Low Concentrations of the Histone Deacetylase Inhibitor, Depsipeptide (FR901228), Increase Expression of the Na\(^+\)/I\(^-\) Symporter and Iodine Accumulation in Poorly Differentiated Thyroid Carcinoma Cells

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ABSTRACT
Thyroid carcinoma accounts for the majority of deaths from endocrine cancers. A major cause of treatment failure is the inability to trap iodine. Chemotherapeutic agents with differentiating properties have been tried in an attempt to increase iodine uptake. We examined the ability of the novel histone deacetylase (HDAC) inhibitor, depsipeptide (FR901228), to modulate the expression of thyroid-specific genes. Four cell lines, two derived from follicular thyroid carcinomas (FTC 133 and FTC 236) and two derived from anaplastic thyroid carcinomas (SW-1736 and KAT-4) were used. In these four cell lines, a very low concentration of depsipeptide (1 ng/mL) increased histone acetylation and expression of both thyroglobulin and the Na\(^+\)/I\(^-\) symporter messenger RNAs. After 3 days, messenger RNA levels approached those of a normal thyroid control. Depsipeptide induced increases in \(^{125}\)I accumulation indicated that a functional Na\(^+\)/I\(^-\) symporter protein was induced. Transient transfections indicate that the effects are mediated at least in part by a trans-activating factor. These in vitro results suggest that depsipeptide or other histone deacetylase inhibitors might be used clinically in thyroid carcinomas that are unable to trap iodine as an adjunct to radioiodine therapy.

With both WDTCs that have lost the ability to trap iodine and ATCs, therapeutic options are limited and largely unsuccessful. Palliative or debulking surgery (metastatectomy), external radiation, and chemotherapy have all been tried, with limited success (12–17). Among experimental options, restoration of iodine trapping has been pursued without convincing efficacy until now (18–22). In the present study, we describe the use of very low doses of a histone deacetylase (HDAC) inhibitor to increase the expression of thyroid-specific proteins. In four thyroid carcinoma cell lines, including two derived from anaplastic thyroid carcinomas, treatment with the HDAC inhibitor, depsipeptide (FR901228), led to a marked increase in expression of thyroglobulin and the Na\(^+\)/I\(^-\) symporter (NIS), with a resultant increase in \(^{125}\)I accumulation. Transient transfection studies indicate this increase is mediated at least in part by trans-activation of these genes.

Materials and Methods

Cell lines and culture conditions
Follicular thyroid carcinoma (FTC) 133 and FTC 236 were derived from cultures obtained from the primary tumor (FTC 133) and a nodal metastasis (FTC 236) of a follicular thyroid carcinoma. The anaplastic thyroid carcinoma cell lines were derived from primary cultures of human ATC tumors. SW-1736 was developed by Drs. Leibowitz and McCombs, III, at the Scott and White Memorial Hospital (Temple, TX) in 1977, was maintained by Nils-Erik Heldin (Uppsala University, Uppsala, Sweden), and provided by Kenneth Ain (University of Kentucky, Lexington, KY). KAT-4 was developed and maintained in RPMI media containing 10% FBS in the laboratory of K. Ain. FTC 133 and FTC 236...
were originally maintained in medium containing TSH, but this was discontinued when a difference in growth rate could not be demonstrated in the presence or absence of TSH.

**Fluorescein isothiocyanate staining**

Cytospins were made from trypsinized cells, and the slides were fixed in 95% ethanol/5% acetic acid for 1 min at room temperature. After fixation, slides were washed twice with PBS for 5 min, blocked in 8% BSA in PBS for 1 h at room temperature, and washed 15 min in PBS before incubating overnight at 4°C with 5 μg/mL anti-α acetylated Histone H3 (Upstate Biotechnology, Inc., Lake Placid, NY) in 2% BSA in PBS. Subsequently, cells were washed twice with PBS for 5 min at room temperature and then stained with horse antirabbit fluorescein isothiocyanate conjugated secondary antibody (Vector Laboratories, Inc., Burlingame, CA). After staining with secondary antibody, slides were washed three times with PBS for 15 min and then counterstained with DAPI containing antifade compound (Vector Laboratories, Inc.).

