Induction of Thyroid Gene Expression and Radioiodine Uptake in Thyroid Cancer Cells by Targeting Major Signaling Pathways

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Context: Radioiodine ablation is commonly used to treat thyroid cancer, but a major challenge is often the loss of radioiodine avidity of the cancer caused by aberrant silencing of iodide-handling genes.

Objectives: This study was conducted to test the therapeutic potential of targeting the aberrantly activated MAPK and PI3K/Akt/mTOR pathways and histone deacetylase to restore radioiodine avidity in thyroid cancer cells.

Experimental Design: We tested the effects of specific inhibitors targeting these pathways/molecules that had established clinical applicability, including the MAPK kinase inhibitor RDEA119, mTOR inhibitor temsirolimus, Akt inhibitor perifosine, and histone deacetylase inhibitor SAHA, individually or in combinations, on the expression of iodide-handling genes and radioiodide uptake in a large panel of thyroid cancer cell lines.

Results: The expression of a large number of iodide-handling genes could be restored, particularly the sodium/iodide symporter, TSH receptor, and thyroperoxidase, by treating cells with these inhibitors. The effect was particularly robust and synergistic when combinations of inhibitors containing SAHA were used. Robust expression of sodium/iodide symporter in the cell membrane, which plays the most important role in iodide uptake in thyroid cells, was confirmed by immunofluorescent microscopy. Radioiodide uptake by cells was correspondingly induced under these conditions. Thyroid gene expression and radioiodide uptake could both be further enhanced by TSH.

Conclusions: Targeting major signaling pathways could restore thyroid gene expression and radioiodide uptake in thyroid cancer cells. Further studies are warranted to test this therapeutic potential in restoring radioiodine avidity of thyroid cancer cells for effective ablation treatment. (*J Clin Endocrinol Metab* 95: 820–828, 2010)

Thyroid cancer is a common endocrine malignancy that has seen a nearly linear increase in incidence in recent decades (1–3). After thyroidectomy, radioiodine ablation therapy is commonly pursued as the mainstay of medical treatment for this cancer (4, 5). This radioiodine treatment takes advantage of the unique function of thyroid follicular cells to take up and concentrate iodide, a process that involves several key thyroid iodide-handling protein molecules, including sodium/iodide symporter (NIS), TSH receptor (TSHR), thyroperoxidase (TPO), thyroglobulin (Tg), and various thyroid transcription factors (6, 7). NIS is localized in the basal membrane of follicular thyroid cells and transports iodide from blood stream into the thyroid cell. TPO is involved in organification and incorporation of iodide into Tg for iodide accumulation and thyroid hormone synthesis in the thyroid gland. Several

Abbreviations: 7-AAD, 7-Amino-actinomycin D; HDAC, histone deacetylase; MEK, MAPK kinase; mTOR, mammalian target of rapamycin; NIS, sodium/iodide symporter; PI3K, phosphatidylinositol-3-kinase; Tg, thyroglobulin; TPO, thyroperoxidase; TSHR, TSH receptor; TTF, thyroid transcription factor.

thyroid transcription factors, including thyroid transcription factor 1 (TTF1 or TITF1) (8) and 2 (TTF2 or FOXE1) (9) and PAX8 (10) are involved in the regulation of thyroid genes. TSH, acting on TSHR, in thyroid cell membrane plays a central role in up-regulating these thyroid iodide-handling genes and iodide uptake in thyroid cells.

Thyroid cancer sometimes loses the ability to take up iodide, creating a major obstacle for radioiodine treatment to which there is currently no solution. The 10-yr survival rate of patients with nonradioiodine avid metastatic thyroid cancer is only 10% (11). In fact, thyroid cancer-caused death virtually always occurs in cases that have lost radioiodine avidity. The underlying molecular basis is aberrant silencing of iodide-handling genes. Numerous studies demonstrated impaired or lost expression of the genes for NIS, TSHR, TPO, Tg, and certain thyroid transcription factors (12–17). Interestingly, silencing of these genes is often associated with *BRAF* mutation in thyroid cancer, which promotes overactivation of the MAPK pathway (7, 11, 18–20). A recent study demonstrated that LY294002, an inhibitor of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, increased NIS expression in rat thyroid cells (21), suggesting that the PI3K/Akt pathway may also play a role in the regulation of thyroid iodide-handling genes. Inhibition of histone deacetylase (HDAC) was previously shown to induce expression of NIS (22–24), but many of the cell lines used in these studies are now known to be of nonthyroid origin (25). It therefore remains to be established whether targeting these major signaling pathways/molecules could restore expression of thyroid genes in thyroid cancer cells and hence may be therapeutically applicable in radioiodine treatment of thyroid cancer patients. In the present study, we used a large panel of authenticated thyroid cancer cell lines to test the therapeutic potential of targeting major signaling pathways to restore thyroid gene expression and radioiodide uptake using several clinically applicable inhibitors.

