or absence of GC-1 (10 nM) to evaluate the effects of liganded TRβ on thyrosphere growth alone or subsequent to a therapeutic agent treatment. Tumorspheres grew for seven days and were then counted with an inverted microscope (Carl Zeiss, Oberkochen, Germany).

Immunoblot Analysis. Proteins were isolated from whole cells in lysis buffer (20mM Tris-HCl [pH 8], 137mM NaCl, 10% glycerol, 1% Triton X-100, and 2mM EDTA) containing Protease Inhibitor Cocktail 78410 (Thermo Fisher Scientific). Proteins were resolved by polyacrylamide gel electrophoresis on 10% sodium dodecyl sulfate gels EC60752 (Thermo Fisher Scientific) and immobilized onto nitrocellulose membranes (GE Healthcare, Chicago, IL, USA) by

electroblot (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% w/v BSA in TBS and 0.1% v/v Tween20 (Gold Bio) for one hour at room temperature. Specific proteins were detected by immunoblotting with the indicated antibodies (Table 1); immunoreactive proteins were detected by enhanced chemiluminescence (Thermo Fisher Scientific) on a ChemiDoc XRS+ (Bio-Rad Laboratories).

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted using RNeasy Plus Kit (Qiagen, Hilden, DE) according to manufacturer's protocol. cDNA was then generated using 5X LunaScript RT SuperMix, and mRNA expression was quantified by qRT-PCR using 2X Luna Universal qPCR Master Mix (NEB, Ipswich, MA, USA) on a QuantStudio 3 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Fold change in gene expression compared to endogenous controls was calculated using the ddCT method. Primer sequences are indicated in Table 2.

Iodide Uptake Assay. Cells were seeded at a density of 40,000 cells per well in a 96 well plate with or without 10 nM GC-1 for 48 hours. Media were discarded, and cells were washed four times with warm PBS before incubation with 1 μM potassium iodide (KI, Sigma) in PBS for 1 hour at 37°C. Wells were washed an additional four times with warm PBS and 100 μL of ddH_2O were added to each well. A standard curve ranging from 100-700 nM KI was assayed in parallel. 100 μl of 24 mM ammonium cerium (IV) sulfate hydrate with 5.7% concentrated H_2SO_4 (Sigma) and 100 μl of 24 mM sodium arsenite (III) with 205 mM sodium chloride (NaCl) and 50 mM sodium hydroxide (NaOH, Sigma) were added to each well. The plate was incubated in the dark at room temperature for 30 minutes, and absorbance at 415 nm was measured with a Synergy 2 Multi-Detection Microplate Reader (Agilent Technologies, Santa Clara, CA, USA).

Statistics. All statistical analyses were performed using GraphPad Prism software. Paired comparisons were by T-test and group comparisons were made by a 2-way ANOVA followed by

a Tukey multiple comparisons test or Sidak's multiple analyses where indicated. Data are represented as mean ± standard deviation. Significance is indicated * $p<0.05$. Area under the curve (AUC) at the 95th confidence interval was used to evaluate statistical differences in growth and migration assays.

RESULTS:

Sobetirome, GC-1, blocks the tumorigenic phenotype and cancer stem cell growth in ATC cells transduced with TRβ.

We have previously demonstrated that T_3 (10 nM) treatment of transduced SW1736 cells, in which TRβ is re-expressed (SW-TRβ), induced a tumor suppression transcriptomic program and reduced cell growth when compared to cells transduced with empty vector (SW-EV) with low levels of endogenous TRβ (10). In the present studies, we tested the ability of GC-1 (10 nM) to alter the ATC phenotype and cancer cell stemness. We found that GC-1 significantly decreased cell growth in four days (**Fig. 1A**). There was a 50% reduction in the overall growth rate of SW-EV cells and 75% reduction in SW-TRβ cells, indicating potent activation of endogenous TRβ (**Fig. 1B**).

As cancer stem cells are considered to be responsible for tumor initiation and tumor recurrence after chemotherapy, targeting these cells is a key for successful treatment. Therefore, a critical question is whether or not thyroid cancer stem cells are responsive to GC-1. Thyrosphere formation is robust in SW-EV cells, and GC-1 induced a significant reduction in growth (**Fig. 1C**). Re-expression of TRβ (SW-TRβ) reduces thyrosphere growth and the addition of GC-1 induces a greater than 80% reduction in thyrosphere formation (**Fig. 1C**). These results indicate that GC-1 alone can activate TRβ to reduce the thyroid cancer stem cell population.

Apoptotic signaling is a key factor in mediating ligand-activated TRβ modification of the

ATC aggressive phenotype (10). Thus, we assessed the effects of GC-1 on PARP and caspase 3 cleavage by immunoblot as markers of late-stage apoptosis (**Fig. 1D**). GC-1 significantly increased the presence of cleaved PARP and cleaved caspase 3 in SW-TRβ consonant with increased apoptotic signaling.

These results indicate that GC-1 can activate TRβ to reduce the aggressive phenotype in transduced ATC cells with TRβ re-expressed and recapitulate the effects seen with T_3 at comparable concentrations.

GC-1 slows growth of parental ATC cells.

