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Effect of Combined Epigenetic Treatments and Ectopic NIS Expression on Undifferentiated Thyroid Cancer Cells

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Abstract. *Background: Poorly differentiated (PDTC) and anaplastic thyroid (ATC) cancer cells are characterized by the acquisition of epigenetic abnormalities, leading to the silencing of both the sodium iodide co-transporter and the Coxsackie adenovirus receptor. As aberrant histone acetylation and DNA methylation represent epigenetic mechanisms involved in neoplastic transformation, our study investigated the anticancer properties of epigenetic modifiers in thyroid carcinoma. Materials and Methods: The cytotoxicity and gene expression modulation of histone deacetylase and DNA methyltransferase inhibitors were evaluated in both PDTC and ATC. Results: Epigenetic treatments were cytotoxic to tumor thyrocytes and restored sodium iodide co-transporter and Coxsackie adenovirus receptor, expression as well as radioiodine uptake, in PDTC but not in ATC. However, ectopic expression sodium iodide co-transporter re-activated radioiodine incorporation in ATC. Conclusion: The ability of epigenetic treatments to interfere with tumor proliferation and induce Coxsackie adenovirus receptor expression, coupled with the ability of ectopic sodium iodide co-transporter to restore radioiodine uptake, raise the possibility that these therapeutic approaches may provide clinical benefit to patients with thyroid carcinoma refractory to radioiodine treatment.*

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Thyroid cancer is the most frequent form of endocrine malignancy, accounting for about 1.7% of all human cancer in the US. Neoplastic transformation of the follicular epithelium can give rise to differentiated (DTC), poorly differentiated (PDTC) or anaplastic (ATC) thyroid carcinomas with an aggressive clinical behavior associated with a dismal prognosis (1, 2). Conventional therapy for DTCs and PDTCs consists of total thyroidectomy and radioiodine (^{131}I) therapy driven by expression of the sodium iodide co-transporter (solute carrier family 5 member 5, SLC5A5; NIS), with >95% survival rates at 5 years. However, 15-20% of PDTCs and all ATCs display loss of NIS expression, compromising their ability to concentrate radioiodine. Hence, the therapeutic options for these patients become limited and largely unsuccessful (3, 4). Increasing knowledge of the molecular alterations underlying thyroid carcinogenesis has led to the identification of several genetic events contributing to neoplastic transformation such as activating mutations in V-raf murine sarcoma viral oncogene homolog B1 (*BRAF*), V-Ha-ras Harvey rat sarcoma viral oncogene homolog (*HRAS*), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and neuroblastoma RAS viral (v-ras) oncogene homolog (*NRAS*); rearranged during transfection/papillary thyroid cancer (*RET*/*PTC*); and additional alterations involving genes of the p53 family and interferon regulatory factor 5 (*IRF5*) (1, 5-8). While each of these genes represents a potential therapeutic target, to date only anti-BRAF-directed drugs have been approved for this aggressive disease (4, 9).

In the search for novel therapeutic strategies, several reports have focused on the role of epigenetic modifications

Table I. *Primers used in reverse transcriptase-polymerase chain reaction in this study.*

Gene	Encoded protein	Forward	Reverse
NIS	Sodium iodide co-transporter	GGCGTCGCTCCTGTCCAC	CGCCCACAAGCATGACAC
CAR	Coxsackie adenovirus receptor	GCCTTCAGGTGCGAGATGTTAC	TCGCACCCATTCGACTTAGA
NCOA4	Nuclear receptor coactivator 4	TGGAGAAGAGAGGCTGTATCT	ATTGAAGAAATTGCAGGCTC

in thyroid tumorigenesis. Indeed, aberrant DNA methylation, improper chromatin remodeling and microRNA-dependent silencing of phosphatase and tensin homolog (*PTEN*), ras-related protein (*RAPB*), paired box 8 (*PAX8*) and *NIS* have been frequently reported in PDTCs and ATCs (10, 11). With the advent of an ever-increasing number of epigenetic modulators, targeting the epigenetic machinery is considered a potential therapeutic approach in cancer treatment (12-14) including undifferentiated thyroid carcinomas (15). Likewise, the use of appropriately engineered viral vectors to perform gene therapy approaches has become an additional therapeutic possibility for these patients (16). To this end, recombinant adenoviral vectors have been preferred to other viral gene delivery systems as they offer multiple advantages in terms of safety (lack of genotoxicity), high gene expression and successful clinical use for suicide gene therapy strategies (17-19). However, many PDTCs and all ATCs exhibit a progressive loss in cell differentiation promoted by epigenetic alterations or by constitutive V-raf murine sarcoma viral oncogene homolog B1(RAF)/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated protein kinases (ERK) activation that leads to a deficit in expression of the Coxsackie and adenovirus receptor (CAR), the high affinity receptor required for effective adenoviral-mediated gene therapy (10, 20-23). We therefore employed two epigenetic modifiers valproic acid (VPA) and 5-azacytidine (5-AC) to establish whether these drugs would lead to the re-expression of both NIS and CAR, thereby resensitizing PDT and ATC cells to radioiodine therapy.