Materials and Methods

Human thyroid cancer cell lines

The thyroid cancer cell lines C643, Hth7, Hth74, and SW1736 were originally from Dr. N. E. Heldin (University of Uppsala, Uppsala, Sweden); KAT18 from Dr. Kenneth B. Ain (University of Kentucky Medical Center, Lexington, KY); OCUT-1 from Dr. Naoyoshi Onoda (Osaka City University Graduate School of Medicine, Osaka, Japan); BCPAP from Dr. Massimo Santoro (University of Federico II, Naples, Italy); K1 from Dr. David Wynford-Thomas (University of Wales College of Medicine, Cardiff, UK); WRO from Dr. G. J. F. Juillard (University of California-Los Angeles School of Medicine, Los Angeles, CA); and FTC133 from Dr. George Brabant (University of Manchester, Manchester, UK). The TPC1 cell line was provided by Dr. Alan P. Dackiw (The Johns Hopkins University,

Baltimore, MD). These cancer cells have been recently authenticated to be unique thyroid cancer cell lines (25), except for the OCUT1 cell that has been used in other recent thyroid cancer studies (26, 27). Their tumor origins and genetic alterations are presented in Supplemental Table 1 (published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). All these cells were grown at 37 C in RPMI 1640 medium with 10% fetal bovine serum, except for FTC133, which was cultured with DMEM/F-12 medium. In some experiments, cells were treated with the MAPK kinase (MEK) inhibitor RDEA119, the mammalian target of rapamycin (mTOR) inhibitor temsirolimus (CCI779), the Akt inhibitor perifosine, and the HDAC inhibitor SAHA with the indicated concentrations for 30 h, individually or in different combinations. Dimethylsulfoxide or PBS was used as the vehicle control. Cells were additionally treated with 20 mU/ml bovine TSH (Sigma, St. Louis, MO) in some experiments as indicated. RDEA119 was obtained from Ardea Biosciences, Inc. (San Diego, CA). Temsirolimus, perifosine, and SAHA (Vorinostat) were obtained from Cayman Chemical (Ann Arbor, MI).

Western blotting assay

Cells were lysed in RIPA buffer. Cellular proteins were collected by centrifugation at 4 C for 20 min, subjected to 10% SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ), and membranes were probed with the indicated primary antibodies. Anti-phospho-ERK (sc-7383), anti-ERK1 (sc-94), anti-phospho-Akt (sc-7985-R), anti-phospho-p70S6 kinase (p70S6K) (sc-230), anti-Ac-histone (sc-8655-R), and anti-Actin (sc-1616-R) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antigen-antibody complexes were visualized using horseradish peroxidase-conjugated antimouse (sc-2005; Santa Cruz Biotechnology) or antirabbit (sc-2004; Santa Cruz Biotechnology), IgG antibodies, and the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech).

RNA extraction and real-time quantitative RT-PCR analysis

Total RNA was isolated from cells using TRIzol reagent according to the instructions of the manufacturer (Invitrogen, San Diego, CA). Total RNA (2 μg) was converted to cDNA on an iCycler Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using Oligo-dT and SuperScript II according to the instructions of the manufacturer (SuperScript First-Strand Synthesis kit; Invitrogen). Real-time quantitative RT-PCR analysis was performed to evaluate the expression of thyroid genes on an ABI Prism 7900HT Sequence Detector (Applied Biosystems, Foster City, CA), using SYBR GreenER qPCR SuperMix according to the instructions of the manufacturer (Applied Biosystems). Primer pairs of thyroid-specific genes, *NIS*, *TSHR*, *TPO*, *Tg*, *PAX8*, *FOXE1*, and *TTF1* were designed using Primer Express (Applied Biosystems). PCR primer pairs were designed to span a large intron, and the sequences are presented in Supplemental Table 2. The expression value of each gene was normalized to β-actin cDNA to calculate the relative amount of RNA present in each sample according to the $2^{-\Delta\Delta Ct}$ method (28). The specificity of real-time quantitative PCR for all these genes was confirmed by running the PCR products on a 1.5% agarose gel to show single specific bands at the expected sizes and by sequencing (data not shown). Each sample was run in triplicate.
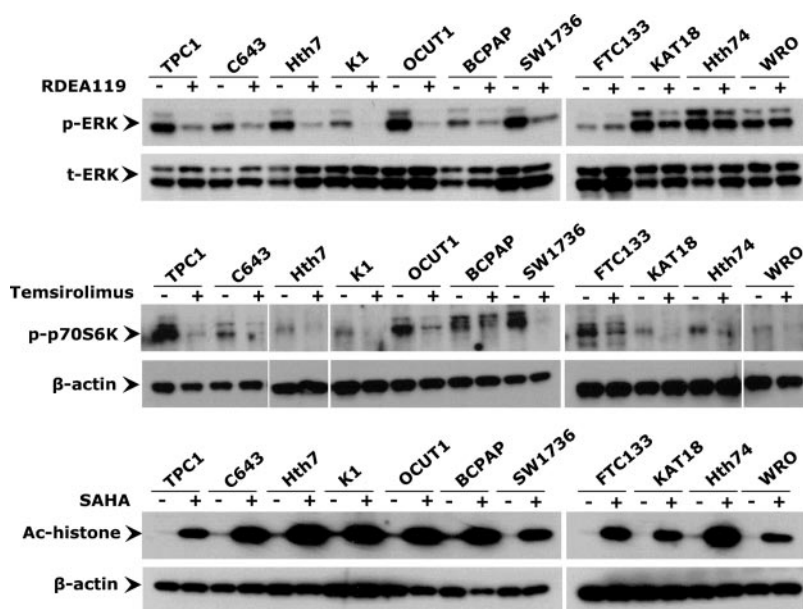
FIG. 1. Effects of various inhibitors on the MAPK pathway, PI3K/Akt/mTOR pathway, and HDAC in thyroid cancer cells. Inhibition of ERK and p70S6K phosphorylation and histone deacetylation was achieved using the MEK-specific inhibitor RDEA119, mTOR-specific inhibitor temsirolimus (CCI779), and HDAC-specific inhibitor SAHA, respectively. Thyroid cancer cells were treated with 0.5 μM RDEA119, 10 nM temsirolimus, or 5 μM SAHA for 30 h. Dimethylsulfoxide or PBS was used as the vehicle control. After treatments, cells were lysed for Western blotting analyses. The effect of inhibition is reflected by the level of phosphorylated ERK and p70S6K and acetylated histone detected with specific anti-phosphorylated ERK (p-ERK), anti-phosphorylated p70S6K (p-p70S6K), and anti-acetylated histone H3 (Ac-histone) antibodies. Immunoblotting with antibodies against t-ERK or β-actin was used for quality control.