Although it was confirmed that GC-1 could induce the same phenotypic changes in our transduced ATC cell line model, it was critical to assess whether agonist-selective activation of endogenous TRβ could induce similar effects in unmodified parental ATC cell lines with diverse mutational backgrounds (17). SW1736, 8505C, OCUT2, and KTC-2 cells all harbor *BRAF*^{V600E} and *TERT* driver mutations. SW1736 and 8505C have additional *TP53* mutations. SW1736 has an additional *TSHR* mutation, and OCUT2 has a gain-of-function *PIK3CA* mutation (summarized in **Fig. 2A**). Endogenous TRβ expression is low in all four ATC cell lines when compared with normal thyroid cells (18). Treatment of each of these cell lines with 10 nM GC-1 for four days yielded a modest but significant decrease in cell growth in all four cell lines (**Fig. 2B-E**). Treatment with 10 nM T_3 did not significantly alter the growth of any of these four cell lines. This result suggests that even low levels of endogenous TRβ can be selectively activated with GC-1, and it is more effective at promoting tumor suppressor activity than T_3.

GC-1 increases the efficacy of selected therapeutic agents

As agonist activation of TRβ can induce a tumor suppression program and reduce the aggressive tumor phenotype in ATC cells, a critical question is whether GC-1 can enhance the

efficacy of therapeutics that target selective pathways. Cell viability was calculated following treatment for three days with increasing concentrations of the PI3K inhibitor buparlisib (pan-PI3K inhibitor) or alpelisib (PI3K mutant inhibitor) for OCUT2 cells which harbor a gain-of-function *PIK3CA* mutation (**Fig. 3A**), the cell cycle inhibitor palbociclib (**Fig. 3B**), or the multi-tyrosine kinase inhibitor sorafenib (**Fig. 3C**), with or without 10 nM GC-1. Each of the therapeutic agents decreased cell viability in three days in a dose-dependent manner. The addition of GC-1 significantly increased the efficacy of these agents at lower concentrations. For example, 1 μM buparlisib was required to reduce the cell viability below 50% in the absence of GC-1, but when combined with 10 nM GC-1 this could be achieved with 0.1-0.5 μM buparlisib (**Fig. 3A**). Similar trends were observed with all four therapeutics and across the panel of cell lines used.

GC-1 enhances the effects of therapeutic agents on cell migration

In addition to reducing cell viability, many therapeutics target pathways that are involved in cell migration. To further evaluate the ability of GC-1 to enhance the efficacy of select therapeutics, cells were treated with the indicated inhibitors at concentrations determined to be effective based on the cell viability assays (**Fig. 3**), and migration was observed by assessing wound closure. Each inhibitor was able to successfully prevent wound closure, and this effect was increased when each of the inhibitors were combined with GC-1, with the exception of buparlisib in the KTC-2 cells (**Fig. 4B-E**). Notably, GC-1 alone was able to prevent wound closure of all tumor cell lines with similar success as each inhibitor alone (**Fig. 4A-E**). These findings are similar to what we previously observed in experiments using endogenous thyroid hormone, T_3, indicating GC-1 is a suitable alternative for TRβ activation and mitigation of malignant phenotype.

GC-1 blocks tumorsphere outgrowth and increases the efficacy of therapeutic agents.

Current treatment options for ATC primarily target hyperproliferative cells, but aggressive cancers like ATC are enriched for long-lived cancer stem cells that are thought to be a major mechanism for therapy resistance and tumor recurrence. The signaling pathways that govern stemness and proliferation are separate and frequently oppose each other. We previously demonstrated that ligand-activated TRβ regulates genes related to stemness in ATC cells and that GC-1 could block thyrosphere outgrowth in our modified ATC cells (**Fig 1D**). We therefore tested the hypothesis that GC-1 could prevent thyrosphere outgrowth after an initial treatment with an inhibitor that targets proliferation.

ATC cells were treated with the indicated inhibitor or vehicle for three days, followed by re-plating in non-adherent serum-free conditions. We again observed that GC-1 alone could reduce thyrosphere formation in unmodified ATC cell lines (**Fig. 5A-C**). None of the inhibitors could completely prevent thyrosphere outgrowth when used alone; however, sorafenib was the most effective overall (**Fig. 5C**). Strikingly, the addition of GC-1 upon removal of each inhibitor blocked nearly all thyrosphere outgrowth. This effect was consistent across all four cell lines and all three inhibitors used in this experiment. This result suggests that GC-1 may be particularly effective at blocking cancer stem cell expansion

GC-1 promotes re-differentiation of ATC cell lines.

In transduced cells, TRβ activation with T_3 reduces the aggressive phenotype partially through induction of re-differentiation in ATC cell. Therefore, we investigated the potential for GC-1 alone to promote re-differentiation in parental SW1736 cells. Gene expression analysis using RT-qPCR revealed that 24 hours of GC-1 exposure increased levels of thyroid-specific gene transcripts (**Fig. 6A**). This transcriptomic program is summarized by the thyroid differentiation score (TDS), which significantly increased in the GC-1-treated cells (**Fig. 6B**). Additionally, GC-1 alone decreased the overall number of $CD24^-/CD44^+$ cells present in the

monolayer population as evident by RT-qPCR (**Sup. Fig. 1**). We also observed a decrease in the ATC markers *ALCAM* (CD166) and *ALDH1A1* (**Sup. Fig. 1**).

Intriguingly, *SLC5A5* expression was greatly increased with 24 hours of GC-1 (**Fig. 6A**). Therefore, we further investigated NIS protein levels after 48 hours of GC-1 treatment. Not only were nascent and mature, glycosylated NIS protein significantly increased following GC-1 treatment (**Fig. 6C-D**), iodide uptake increased as well (**Fig. 6E**). This indicated that the increased NIS protein was functional. These findings reveal a novel function of GC-1 to induce re-differentiation in an ATC cell line model and offers a promising target to increase NIS levels and iodide uptake.