Materials and Methods

Cell lines and treatment. Human immortalized poorly differentiated NPA and FTC-133, and anaplastic C-643 and SW-1736 thyroid carcinoma cell lines (DSMZ; Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures) were used in this study. NPA cells were grown in RPMI-1640 medium; C-643 and SW-1736 in minimum essential medium, while FTC-133 were kept in Dulbecco's modified Eagle's medium-F12 (DMEM-F12). All media were supplemented with 2 mM glutamine, 100 U/mI penicillin and 100 μg/mI streptomycin (all from Sigma-Aldrich, St. Louis, MO USA) and 10% heat-inactivated fetal bovine serum (FBS) (EuroClone, Milan, Italy). The FRTL-5 cell line derived from normal thyroid glands of 5-to 6-week-old Fischer rats was cultivated in DMEM-F12. All cells were maintained in a humidified 5% CO_2 incubator at 37˚C. In all experiments performed with drug treatment, the cells were either left untreated or exposed to VPA (3 mM) alone, 5-AC (1 μM) (all from Sigma-Aldrich) alone, or treated with a combination of the two drugs as specified in Figure 1A. Fresh culture medium with the drugs was replaced every 24 hours.

Primary cultures. Normal thyrocytes were derived from tissues obtained at surgery with the informed consent of each donor. Primary cultures were obtained as previously described (24).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted by Trizol reagent from each cell line and reverse-transcribed with Superscript III according to the manufacturers protocol. RT-PCR reactions were performed using Pfx platinum polymerase (Invitrogen, Paisley, UK) using the primers given in Table I.

Cell proliferation, cell death and cell-cycle analyses. Thyroid cells were incubated in 96-well plates and their proliferation rates determined by the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, WI, USA). Apoptosis was measured staining the cells with Annexin V-fluorescein isothiocyanate (FITC) (BD Pharmigen, Milan, Italy), while cell-cycle analyses were performed as previously reported (25).

Migration assays. Migration assays were performed as previously described (26, 27).

Plasmid constructs and cell transfection. Full-length *NIS* cDNA (kindly provided by Dr. S. M. Jhiang, Ohio State University) was removed from the pcDNA3 expression vector and ligated in the *p3XFLAG-CMV-14* vector (Sigma-Aldrich) using *Eco*RI as a unique restriction site. pNIS-3XFLAG was employed for transient transfection into C-643 and SW-1736 cell lines using Lipofectamine 2000 (Invitrogen).

Isolation of plasma membrane protein and immunoblot analysis. C-643 and SW-1736 cell lines, transfected with empty vector or with a plasmid expressing pNIS-3xFLAG, were used to obtain plasma membrane samples using the Qproteome plasma membrane protein kit (Qiagen, KJ Venlo, the Netherlands). Protein extraction and transfer to appropriate blotting membranes were performed as previously described (28). Nitrocellulose membranes were then hybridized with anti-FLAG M2, anti-actin (both from Sigma-Aldrich), anti-CAR, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-Na/K-ATPase (all from Santa Cruz Biotechnology, Dallas, TX, USA). Appropriate horseradish peroxidase-conjugated secondary antibodies were added (Amersham, GE Healthcare, Chicago, IL, USA) and proteins were visualized using the LiteAblot enhanced chemi-luminescence reagent (EuroClone).