Immunofluorescent localization of NIS

Cells treated with specific inhibitors, individually or in different combinations, to induce the expression of NIS were incubated with VJ2 α-hNIS mAb (a gift from Dr. Sabine Costagliola at the Free University of Brussels) (29), and diluted at 1:20 in FACS buffer (3% fetal bovine serum, 0.02% NaN$_3$ in PBS) at 4 C for 40 min. Cells were then washed twice with FACS buffer and incubated with fluorescein isothiocyanate-conjugated α-mouse IgG (Sigma) diluted at 1:100 in FACS buffer at 4 C for 40 min. Cells were washed again in FACS buffer, and then resuspended in 500 μl of FACS buffer with 7-amino-actinomycin D (7-AAD). Fluorescent microscopic examination was conducted to monitor NIS expression (Nikon Corporation, Tokyo, Japan).

Radioiodide uptake assay

Thyroid cancer cells (5×10^5 cells per well) were seeded in 12-well plates and treated with the indicated inhibitors for 30 h. After treatment with inhibitors, one well was counted for cell number for each group, and the remaining wells were incubated with 1 ml serum-free RPMI 1640 medium containing 1 μCi Na^{125}I in 5 μM nonradioactive NaI for 1 h at 37 C with 5% CO$_2$. To determine NIS-specific uptake, a group of wells were preincubated with NaClO$_4$ at 300 μM for 30 min before the start of incubation with Na^{125}I. The medium was subsequently aspirated, and cells were quickly washed twice with ice-cold Hank's balanced salt solution. Cells were trypsinized with 200 μl of 0.25% trypsin and, once lifted, were lysed with 500 μl of NaOH (0.33 M) containing 1% sodium dodecyl sulfate. Cells were col-

lected, and radioactivity was counted by a gamma-counter. Experiments were performed in triplicate.

Statistical analysis

All the experiments were similarly performed at least two or three times. Most of the measurements were conducted in triplicate and some in duplicate. Data were compared using the *t* test. Significance was defined as $P < 0.05$. Unless indicated, data shown in the figures are representatives.

Results

Induction of the expression of thyroid iodide-handling genes in thyroid cancer cells by suppressing the MAPK pathway, PI3K/Akt/mTOR pathway, and HDAC