**Protein collection and Western blot analysis**

Cells were scraped into lysis buffer A containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 1.5 mM PMSF and centrifuged at 11,000 g for 10 min at 4°C. Pellets thawed in buffer B containing 50 mM HEPES (pH 7.9), 420 mM KCl, 0.1 mM EDTA (pH 8.0), 5 mM MgCl₂, 20% glycerol, 0.5 mM DTT, and 1.5 mM PMSF were rotated for 30 min at 4°C, and centrifuged at 11,000 g for 15 min at 4°C. The supernatants were collected as nuclear extracts. Ten micrograms of protein were separated on an 11% SDS-PAGE gel, and electrophotoblotting to Immobilon-P transfer membrane (Millipore Corp.) was performed. Nonspecific protein binding was blocked using 5% milk in TNE buffer (20 mM Tris (pH 7.4), 2 mM NaCl, 1 mM EDTA, and 0.15% Tween 20) for 30 min. The membrane was incubated for 30 min with a rabbit polyclonal peroxidase-linked secondary antibody (Amersham Pharmacia Biotech). After washing, DAPI containing antifade compound (Vector Laboratories, Inc.) was added and incubated for 30 min. After washing, the membrane was developed in ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

**Quantitative PCR amplification of the thyroglobulin and sodium iodide symporter**

Quantitative RT-PCR for thyroglobulin (TG) and NIS was performed as previously described (23, 24). Total RNA was extracted using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). Single-stranded oligo (dT)-primed complementary DNA (cDNA) was generated using MMLV reverse transcriptase (Life Technologies, Inc., Eggenstein, Germany). Oligonucleotide primers, used for analysis of human TG RNA expression, were: TG 3′ (sense), 5′-CGTCGTACAGGGATAGCAC-3′, and TG 3′ (antisense), 5′-ATATCGCAGACAGTGGCAATA-3′. These primers should generate a product that is 219 bp in length. The amplification reaction was carried out for 35 cycles, and each cycle consisted of 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min, followed by a final 10-min elongation at 72°C.

Oligonucleotide primers for human NIS RNA amplification were: NIS (1) 5′ (sense), 5′-GTGCCGGCCAGTTACTCATT-3′, and NIS (1) 3′ (antisense), 5′-TGACGGTGAAGGAGCCCTGGAACATTTCCAG-3′. The expected human NIS product from a cDNA template is 203 bp. The amplification reaction was run for 30 cycles, and each cycle consisted of 94°C for 20 sec, 64°C for 30 sec, and 72°C for 60 sec, followed by a final 7-min elongation at 72°C. All quantitations were performed by densitometry. Quantitations were based on measured β-actin levels. Oligonucleotide primers for human β-actin RNA amplification were: β-actin 5′ (sense), 5′-GAGGCTACGAGGATGATG-3′, and β-actin 3′ (antisense), 5′-GAGCCGTACAGGATGCCAC-3′.

**Northern blot analysis of NIS**

RNA was electrophoresed on a 1% agarose gel containing 2 μM formaldehyde and was transferred overnight in 20× saline-sodium citrate (SSC) to a nylon membrane (Amersham Pharmacia Biotech). The cDNA probes for Northern blot analysis were generated by PCR using a pair of NIS gene-specific primers: NIS (2) 5′ (sense), 5′-GCTGCGCCTGTCACTCAGAC-3′, and NIS (2) 3′ (antisense), 5′-CCAGGGCCGGAGCAGG-AACATTTCCAG-3′. The NIS gene-specific cDNA fragment was radiolabeled with [α-32P]deoxyctosine-5′-triphosphate by random priming and was used as a hybridization probe. The blot was prehybridized at 42°C in a hybridization mix containing 50% formamide for 2 h, followed by hybridization at 42°C for 12 h. The blot was then rinsed twice in 2× SSC/0.1% SDS at room temperature for 10 min and twice in 0.1× SSC/0.1% SDS at 50°C for 20 min.