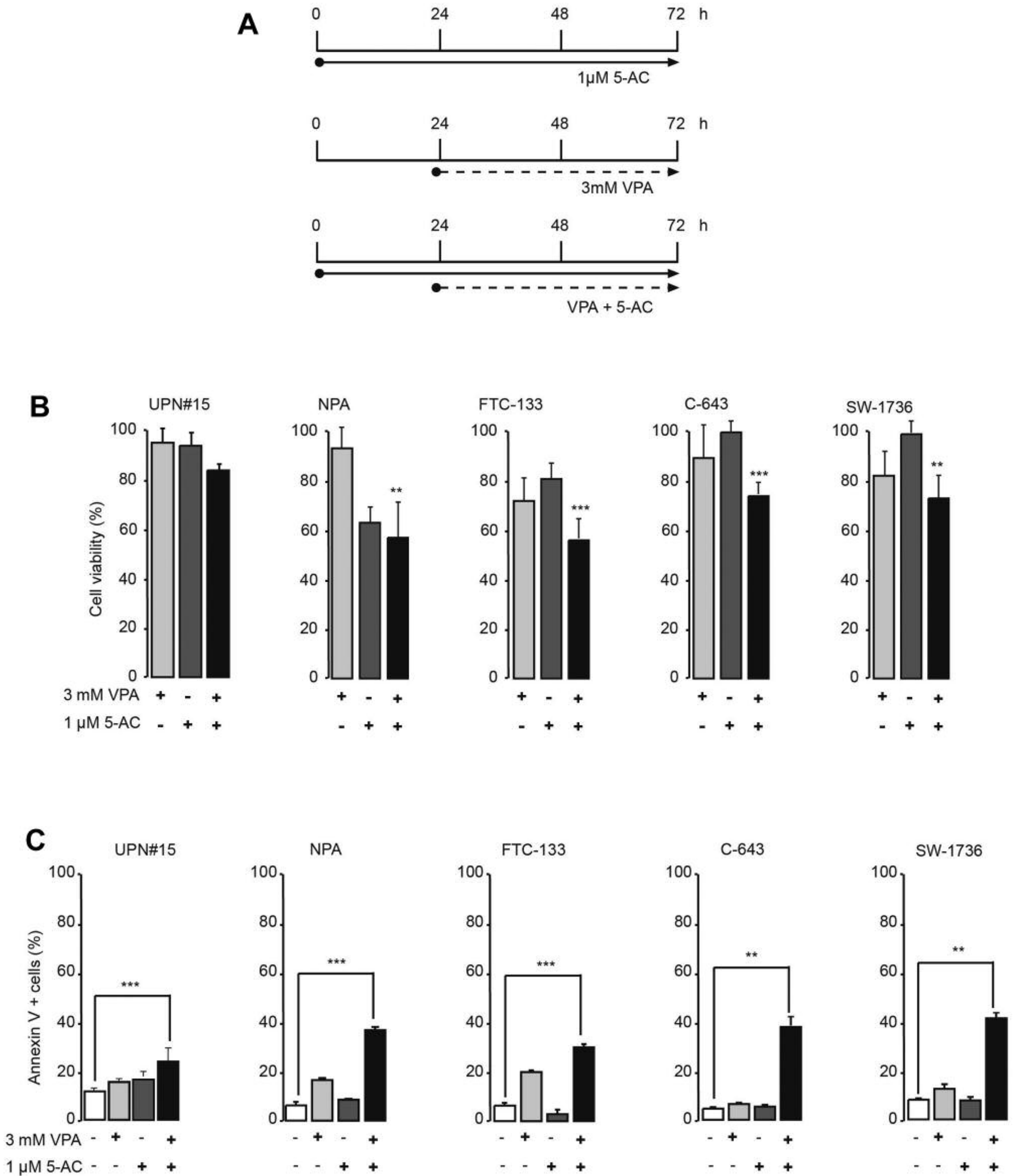

Figure 1. *The sequential combination of 5-azacytidine (5-AC) and valproic acid (VPA) had cytotoxic effects on thyroid cancer cells and tolerable toxicity in normal thyrocytes. A: Schematic representation of the VPA and 5-AC combination. B: The indicated cell lines or normal thyrocytes (UPN#15) were exposed to VPA or 5-AC alone or in combination as reported in (A). B: Histograms show proliferation evaluated by MTS assay compared to the untreated condition arbitrarily set at 100%. C: Thyroid cancer cell lines and primary thyrocytes (UPN#15) were left untreated or exposed to VPA and 5-AC, alone and in combination, and then subjected to annexin-V staining. Histograms show the percentage of annexin-V-positive apoptotic cells as analyzed by flow cytometry. In (B) and (C) bars indicate standard deviation derived from three (NPA, FTC-133, C-643, SW-1736) or two (UPN#15) independent experiments. Significantly different at **p<0.01 and ***p<0.001.*