We tested the effects of the MEK inhibitor RDEA119, the mTOR inhibitor temsirolimus, and the HDAC inhibitor SAHA on 11 thyroid cancer cell lines, including TPC1, C643, Hth7, K1, OCUT1, BCPAP, SW1736, FTC133, KAT18, Hth74, and WRO cells. As shown in Fig. 1, treatment of cells with RDEA119 at 0.5 μM for 30 h strongly inhibited phosphorylation of ERK (p-ERK) in most thyroid cancer cells, particularly TPC1, C643, Hth7, K1, OCUT1, BCPAP, and SW1736 cells that harbored genetic alterations in the MAPK pathway, including RET/PTC, *Ras* mutation and *BRAF* mutation. In thyroid cancer cell lines FTC133, KAT18, Hth74, and WRO cells that did not harbor these genetic alterations, there was modest or no effect on p-ERK. At 10 nM for 30 h, temsirolimus remarkably inhibited phosphorylation of p70S6K, a substrate of mTOR that is involved in cell cycle control (30), in all of the 11 thyroid cancer cells (Fig. 1). Treatment of cells with the HDAC inhibitor SAHA at 5 μM for 30 h resulted in a marked increase in histone acetylation in all cell lines (Fig. 1). We next explored the ability of these inhibitors to induce the expression of thyroid genes in cells treated with these inhibitors, individually or in different combinations under the conditions used for the studies in Fig. 1. As shown in Figs. 2A and 3, expression of *NIS*, *TSHR*, *TPO*, and *Tg* was restored to various extents by RDEA119, temsirolimus, or SAHA in most of the 11 thyroid cancer cells. *FOXE1* and *TTF1* genes were also reexpressed to various extents in different cells. However, expression of *PAX8* seemed somehow to be negatively affected by these inhibitors (Supplemental Fig. 1A). Among the three inhibitors, SAHA showed the
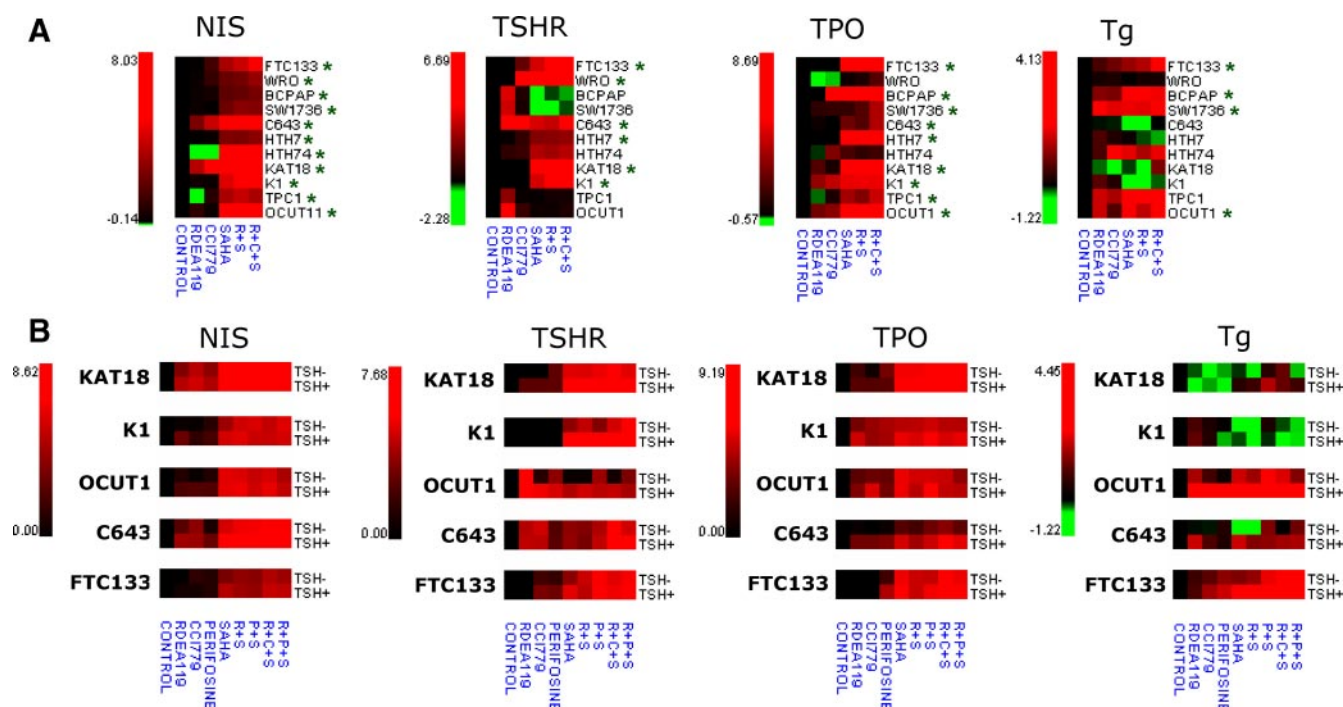
FIG. 2. Induction of the expression of *NIS*, *TSHR*, *TPO*, and *Tg* genes in thyroid cancer cells using various inhibitors, individually or in different combinations. A, Eleven authenticated thyroid cancer cells, as indicated, were treated with inhibitors (RDEA119, CCI779, and SAHA) as described in Fig. 1, individually or in different combinations, to induce the expression of *NIS*, *TSHR*, *TPO*, and *Tg* genes. Data were obtained using real-time RT-PCR and are presented as the mean of values from three assays for the ratios of specific gene expression with natural log conversion. The *red color* represents the highest expression, *green* is the lowest expression, and *black* is the intermediate. B, Effect of TSH stimulation on *NIS*, *TSHR*, *TPO*, and *Tg* expression induced by specific inhibitors (RDEA119, CCI779, perifosine, and SAHA), individually or in different combinations. Details are as described in *Materials and Methods*, and data were obtained and are presented as in panel A. *Green asterisks* show that the basal level of gene expression is low. R, RDEA119; C, CCI779 (temsirolimus); P, perifosine; S, SAHA.

most pronounced effect on the expression of thyroid genes. Interestingly, although RDEA119 and temsirolimus each alone had a small effect, a remarkable synergistic effect was seen between RDEA119 or temsirolimus and SAHA on most genes in most cells (Fig. 2A, Fig. 3, and Supplemental Fig. 1A). Combination of temsirolimus with RDEA119 even better synergized the effect of SAHA. Combined use of inhibitors of MAPK and PI3K/Akt pathways without SAHA did not show significant synergistic effects in thyroid cancer cells (data not shown).