**Growth inhibition and cell viability**

Cells were plated at a density of 3000 per well in 96-well plates in 180 μL medium in triplicate. Drug sensitivity was measured by exposing the cells to graded concentrations of desippeptide in a final volume of 200 μL. At 72 h viable cells were estimated in a colorimetric assay that measures the formazan reduction product of MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenylytetrazolium bromide), which is produced by mitochondrial activity of viable cells. The reduction product was dissolved in dimethyl sulfoxide, and absorbance was quantitated using a plate reader spectrophotometer.

**125I accumulation**

Cells (3×10⁴) were seeded into 24-well dishes in 1 mL medium. After a 1-day incubation period at 37°C with 5% CO₂, the medium was aspirated and fresh medium was added with or without desippeptide (final concentration 1 ng/mL). Iodide uptake was initiated by adding 0.5 mL HBSS (Life Technologies, Inc.) containing approximately 2 μCi carrier-free Na¹²⁵I (NEN Life Science Products, Boston, MA) and 30 μμ-gal Sodium. Incubations were performed for 10 min. For perchlorate inhibition studies, NaClO₄ was added as a 100× solution in HBSS to a final concentration of 30 and 100 μM, immediately after the addition of radiolabeled iodine. Reactions were rapidly terminated by removing the radioactive HBSS and washing the cells twice with ice-cold HBSS. Cells were then solubilized by incubation for 20 min in 0.4 mL of 1.0% Triton X-100 (Sigma, Allentown, PA) in HBBS, and accumulated iodide was measured in a gamma counter. The number of cells per well was determined by harvesting and counting (at the time of experiment) three additional wells of cells.
**Construction of reporter plasmids**

The promoter of the TG gene was isolated using the PCR and DNA from FTC 236 cells. Primers used were: 5\(^9\) (sense), 5\(^{500}\)GAGCTCTAA-GAGGTTGTTAGAG\(^{479}\); and 3\(^9\) (antisense), 5\(^{479}\)TTTCTGGGCCCT-TCTGGAGGAA\(^{17}\).

The amplified fragment was subcloned into the pCRII TA vector (Invitrogen, Carlsbad, CA), and its sequence was confirmed. After digestion with \(KpnI\) and \(XhoI\), the 540-bp promoter fragment was ligated to the pGL3-B luciferase (Luc) vector (Promega Corp., Madison, WI). This construct was designated TG promoter-Luc.

The enhancer element of the TG gene was amplified using the PCR and the following primers: 5\(^9\) (sense), CGGGGTACC\(^{2698}\)GTTCTCA-CGAGCTCAGTGGAG\(^{2677}\); and 3\(^9\) (antisense), CGGACTAGT\(^{2172}\)CC-CATTGCCCTAAAATGCATGC\(^{2193}\).

\(KpnI\) (sense) and \(SpeI\) (antisense) restriction sites flanked the TG enhancer sequence. The amplified fragment was inserted into the TG promoter-Luc plasmid digested with \(KpnI\) and \(SpeI\). This construct was designated TG enhancer/promoter-Luc.

In addition, the HSV-thymidine kinase (TK) minimum promoter was obtained by digesting pRL-TK (Promega Corp.) with \(HindIII\) and \(BglII\); this was subcloned into pGL3-B luciferase vector, and designated TK-Luc. TK-Luc was used as the positive control.

**Transfections and luciferase assays**

Transient transfections were performed using a liposome-mediated method. For all cell lines, \(3 \times 10^4\) cells were plated 24 h before transfection, after which 0.5 \(\mu g\) plasmid DNA and 4.5 \(\mu L\) of TransFast (Promega Corp.) mixed with 200 \(\mu L\) of medium were added to each well. After incubating 1 h in the above mixture, cells were cultured in the presence or absence of depsipeptide (1 ng/mL) for 2 days. After harvesting, total protein concentration was measured using the protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Firefly luciferase activity was assessed using the luciferase assay system (Promega Corp.) and normalized to protein. All transfections were performed in triplicate. In all experiments, TK-Luc was used as the positive control. The result with the TK-Luc vector was assigned a value of 100\%, and all other values were expressed relative to this as relative luciferase units.