DISCUSSION:

Nuclear receptors (NRs) are master regulators of growth and differentiation and are increasingly recognized as diagnostic and therapeutic targets in thyroid, breast, and other hormone-dependent cancers. Yet, the actions of non-steroidal NRs in these cancers are not well characterized (19).There is compelling evidence that loss of expression of TRβ, a member of the thyroid hormone receptor (TR) family, through genomic modifications and epigenetic silencing is characteristic of thyroid, breast and other endocrine-related tumors (20-26).

Multiple lines of evidence demonstrate the tumor suppressive activity of TRβ in thyroid cancer. Numerous pre-clinical studies demonstrate the potent anti-tumorigenic effect of robust expression of the TRβ gene. Re-expression of silenced TRβ using demethylating agents delays thyroid tumor progression *in vivo* (27), mice expressing a c-terminal frameshift of TRβ ($Thrb^{PV}$) spontaneously develop follicular thyroid cancer (28), and $Thrb^{PV/PV}$ $Kras^{G12D}$ mice develop thyroid tumors with features of de-differentiated thyroid cancer (29). Our studies in anaplastic thyroid cancer cells revealed that activation of TRβ induces a tumor suppression transcriptomic program and reduces the aggressive phenotype (9,10).

Agonists have been developed to target tissue specific expression of the most abundant isomers (30). Selective activation of TRβ in pre-clinical and clinical studies have yielded exciting results with great promise for novel interventions. Patients who were rendered hypothyroxinemic and treated with T_3 alone had longer survival with end-stage solid tumors (31) and T_3 treatment enhanced chemosensitivity (32). Following tyrosine kinase inhibitor therapy, patients demonstrated improved survival with treatment of hypothyroidism (33). Mimetics of T_3 that selectively act as TRβ agonists have been clinically successful for treatment of metabolic disorders, hyperlipidemia, hypercholesterolemia and non-alcoholic steatohepatitis without the cardiovascular effects mediated by TRα (11,12). Those in clinical trials include TRβ selective sobetirome (GC-1) (14) with comparable transcriptomic effects of T_3 (16). Our findings demonstrate that GC-1 alone decreases the ATC aggressive phenotype in transduced cells in which TRβ is re-expressed and parental cell lines with diverse genetic backgrounds. These results emphasize the generalizability of GC-1 to activate a TRβ tumor suppression program.

In anaplastic thyroid cancer tumors, several genetic alterations and disrupted signaling pathways are usually found. Therefore, the treatment aim is to inhibit or disrupt several pathways with combination therapies that block cell survival, proliferation typically by targeting the MAPK, PI3K, and cell cycle pathways (7,34). There are many therapeutics that are in use in combination or alone including multi-kinase inhibitors, epigenetic modulators, apoptosis-inducing agents, and others. However, to date, none have been successful in long-term treatment. Development of resistance and toxicity are limitations to their use.

In this study, we tested representative drugs that targeted the PI3K, MAPK, and cell cycle pathways to assess whether GC-1 could alter the effectiveness of these drugs on their targets. Since we have demonstrated that activation of TRβ in ATC represses the PI3K pathway through genomic mechanisms (10,18), GC-1 with either buparlisib or alpelisib would be

suppressing PI3K activity in concert. However, TRβ does not directly impact MAPK or cell cycle signaling through transcriptomic modulation so GC-1 in combination with either sorafenib or palbociclib demonstrated concordant effects. In all studies, GC-1 reduced the effective concentrations required to observe a reduction in cell viability. Not surprisingly, not all effects were noted for cell migration as these events are mediated by distinct cell signaling pathways modulated by TRβ. One of our most intriguing observations is the impact of GC-1 alone and in combination with inhibitors on cancer stem cell growth in thyrosphere formation. In all ATC cell types, GC-1 alone significantly reduced thyrosphere formation. Remarkably, GC-1 in combination with any of the drug treatments nearly eliminated thyrosphere formation.

GC-1 induced re-differentiation in the SW1736 cell line in 24 hours, demonstrating potent transcriptional reprogramming. While we have previously demonstrated the potential for TRβ to promote a more differentiated phenotype in ATC cells, this is the first time we have been able to demonstrate the profound impact of GC-1 in a parental cell line with low TRβ expression. Importantly, we observed a concomitant increase in the sodium iodide symporter that led to a higher intake of iodide. Since PDTC and ATC tumors often exhibit a marked decrease in NIS expression and resistance to radioactive iodide (RAI) therapy, the results presented here raise a provocative implication for the use of GC-1 in combination with RAI.

Our findings in this study demonstrate for the first time that selective activation of TRβ with GC-1 (Sobetirome) can reduce the aggressive phenotype, reduce the cancer stemness in anaplastic thyroid cancer cells, increase the sensitivity of these cells to therapeutic agents, and promote re-differentiation and iodide uptake. These studies provide the foundation for further establishing the broad impact of GC-1 alone and in combination with various therapeutics in mitigating toxicity and the development of resistance to treatment.

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Figure 1. GC-1 blocks tumorigenic phenotypes in ATC cells transduced with TRβ. A. Growth curves demonstrate that SW-TRβ growth is significantly decreased after treatment with GC-1 for 4 days (n=3). Significance (* $p<0.05$) was determined by a two-way ANOVA and Tukey's multiple comparisons test. **B**. Area under the curve (AUC) analysis of growth curve data. Significance (* $p<0.05$) was determined by t-test. **C.** Treatment with GC-1 slows migration of SW-TRβ and SW-EV cells measured by scratch assay. Significance (* $p<0.05$) was determined by t-test. D. GC-1 repressed thyrosphere formation in SW-TRβ and SW-EV cells (n=4). Significance (* $p<0.05$) was determined by t-test. E. Immunoblot demonstrates that 5 days of GC-1 treatment induces apoptosis in SW-TRβ cells assessed by cleavage of PARP and caspase 3 (n=3).