TSH stimulation enhanced thyroid gene expression induced by SAHA synergized by perifosine and other inhibitors in thyroid cancer cells

Because Akt is upstream of mTOR in the PI3K/Akt pathway and is coupled also to other signaling molecules, targeting Akt may also be a reasonable strategy in restoring the expression of thyroid genes. We therefore also examined the effect of the Akt inhibitor perifosine on selected thyroid cancer cells, including KAT18, K1, OCUT1, C643, and FTC133 cells. These cells were treated with 5 μM perifosine for 30 h. As shown in Supplemental Fig. 2, this inhibitor remarkably inhibited Akt phosphorylation in most cells. Correspondingly, like temsirolimus, although perifosine

alone had only a minimal effect, it could enhance the effect of SAHA on the expression of the *NIS*, *TSHR*, *TPO*, and *Tg* genes in many of these cells (Figs. 2B and 3). The *PAX8*, *FOXE1*, and *TTF1* genes were also reexpressed to various extents by treatment with perifosine in combination with SAHA in different cells (Supplemental Fig. 1B). Like temsirolimus, perifosine, when combined with RDEA119, even better synergized the effect of SAHA (Fig. 2B, Fig. 3, and Supplemental Figs. 1B and 3). Because TSHR plays an important role in up-regulating the expression of iodide-handling genes in thyroid cells (6, 7), we investigated whether TSH treatment could affect the expression of thyroid genes in these cells. As shown in Fig. 2B, expression of the *NIS*, *TSHR*, *TPO*, and *Tg* genes induced by RDEA119, temsirolimus, perifosine, SAHA, and their various combinations was enhanced by TSH to various extents in most cells. The expression of the *PAX8*, *FOXE1*, and *TTF1* genes induced by various inhibitors was also enhanced by TSH in many cases (Supplemental Figs. 1B and 3). A striking finding was the dramatic enhancing effect of TSH on thyroid gene expression induced by SAHA or SAHA combined with the inhibitors of the MAPK and PI3K/Akt pathways as shown in Figs. 2B and 3 for *NIS*, *TSHR*, *TPO*, and *Tg* genes and in Supplemental Figs. 1B and 3
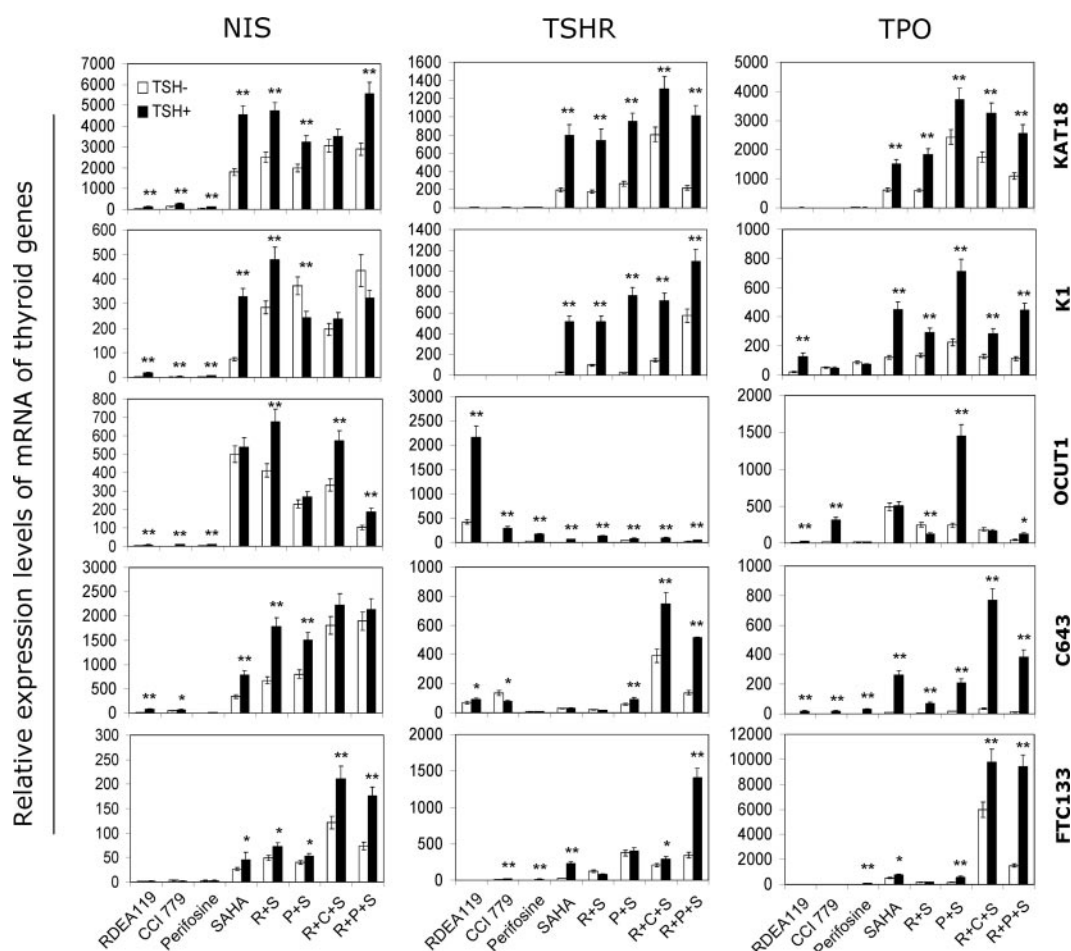
FIG. 3. TSH enhancement of expression of *NIS*, *TSHR*, and *TPO* genes induced by various inhibitors, individually or in different combinations, in thyroid cancer cells. Various thyroid cancer cells were treated with specific inhibitors (RDEA119, CCI779, perifosine, and SAHA) as described in Fig. 1 and in *Materials and Methods*, individually or in different combinations, supplemented with or without 20 mU/ml TSH. Data are presented as the mean ± SD of values from three assays. R, RDEA119; C, CCI779 (temsirolimus); P, perifosine; S, SAHA. *, $P < 0.05$; **, $P < 0.01$ for comparison with control.