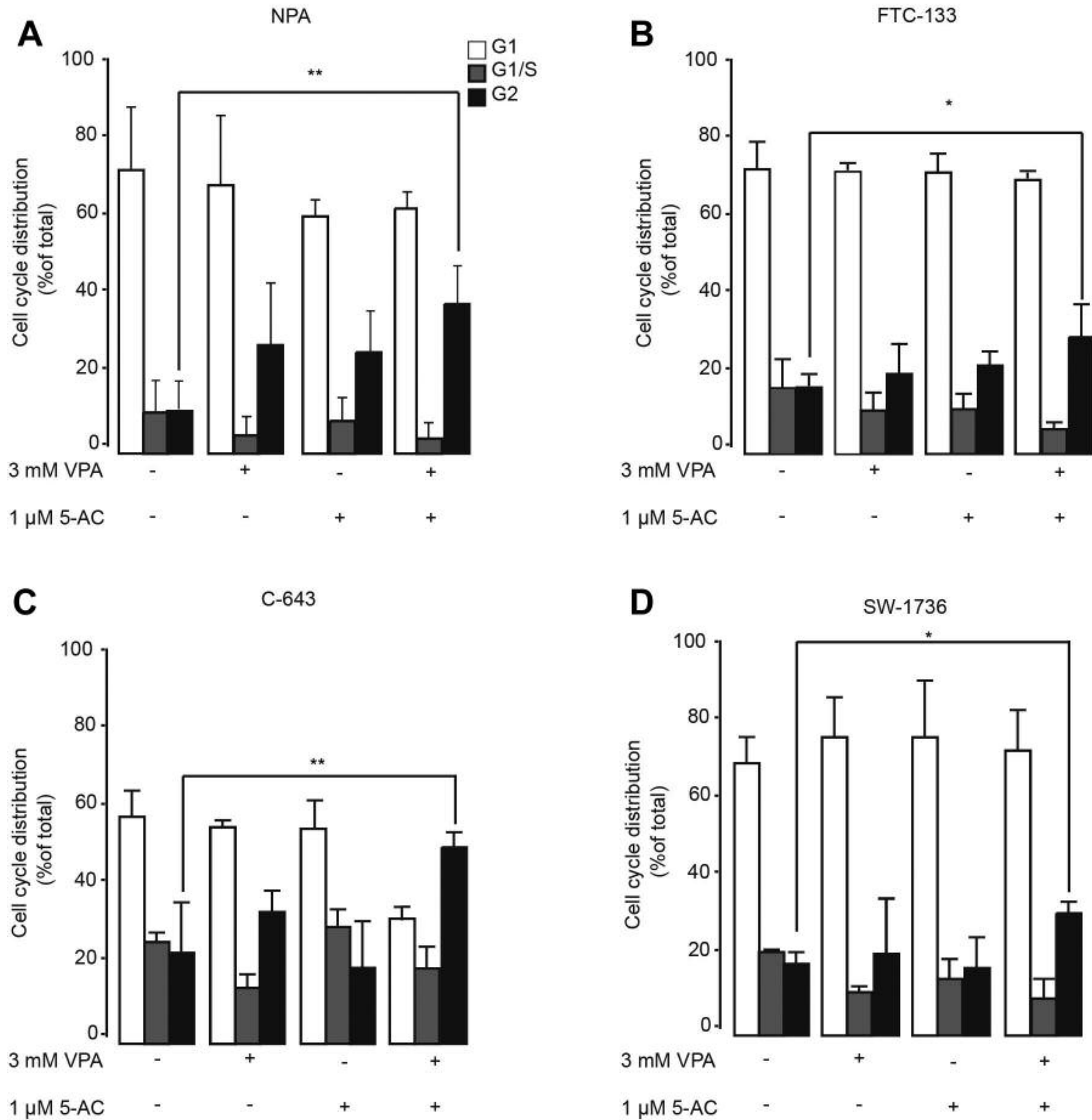

Figure 2. *Exposure to 5-azacytidine (5-AC) and valproic acid (VPA) arrested thyroid cancer cells in the G_2/M phase of the cell cycle. Cancer cells were left untreated or incubated with VPA and 5-AC as reported in Figure 1A and then stained with propidium iodide. Histograms, gated for subG_0-G_1 populations in order to normalize for treatment-induced cell death, show the cell-cycle distribution of thyroid cancer cells as analyzed by flow cytometry. Bars indicate the standard deviation obtained from three independent experiments. Significantly different at *p<0.05 and **p<0.01.*

Iodine uptake. Uptake of ^{125}I by cancer cells NPA, FTC-133, C643, SW-1736 or FTRL-5 in each experimental condition was measured as previously described (29) with the following modifications. Briefly, after exposure to drugs, as indicated in the Materials and Methods, cells were split and seeded onto 12-well plates. The culture medium (with or without drugs) was aspirated, and cells were washed with 1 mI of Hank's balanced salt solution (HBSS) (Life Technologies) supplemented with HEPES 10 mM (pH 7.3). To each well, 500 µl of buffered HBSS containing 0.1 µCi carrier-free Na^{125}I and 10 µm NaI (specific activity, 20 mCi/mmol) were added. In half of the wells, the buffer also contained the NIS inhibitor, $KClO_4$ (100 µm), as a control for non-specific uptake. After a 40-min incubation at 37˚C in a humidified atmosphere, the radioactive medium was aspirated, and cells were washed with 1 mI of ice-cold HBSS. One milliliter of 95% ethanol was added to each well for 20 min, and then transferred into vials for gamma-counting to measure the amount of ^{125}I associated with the cells. Each experiment was performed at least twice in quadruplicates, and rat FRTL-5 cells were included as a positive control. Radioactivity was then normalized to total cell number.