Figure 2: GC-1 slows growth of parental ATC cells. A. Summary of mutated genes in four ATC cell lines implemented in this study (17). B-E. Relative cell growth of unmodified ATC cell lines was measured by cell counting assay after 4 days of treatment with 10 nM GC-1 or 10 nM T_3. Significance (* $p<0.05$) was determined by a two-way ANOVA followed by Dunnett's multiple comparisons test; 3 independent experiments were performed per treatment group.

Figure 3. GC-1 increases efficacy of inhibitors. Cell viability of unmodified ATC cell lines was measured by SRB assay after 3 days of treatment with increasing concentrations buparlisib or alpelisib (A), sorafenib (B), or palbociclib (C) simultaneously in combination with 10 nM GC-1. Significance (* $p<0.05$) was determined by a two-way ANOVA followed by Sidak's multiple comparisons test; 3 independent experiments were performed per each treatment group.

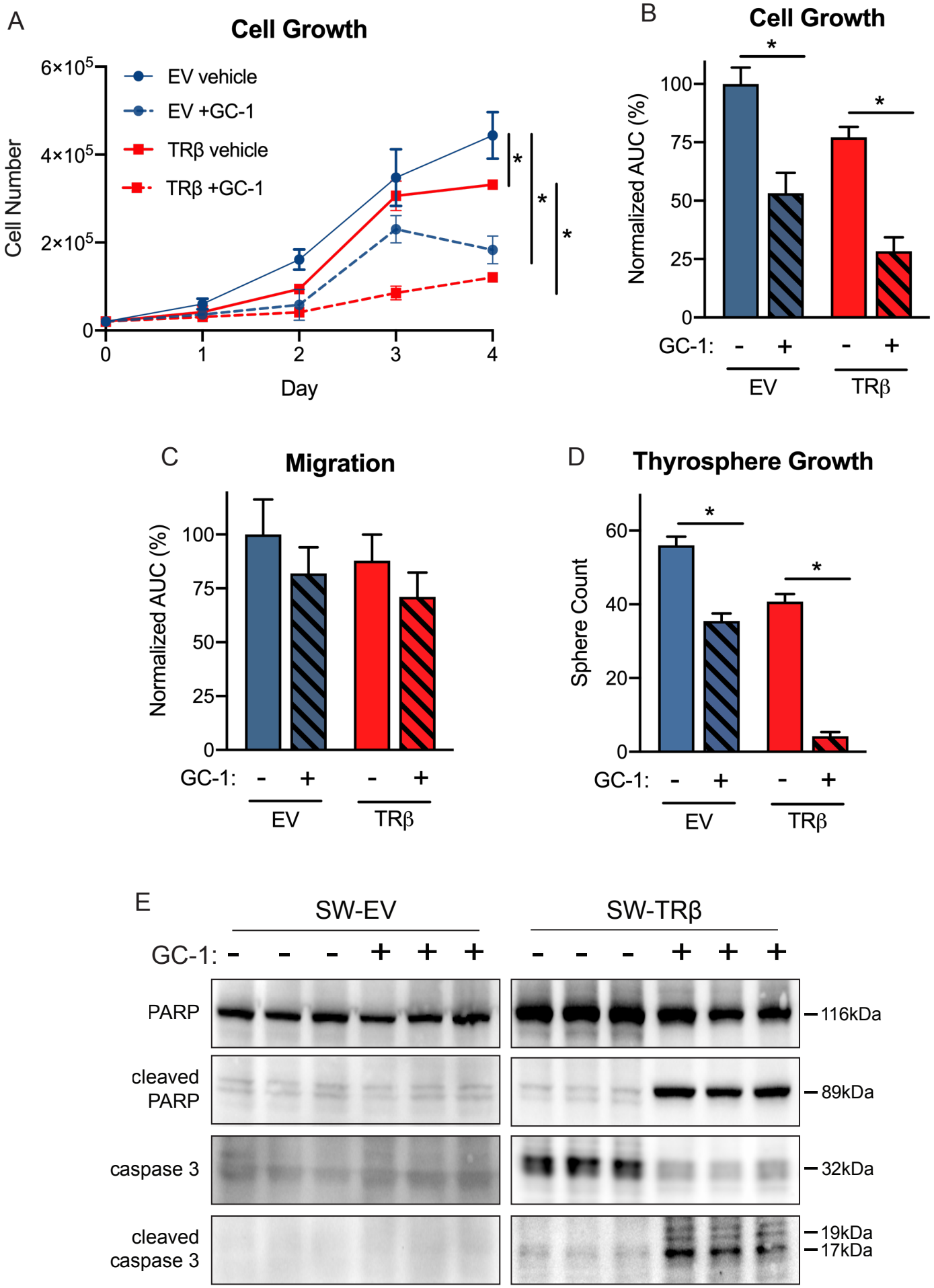
Figure 4. GC-1 enhances the effects of inhibitors on cell migration. Cell migration was measured by scratch assay. Representative images demonstrate reduced scratch closure after two days when ATC cells are treated with GC-1 (A). Area under the curve (AUC) analysis shows SW1736 (B), 8505C (C), OCUT2 (D), and KTC-2 (E) cells all exhibit lower rates of

scratch closer after treatment with GC-1 alone and further reduced scratch closure rates after combined treatment with GC-1 and inhibitors. Significance (* $p<0.05$) was determined by t-test.