for other thyroid genes. There was also an interesting dramatic synergistic effect of TSH on the expression of its own receptor TSHR induced by SAHA or by treatment combinations containing SAHA in most cells (Figs. 2B and 3).

NIS protein expression in the cell membrane and radioiodide uptake in thyroid cancer cells induced by simultaneously suppressing the MAPK and PI3K/Akt pathways and HDAC

Because NIS plays a central role in thyroid cellular iodide uptake, we next investigated further the expression of NIS, particularly its expression on cell membranes, using immunofluorescent microscopy in selected thyroid cancer cells, including C643, K1, and KAT18 cells. As a representative test, we chose to use the combination of RDEA119, perifosine, and SAHA because this induced a robust expression of NIS in many thyroid cancer cells per real-time quantitative RT-PCR analysis (Figs. 2 and 3). We used double immunofluorescence to

examine NIS protein expression. The red color represented nuclear staining by 7-AAD, and the green color represented NIS expression. Because 7-AAD does not readily pass through intact cell membranes, cells with the green color alone were thus intact viable cells that did not have 7-AAD nuclear staining. NIS staining in these cells represented NIS protein expression on cell membranes. As shown in Fig. 4, robust expression of the NIS protein in cell membranes was induced by simultaneous treatment of cells with the three inhibitors.

Given the induction of expression of thyroid iodide-handling genes in thyroid cancer cells by suppressing multiple signaling pathways/molecules, we finally tested the functional relevance of such gene expression by examining the ability of cells to take up radioiodide in three selected thyroid cancer cells C643, K1, and KAT18. As shown in Fig. 5, combined treatment of cells with RDEA119, perifosine, and SAHA robustly induced radioiodide uptake in all three cells. Additional treatment of cells with TSH significantly enhanced radioiodide uptake induced by treat-
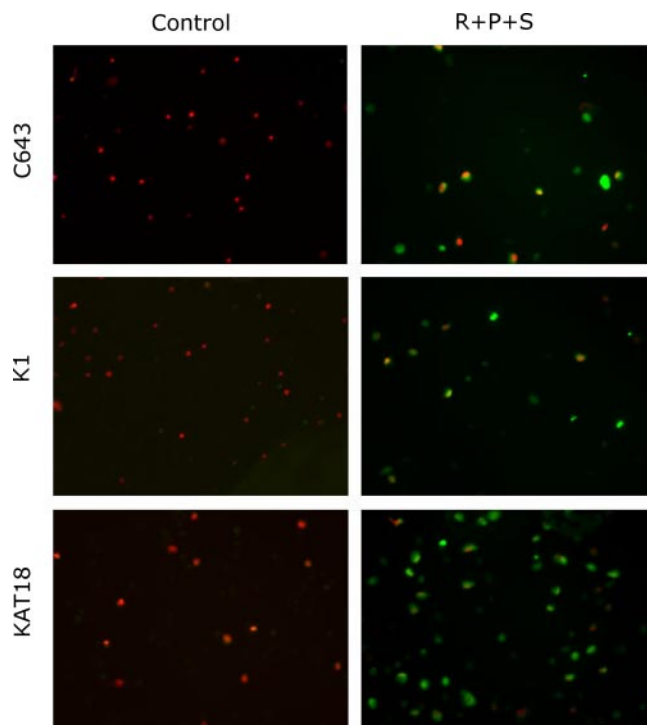
FIG. 4. Immunofluorescent microscopic analysis of NIS expression induced by inhibitors in thyroid cancer cells. After a 30-h combined treatment with RDEA119, perifosine, and SAHA, cells were analyzed by immunofluorescent microscopy using anti-NIS antibody and fluorescein isothiocyanate-coupled secondary antibody. Double immunofluorescence was displayed with the *red color* representing 7-AAD nuclear staining and the *green color* representing NIS expression. Cells with the *green color alone* are intact living cells that do not have 7-AAD nuclear staining. NIS staining in these cells represents NIS protein expression on the cell membrane. Cells with *double colors* suggest that the cells were not intact and therefore both cell membrane NIS staining and 7-AAD nuclear staining occurred. Control cells did not show significant NIS staining, and the dead cells mostly showed nuclear staining with 7-ADD. R, RDEA119; P, perifosine; S, SAHA.