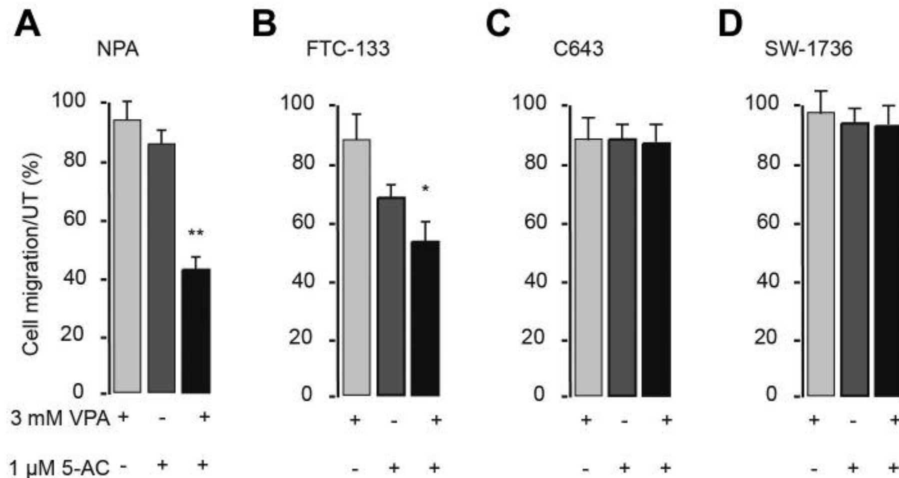

Figure 3. *Exposure to 5-azacytidine (5-AC) and valproic acid (VPA) reduced the invasiveness of poorly differentiated carcinoma cell lines. Thyroid cell lines were subjected to an invasion assay that measured their ability to cross an 8 μm transwell in the presence or absence of VPA/5-AC. Histograms indicate the percentage of migrated cells compared to the untreated condition arbitrarily set at 100%. Bars represent the standard deviation derived from three independent experiments. Significantly different at *p<0.05 and **p<0.01.*

Statistical analysis. Unpaired, single-tailed *t*-tests with 95% confidence intervals were used to perform statistical analysis employing PRISM software (GraphPad Software Inc.) with significance accepted at $p<0.05$.

Results

The sequential combination of VPA and 5-AC had cytotoxic effects on thyroid carcinoma cells. To assess the anti-proliferative effects of epigenetic modulators on thyroid cancer cells and normal thyrocytes, cells were exposed to 3 mM VPA (30) and 1 μM 5-AC (31) alone, or in a sequential combination as summarized in Figure 1A. VPA and 5-AC as single agents only reduced the proliferative rate of NPA and FTC-133 cells. On the contrary, we observed that the combination significantly impaired the proliferative rates of the entire panel of thyroid carcinoma cells. Interestingly, the proliferative ability of primary healthy thyrocytes (UPN#15) was not significantly affected (Figure 1B). Annexin-V staining showed that VPA and 5-AC alone failed to induce significant apoptosis of NPA, FTC-133, C-643 and SW-1736 cancer cells, while their combination increased the apoptotic rate of these cells by 3-4-fold. Lower, albeit significant, cytotoxicity was also observed in primary normal thyrocytes (UPN#15) (Figure 1C).

Combination VPA and 5-AC arrested thyroid cancer cells in the G_2/M phase of the cell cycle. Epigenetic modifications alter cell-cycle distribution thus influencing cell fate (32). We therefore wanted to evaluate if thyroid cancer cells surviving VPA/5-AC treatment were arrested in a specific phase of the cell cycle. To this end, after exposure to VPA with/without 5-AC, we analyzed their cell cycle distribution. After treatment with the combination of VPA and 5-AC, all thyroid cancer lines displayed a significant increase in the population cells in the G_2/M phase of the cell cycle (Figure 2).

Combined epigenetic treatment reduced metastatic potential in PDTC cells. Previous studies have suggested that epigenetic modifications influence tumor cell motility and invasion (33-35). We therefore examined the effect of VPA and 5-AC on the invasive phenotype of poorly differentiated and ATC cells by measuring their ability to migrate across an 8 μm trans-well. Poorly differentiated NPA and FTC-133 cells were sensitive to effect of VPA combined with 5-AC as they displayed a 40-50% reduction in their motility (Figure 3A and B). On the contrary, VPA and 5-AC failed to exert any significant effect on the invasive ability of ATC cells (Figure 3C and D).