Figure 5. GC-1 blocks thyrosphere outgrowth and increases the efficacy of therapeutic agents. Thyrosphere growth was determined for ATC cells after 3 days of treatment with 0.5 μM buparlisib or alpelisib (A), 10 μM sorafenib (B), or 1 nM palbociclib (C) under adherent culture conditions, followed by plating in conditions for spheroid growth in the presence of 10 nM GC-1 for 7 days. GC-1 alone and each therapeutic agent significantly blocked sphere formation in all cell types. GC-1 in addition to each therapeutic agent further inhibited or completely blocked sphere formation. Significance (* $p<0.05$) was determined by two-way ANOVA followed by Sidak's multiple comparisons test; 3 independent experiments were performed per each treatment group; n=3 per analyses.

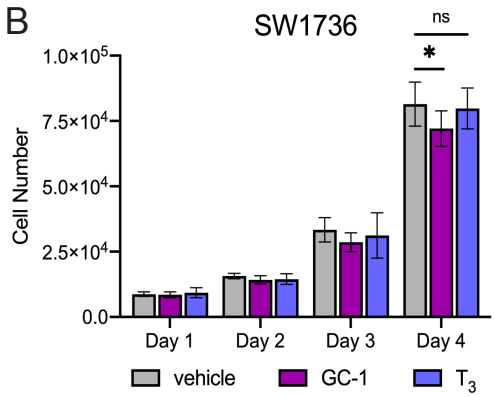
Figure 6. GC-1 induces expression of redifferentiation markers in SW1736 cells. A. RT-qPCR for thyroid differentiation markers was performed on SW1736 cells treated with or without 10 nM GC-1 for 24 hours. mRNA levels in cells treated with GC-1 are relative to the untreated cells. Significance (* $p<0.05$) was determined by Student's unpaired t test; 3 independent experiments were performed per treatment group. B. Thyroid differentiation score (TDS) was calculated using the expression level of the genes in panel A, excluding *THRB*, as previously described (10, 17). Significance (* $p<0.0001$) was determined by Student's unpaired t test. C. SW1736 cells were treated with or without 10 nM GC-1 in triplicate for 48 hours prior to protein extraction and analysis via immunoblot. Normal-like thyroid Nthy-ORI cells were used as a positive control. D. Relative NIS/GAPDH protein levels from (C) were quantified via ImageJ, and significance (* $p<0.01$) was determined by Student's unpaired t test. E. Iodide uptake was measured after 48 hours of GC-1 treatment. Significance was determined by Student's unpaired t test. (* $p<0.001$).

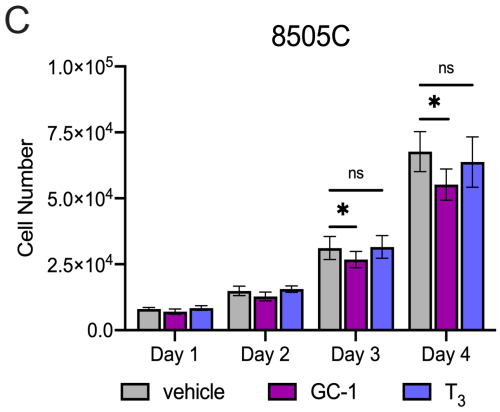
Supplemental Figure 1. RT-qPCR for markers of stemness and proliferation was performed on SW1736 cells treated with or without 10 nM GC-1 for 24 hours. mRNA levels in cells treated with GC-1 are relative to the untreated cells. Significance (* $p<0.05$) was determined by Student's unpaired t test; 3 independent experiments were performed per treatment group.

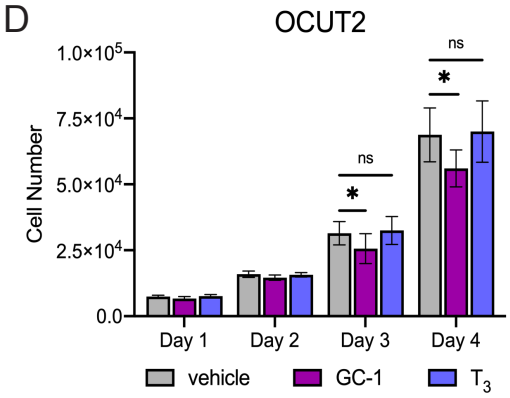

A
Cell Growth
Cell Number
6×10^5
4×10^5
2×10^5
0
EV vehicle
EV +GC-1
TRβ vehicle
TRβ +GC-1
0
1
2
3
4
Day
*
*
*
B
Cell Growth
Normalized AUC (%)
100
75
50
25
0
*
*
GC-1: - + - +
EV
TRβ
C
Migration
Normalized AUC (%)
100
75
50
25
0
GC-1: - + - +
EV
TRβ
D
Thyrosphere Growth
Sphere Count
60
40
20
0
*
*
GC-1: - + - +
EV
TRβ
E
SW-EV
SW-TRβ
GC-1: - - - + + + - - - + + +
PARP
cleaved PARP
caspase 3
cleaved caspase 3
116kDa
89kDa
32kDa
19kDa
17kDa