ment with the inhibitors (Fig. 5). These functional results were consistent with the robust expression of NIS and TSHR under similar conditions in these cells (Figs. 2–4). The induced radioiodide uptake could be inhibited by the NIS blocker $NaClO_4$, suggesting that it was mediated specifically by the newly produced NIS protein. A small basal level of radioactivity was seen in nontreated control cells (Fig. 5). This radioactivity was largely insensitive to $NaClO_4$, suggesting that it was not NIS-specific and probably represented nonspecific binding of radioiodide with cells.

Discussion

Radioiodine ablation therapy after thyroidectomy is the mainstay of medical treatment for thyroid cancer in many patients (4, 5). This treatment takes the advantage of the unique iodide-handling machinery of thyroid cells, involving NIS, TSHR, TPO, Tg, and several thyroid transcrip-
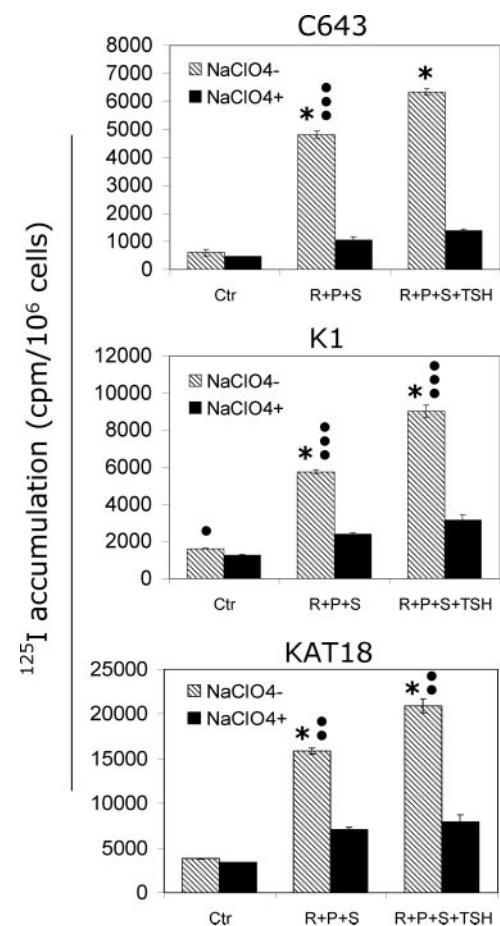
FIG. 5. Radioiodide uptake in thyroid cancer cells induced by inhibitors targeting MEK, Akt, and HDAC in thyroid cancer cells. Thyroid cancer cells C643, K1, and KAT18 were treated with a combination of RDEA119, perifosine, and SAHA as described in *Materials and Methods*, supplemented with or without 20 mU/ml TSH. Cells were subsequently incubated with 1 μCi ^{125}I/1 ml/well on 12-well plates for 1 h. Cells were then washed and harvested for radioactivity measurement using a gamma-counter as described in *Materials and Methods*. Data are expressed as mean ± SD of values from three assays. R, RDEA119; P, perifosine; S, SAHA; Ctr, control. ●●●, $P < 0.001$; ●●, $P < 0.01$; ●, $P < 0.05$, compared with $NaClO_4$-treated cells. *, $P < 0.001$, compared with untreated cells.

tion factors, to take up and accumulate radioiodide. T_4 withdrawal to increase endogenous TSH or use of recombinant human TSH to enhance the ablation efficacy through the action of TSHR is commonly administered (4, 5, 31). This treatment, however, is ineffective in poorly differentiated and undifferentiated thyroid cancer and often even in differentiated thyroid cancer (32, 33). The underlying molecular mechanism is the impairment or even complete loss of the expression of iodide-handling genes and aberrant localization of NIS in these thyroid cancers (12–17, 34). This results in the loss of the ability of thyroid cancer cells to take up and concentrate radioiodide, thus failure of radioiodine treatment. This is a main cause of thyroid cancer-associated morbidity and mortality. There is currently no effective treatment for these thyroid cancer patients when the tumor becomes surgically inoperable.