Combination of VPA and 5-AC restored NIS expression and radioiodine uptake in PDTC cells. Fifteen to 20% of poorly differentiated thyroid cancer cells and all anaplastic thyroid carcinomas display a complete deficit of NIS expression, thereby resulting in their being unresponsive to radio-metabolic therapy (4). Previous evidence has indicated that histone deacetylase inhibition (36) and *BRAF* or *p53* mutational status influence *NIS* transcription (37, 38). Our cell lines display a wide combination of *BRAF* and *p53* alterations as NPA has mutations in both oncogenes (39, 40), FTC-133 and C-643 only carry a *p53* mutation (41, 42), and SW1736 is *p53*-null but expresses wild-type *BRAF* (43). In order to establish the *NIS* expression pattern of our thyroid

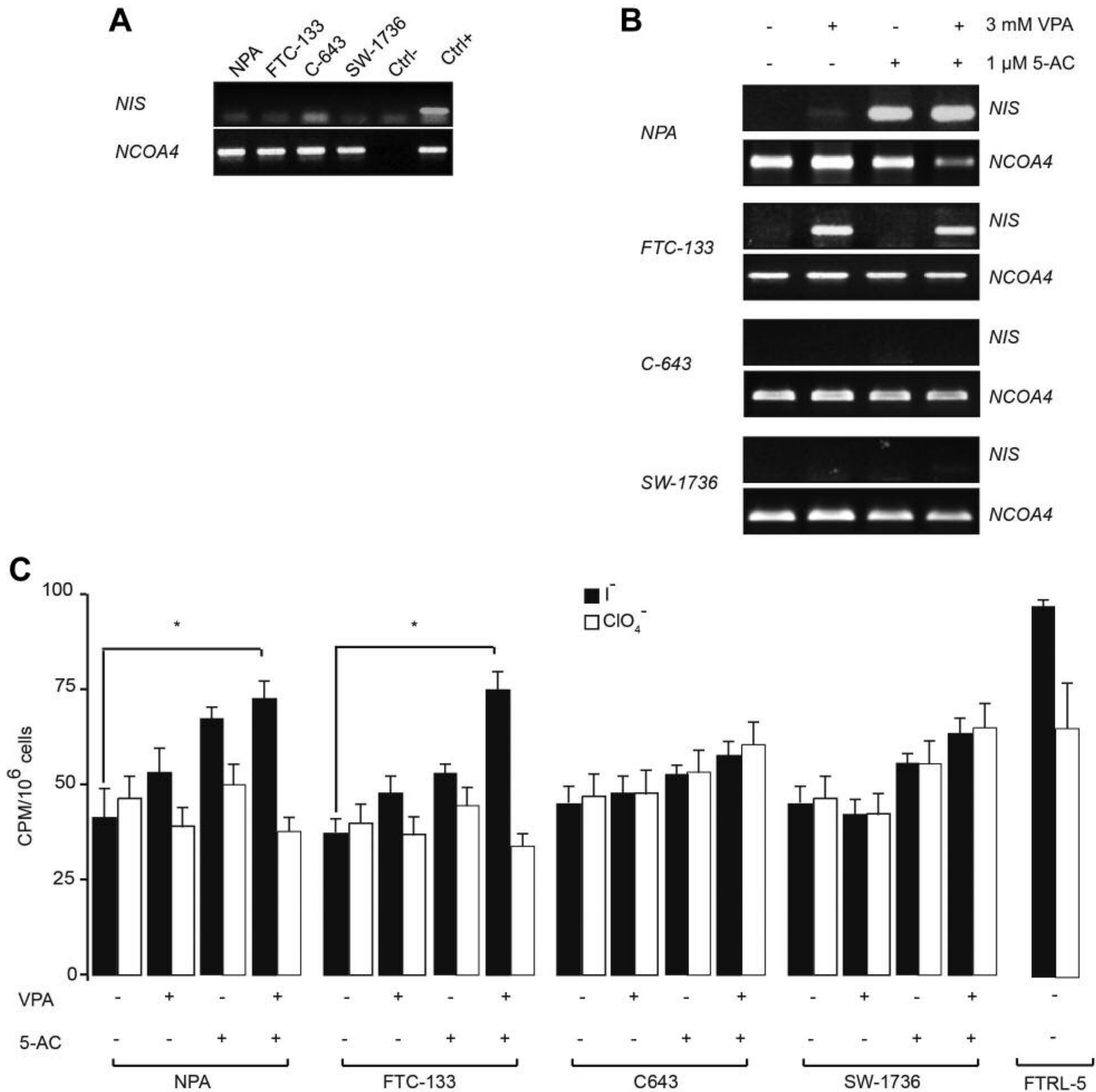

Figure 4. *5-azacytidine (5-AC) and valproc acid (VPA) restored sodium iodide co-transporter (NIS) expression and iodide uptake in poorly differentiated thyroid cells. A: Total RNA was extracted from the indicated untreated thyroid cancer lines and then subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) for NIS gene amplification. Ctrl-and Ctrl+ represent negative and positive controls, respectively. B: The specified thyroid cancer cells were exposed to VPA/5-AC as per Figure 1A then total RNA was extracted subjected to NIS amplification by RT-PCR. C: Thyroid cancer cells were exposed to VPA/5-AC and then a radioiodine up-take assay was performed. Histograms present the counts per minute (CPM) dependent on the amount of 125I incorporated by cells. Nuclear receptor coactivator 4 (NCOA4) was used as loading control. Bars indicate the standard deviation derived from three independent experiments. FTRL-5 cells were used as a positive control and ClO_4^- to measure non-specific binding. Significantly different at *$p<0.05$.*