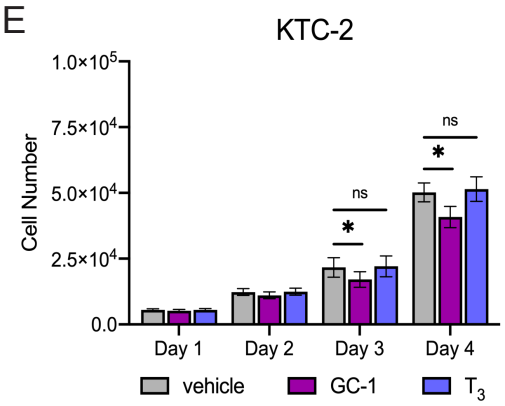
A

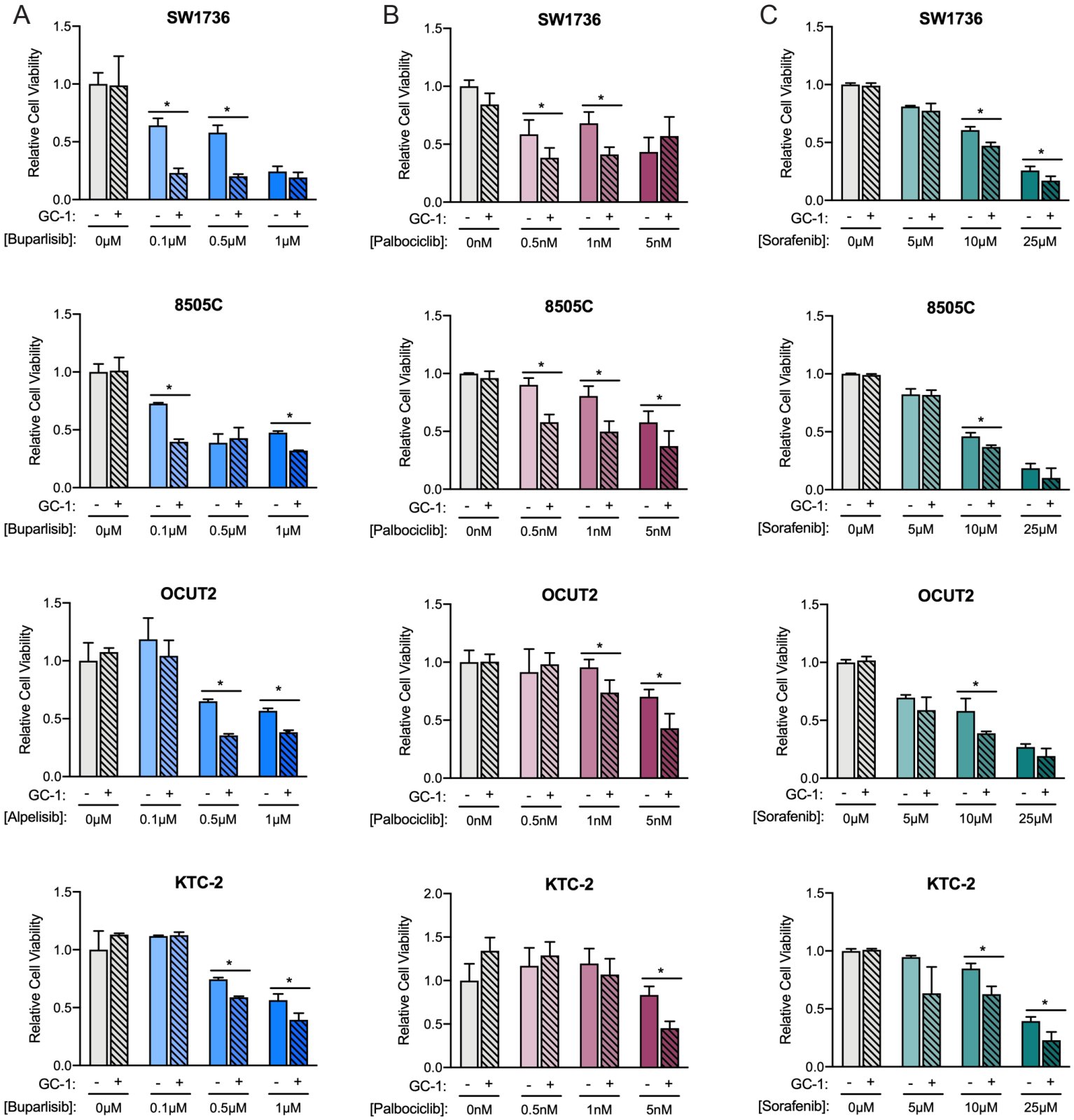
Gene Function	Gene Name	Cell Line: SW1736	8505C	OCUT-2	KTC-2
		Mutation Type			
MAPK Signaling	BRAF	V600E	V600E	V600E	V600E
Telomerase	TERT (promoter)	124C-T	146C-T	146C-T	124C-T
DNA Homeostasis	TP53	Trunc.	Miss.		
PI3K Signaling	PIK3CA			Kinase Domain	
Cell-Cell Contact	NF2		Trunc.		
DNA Homeostasis	ATM		Trunc.		
TSH Signaling	TSHR	Miss.			
Histone Methyltransferase	KMT2C				Miss.
Histone Methyltransferase	KMT2D				Trunc.
Histone Acetyltransferase	EP300	Trunc.			
SWI/SNF Complex	ARID1A				In Frame
SWI/SNF Complex	SMARCA4		Miss.		