The specific molecular mechanism underlying silencing of thyroid iodide-handling genes in thyroid cancer is unclear. Many recent studies demonstrated an association of *BRAF* mutation and, hence, activation of the MAPK pathway with silencing of thyroid genes in thyroid cancer (35, 36). We previously showed that induced expression of the BRAF mutant and consequent activation of the MAPK pathway signaling in rat thyroid cells could silence the expression of thyroid iodide-handling genes and removal of BRAF mutant could restore the expression of these genes (37). We and others also used the luciferase reporting system to show that inhibition of the promoter activities of the *NIS* and *TSHR* genes could be directly linked to the MAPK pathway signaling (34, 37). Thus, overactivation of the MAPK pathway, which is common in thyroid cancer, likely plays an important role in the aberrant silencing of thyroid genes in thyroid cancer. A recent study showed that the PI3K inhibitor LY294002 could induce expression of NIS in rat thyroid cells (21). Although the LY294002 compound is not clinically applicable, these results suggest that the PI3K/Akt pathway may also play a role in the silencing of thyroid genes in thyroid cancer. This may be the case particularly given the common occurrence of activating genetic alterations in the PI3K/Akt pathway in thyroid cancer (38–41). Interestingly, several previous studies demonstrated that HDAC inhibitors could induce expression of NIS in some human cancer cell lines (22–24). Many of the cell lines used in these studies are now known to be nonthyroid in origin (25), and the inhibitors used may not be clinically applicable. Nevertheless, these studies suggest that aberrant histone deacetylation may also play an important role in thyroid gene silencing in thyroid cancer.

We tested the hypothesis in the present study that targeting the MAPK and PI3K/Akt pathways and HDAC could be an effective strategy in restoring the expression of thyroid iodide-handling genes and, hence, uptake of radioiodine in thyroid cancer. Using a large panel of authenticated thyroid cancer cell lines, we indeed demonstrated that inhibitors of these signaling pathways/molecules could induce the expression of thyroid iodide-handling genes. We specifically chose the MEK inhibitor RDEA119, Akt inhibitor perifosine, mTOR inhibitor temsirolimus, and HDAC inhibitor SAHA because these agents have proven acceptable safety profiles and *in vivo* biological activities in clinical trials on other human cancers (42–44). They are therefore ready for clinical use in thyroid cancer if proven to be effective. Among these inhibitors when individually used, SAHA was the most effective in inducing thyroid gene expression. Interestingly, although RDEA119, perifosine, and temsirolimus each alone had only a small effect on thyroid gene expression in thyroid cancer cells, they all significantly synergized the effect

of SAHA. Although a recent clinical trial showed no inhibitory effect of SAHA on the growth and progression of radioiodine-refractory thyroid cancer (44), our present study suggests that SAHA may be effective when used in conjunction with radioiodine ablation therapy for thyroid cancer.

It is particularly important to note that among the various thyroid genes tested, expression of *NIS* was generally most responsive to the treatment with the inhibitors in all the cells. Protein expression and cell membrane localization of NIS was confirmed using immunofluorescent microscopic analysis. This is clinically important because NIS, acting to transport iodide from the blood stream into the thyroid cell, plays the most important role in radioiodide uptake and accumulation in thyroid cancer cells. The functionality of this *NIS* gene expression was clearly shown by our demonstration of robust and $NaClO_4$-sensitive radioiodide uptake in thyroid cancer cells in response to inhibitor treatments. It is worth noting that expression of *TSHR* was also robustly induced by the inhibitors in most cells, and treatment of cells with TSH further enhanced both the expression of thyroid genes and radioiodide uptake induced by the inhibitors targeting the multiple signaling pathways/molecules. This effect of TSH was most dramatic when SAHA was used to treat cells, either alone or in combination with other inhibitors, particularly the latter. These results suggest that use of these inhibitors, particularly in combination with SAHA, would most effectively confer the sensitivity of thyroid cancer cells to radioiodine ablation when the patients undergo T_4 withdrawal to increase endogenous TSH or receive human recombinant TSH. This therapeutic strategy may be expected to be effective even in poorly differentiated and undifferentiated thyroid cancers because most of the cell lines used in the present study were derived from these types of thyroid cancer. In fact, even the thyroid cancer cell lines originally derived from differentiated thyroid cancers that were used in this study likely had lost differentiation because many such human thyroid tumor cell lines have evolved *in vitro* into phenotypes with gene expression profiles that were close to *in vivo* undifferentiated tumors (45). This further supports the idea that restoration of thyroid gene expression and radioiodine avidity are possible in undifferentiated thyroid cancers using the treatment strategies tested in the present study.

In summary, for the first time using a large panel of authentic thyroid cancer cells, we demonstrated robust expression of thyroid iodide-handling genes, particularly *NIS* and *TSHR*, driven synergistically by suppressing the MAPK and PI3K/Akt pathways and HDAC. The functional importance of this thyroid gene expression was demonstrated by restoration of the ability of cells to take up radioiodide that could be dramatically enhanced by

TSH. These results provide important clinical implications for novel therapeutic strategies to restore radioiodine avidity of thyroid cancers. Because the inhibitors tested in the present study are clinically applicable, further studies, including appropriately designed clinical trials in thyroid cancer, with the goal to use them to restore the responsiveness to radioiodine ablation may be warranted.

Acknowledgments

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