cancer panel, total RNA was extracted, reverse transcribed into cDNA and amplified by PCR. No *NIS* transcripts were detected in any of the analyzed cells (Figure 4A). We next exposed these lines to VPA and 5-AC and repeated the anaIysis, observing that these treatments restored *NIS* expression in NPA and FTC-133 cells but not in the anaplastic lines C-643 and SW-1736 (Figure 4B). In detail, VPA had no effect on *NIS* transcription in NPA cells, unlike 5-AC that successfully restored NIS expression both as a single agent and in combination with VPA. The opposite was

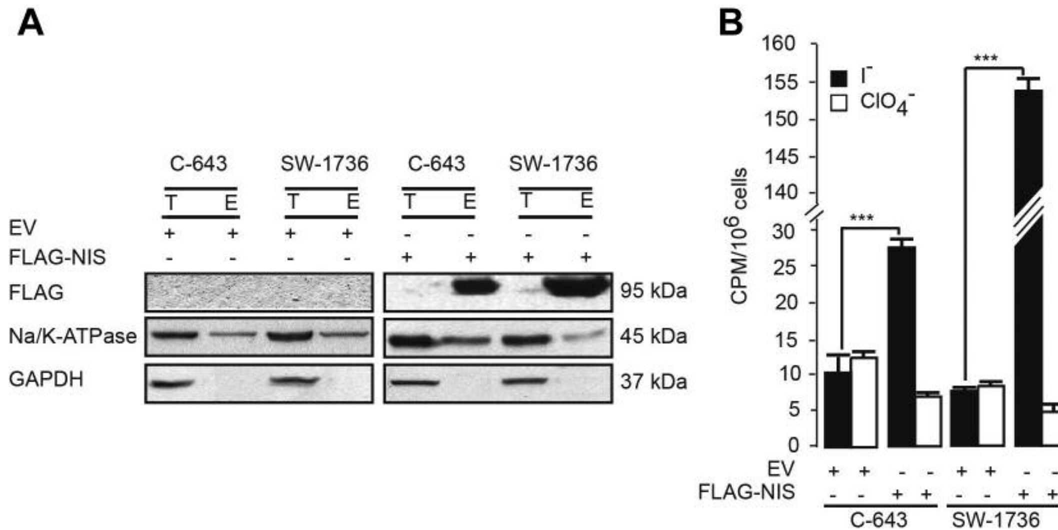

Figure 5. *Ectopic sodium iodide co-transporter (NIS) expression restored radioiodine up-take in undifferentiated thyroid cancer cells. A: C-643 and SW-1736 cells were transiently transfected with empty vector (EV) or 3xFLAG-NIS and then subjected to plasma/membrane protein purification. To confirm 3xFLAG-NIS expression, total cell lysates (T) and the eluted fraction (E) were separated by sodium dodecyl sulphate - polyacrylamide gel electrophoresis and then membranes were hybridized with the indicated antibodies. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Na/K-ATPase were used as a control to confirm correct plasma/membrane protein separation. B: The same cell lines were used to measure ^{125}I uptake in the presence of ClO_4^- to obtain non-specific radioiodine uptake/binding. Histograms represent counts per minute (CPM) dependent on the amount of ^{125}I incorporated by cells. Bars indicate the standard deviation of three independent experiments. Significantly different at ***p<0.001.*

true in FTC-133 cells, suggesting that different epigenetic aberrations are responsible for *NIS* silencing in different thyroid cancer cell lines.

We next wanted to investigate whether *NIS* transcription generated a functional protein capable of radioiodine uptake. We therefore performed iodine uptake assays and found that NPA and FTC-133 cells expressing *NIS* after treatment with both VPA and 5-AC incorporated significant amounts of radioiodine (Figure 4C), demonstrating that drugs targeting a broad spectrum of epigenetic substrates can successfully restore *NIS* expression and function in PDTC cells.

Ectopic NIS expression restored radioiodine uptake in ATC cells. Failure to restore NIS expression in ATC cells after exposure to VPA and 5-AC might be due to microRNA-dependent gene silencing that will not respond to epigenetic modifiers (44). However, even if restoring NIS expression is successful, it is unknown whether ATC cells preserve the molecular machinery required for proper NIS function. We therefore transiently transfected C-643 and SW-1736 cells with a vector coding for a 3xFLAG-NIS and observed both FLAG-NIS protein expression and correct migration to the membrane (Figure 5A). Accordingly, the transfected cells were able to incorporate ^{125}I in radioiodine uptake assays (Figure 5B).