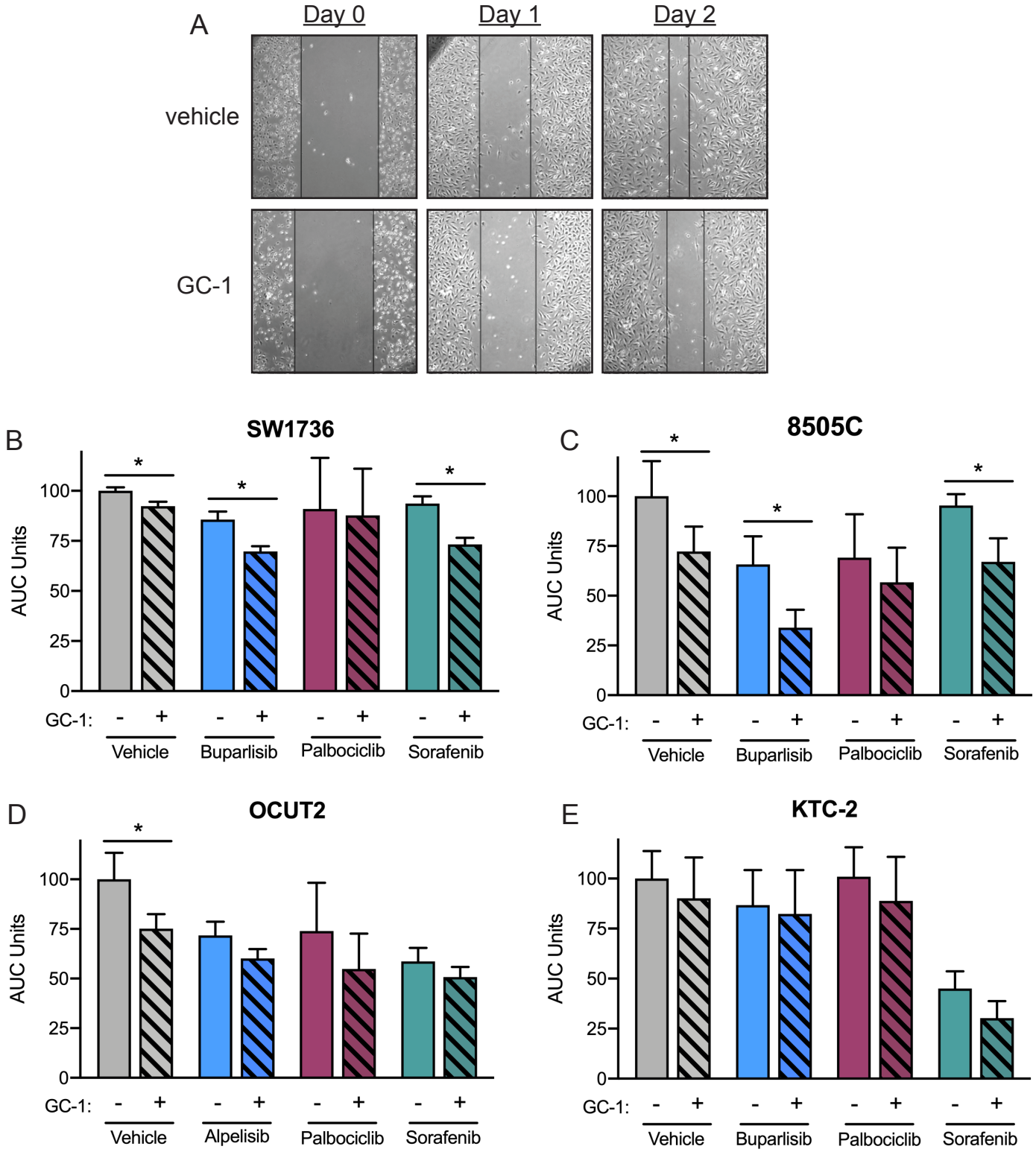


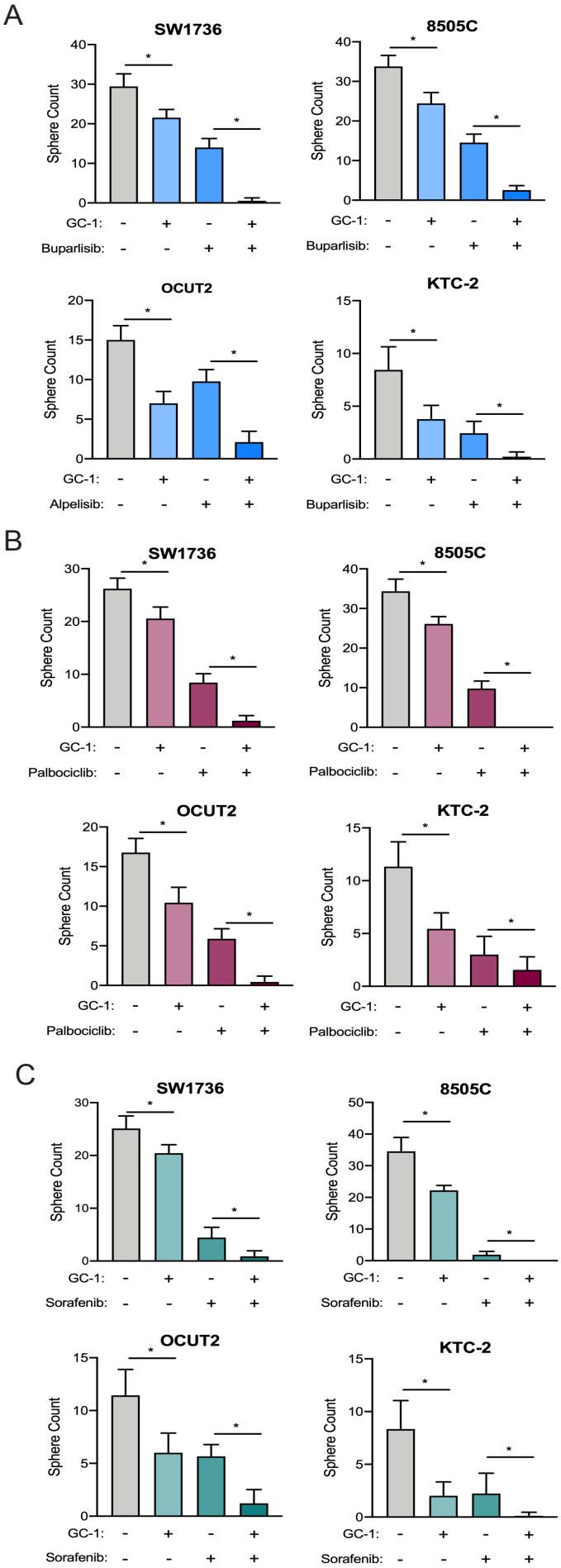


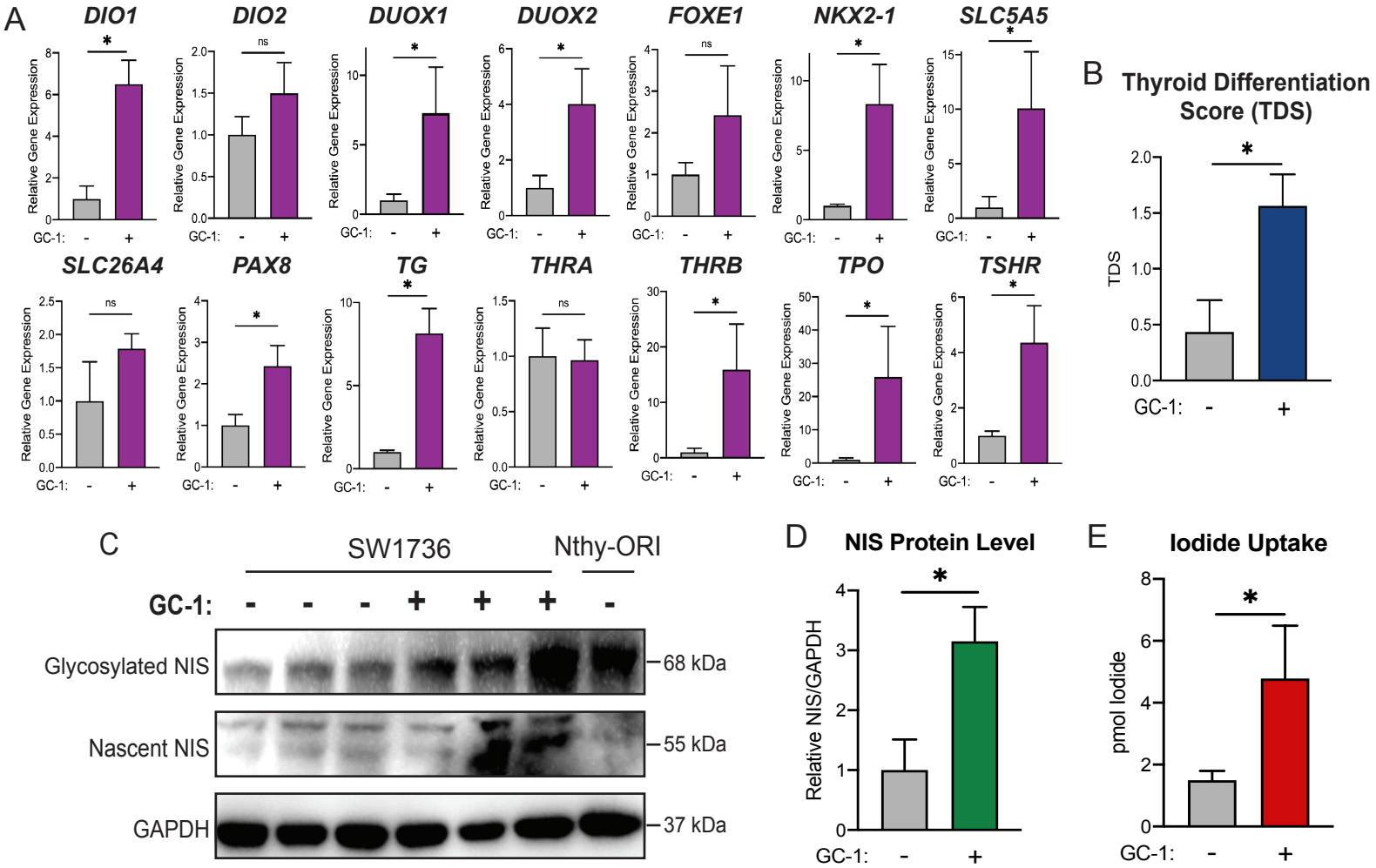





A
B
C
SW1736
8505C
OCUT2
KTC-2
Relative Cell Viability
GC-1:
[Buparlisib]:
0μM
0.1μM
0.5μM
1μM
[Alpelisib]:
[Palbociclib]:
0nM
0.5nM
1nM
5nM
[Sorafenib]:
5μM
10μM
25μM


A
Day 0
Day 1
Day 2
vehicle
GC-1
B
SW1736
AUC Units
0
25
50
75
100
GC-1:
-
+
Vehicle
Buparlisib
Palbociclib
Sorafenib
C
8505C
D
OCUT2
Alpelisib
E
KTC-2


A
SW1736
8505C
OCUT2
KTC-2
Sphere Count
GC-1:
Buparlisib:
Alpelisib:
B
Palbociclib:
C
Sorafenib:


A
DIO1
DIO2
DUOX1
DUOX2
FOXE1
NKX2-1
SLC5A5
SLC26A4
PAX8
TG
THRA
THRB
TPO
TSHR
Relative Gene Expression
GC-1: - +
ns
*
B
Thyroid Differentiation Score (TDS)
TDS
C
SW1736
Nthy-ORI
GC-1: - - - + + + -
Glycosylated NIS
Nascent NIS
GAPDH
68 kDa
55 kDa
37 kDa
D
NIS Protein Level
Relative NIS/GAPDH
E
Iodide Uptake
pmol Iodide