Epigenetic modification induced CAR expression in PDTC and ATC cells. The CAR is a high affinity receptor for adenovirus and previous evidence suggests that its expression is regulated by epigenetic mechanisms (20, 23). Considering the emerging interest in adenovirus-based genetic therapy in cancer (16-19), we evaluated CAR expression before and after thyroid cancer cell exposure to VPA/5-AC. Surprisingly, these epigenetic modifiers induced CAR mRNA re-expression (Figure 6A), with immunoblots confirming the protein translation of the *CAR* transcripts detected in NPA, FTC-133, C-643 and SW-1736 cells exposed to VPA/5-AC (Figure 6B).

Discussion

The concept of precision medicine postulates that effective treatment strategies should be tailored to the distinct variability of both the patient and their disease, thus identifying personalized pharmacological approaches for each individual (45). Indeed, most patients with thyroid cancer diagnosed with well-differentiated thyroid carcinoma undergo surgery and, if needed, ‘targeted’ radioiodine therapy, thus achieving high overall survival rates. However, this approach generates limited benefits for patients with PDTC or ATC. Therefore, additional treatment strategies are urgently needed for these aggressive tumors usually

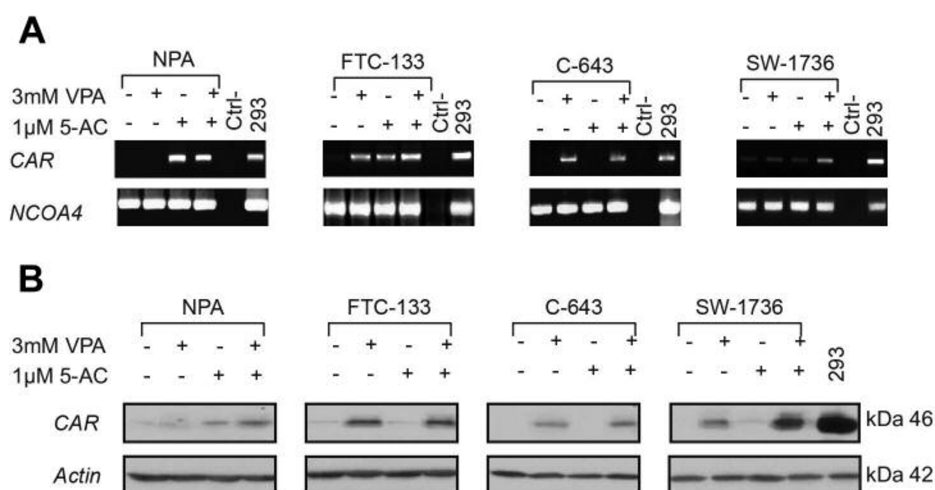

Figure 6. *Valproic acid (VPA) and 5-azacytidine (5-AC) restored Coxsackie adenovirus receptor (CAR) expression in thyroid cancer cells. A: The indicated thyroid cancer cell lines were exposed to the VPA and 5-AC combination and then used to perform reverse transcriptase-polymerase chain reaction for CAR and nuclear receptor coactivator 4 (NCOA4) (loading control). Ctrl: Negative control. B: Protein lysates from the same cells were used to assess CAR expression by immunoblot. Actin and the human embryonic kidney 293 cell line were used as loading and positive controls, respectively.*

associated with a dismal prognosis (4). In this study, we employed an epigenetic treatment for both immortalized and primary thyroid cancer cells representative of PDTCs and ATCs and analyzed its cytotoxic effects and its ability to modulate NIS and CAR expression. We demonstrate that VPA and 5-AC reduced the proliferative potential of thyroid cancer cells, blocking them in the G_2/M phase of the cell cycle and increasing their apoptotic rate. We also show that this drug combination effectively reduced the invasive ability of PDTC cells but faiIed to exert the same effect on ATCs, as in the latter biological context, multiple mechanisms are probably involved in the modulation of cell migration. Finally, we found that our pharmacological chromatin remodeling strategy successfully restored *NIS* gene expression and iodide uptake in PDTCs but not in ATCs. However, as VPA and 5-AC induce CAR re-expression in both PDTC and ATC, CAR re-activation raises the possibility that gene therapy may be employed to exogenously re-establish the NIS level in ATCs. In turn, since ATC cells maintained their ability to locate the NIS protein in the cell membrane, restoring NIS expression may allow the use of radiometabolic therapy for this lethal disease.

In summary, our findings suggest that the use of epigenetic modifiers may represent a useful pharmacological approach for PDTC and ATC as confirmed by several clinical trials demonstrating the antitumor efficacy of different HDCA inhibitors, 5-AC (NCT00134043), (NCT01013597), (NCT00004062) and recombinant adenoviral vectors to induce specific gene expression (NCT00902122) (46, 47). Furthermore, given the well-known safety profile of these drugs and their minimal toxicity to normal thyrocytes, it is conceivable that this strategy may be well tolerated in the clinical setting.

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