**Results**

The experiments described herein evolved from studies attempting to modulate the expression of constructs driven by the TG promoter. The effects of several differentiating agents on the level of expression of constructs containing the TG promoter and on the endogenous TG gene were studied. Preliminary observations indicated that depsipeptide, a novel HDAC inhibitor currently undergoing phase I trials in humans, could modulate expression of TG. This effect was originally observed at concentrations of depsipeptide that were cytotoxic. However, because unpublished observations indicated that inhibition of HDAC could be observed at lower concentrations, the effect of lower doses of depsipeptide on the expression of TG and NIS was investigated _in vitro_. Figure 1 demonstrates cytotoxicity curves with depsipeptide in the four cell lines used in the present study. In these experiments, cells were exposed to depsipeptide for 72 h, after which time MTT assays were performed. As can be seen, in the four cell lines, a depsipeptide concentration of 1 ng/mL for 72 h was not cytotoxic or at most minimally cytotoxic. This concentration was chosen for all subsequent experiments.

Treatment of cells with HDAC inhibitors results in increased chromatin acetylation, and this can be detected using an antibody against acetylated chromatin (25). To confirm that 1 ng/mL depsipeptide could increase histone acetylation in spite of its low to absent cytotoxicity, control cells and cells treated with depsipeptide were stained with an antibody directed against acetylated histones. As shown by the fluorescein isothiocyanate staining and Western blot analysis in Fig. 2, the basal level of acetylated histones is higher in the more differentiated FTC 236 and FTC 133 cells, compared with the anaplastic KAT-4 and SW-1736 cells. More impor-
tantly, treatment with 1 ng/mL depsipeptide for 72 h resulted in a marked increase in histone acetylation in all cell lines.

Having demonstrated increased histone acetylation following depsipeptide, we next examined the expression of two thyroid-specific genes, TG and NIS. Figure 3A and Table 1 demonstrate the results of RT-PCR analysis examining the expression of TG and NIS in the four cell lines before treatment with depsipeptide and after 1, 2, and 3 days of 1 ng/mL depsipeptide. RNA from normal thyroid tissue is included for comparison. Serial dilutions in the exponential range were performed to determine the levels in Table 1. The results of one dilution are shown in Fig. 3A for ease of presentation. As can be seen, neither TG nor NIS expression can be detected, even with RT-PCR, in untreated ATC cells (SW-1736 and KAT-4), while levels detectable only by RT-PCR are found in untreated FTC cells (faint bands seen in original photographs). Following the addition of depsipeptide, increased expression is detected after the first day of treatment in the FTC cells, and in the ATC cells, an increase is apparent after 48 h. In both instances, marked increases in TG and NIS expression are observed after 72 h of depsipeptide. Figure 3B is a Northern analysis demonstrating increased expression of the 4-kb NIS transcript in both SW-1736 and FTC 236 cells following depsipeptide treatment.

To determine whether the induced NIS messenger RNA (mRNA) yielded a functional Na\(^+\)/I\(^-\) symporter, the cellular iodine accumulation studies shown in Fig. 4 were performed. However, a dose response examining the expression of NIS was first performed so as to measure \(^{125}\)I uptake under optimal conditions. Figure 4A shows expression of NIS mRNA increased with increasing depsipeptide concentrations up to 1 ng/mL depsipeptide, with NIS mRNA expression in ATC cells observed only at depsipeptide concentrations of 0.3 and 1.0 ng/mL. Therefore, using 1.0 ng/mL depsipeptide, the accumulation studies shown in Fig. 4, B and C, were performed. The results are expressed as \(^{125}\)I cpm per 10\(^5\) cells. Although differences among cell lines should be interpreted cautiously, the accumulation in the untreated cell lines derived from FTCs is higher than in the cell lines derived from ATCs (Fig. 4B, open bars), consistent with the higher levels of basal NIS expression in the former, as shown by the PCR analysis. More remarkably, however, marked increases in iodine accumulation were observed in all four cell lines following the addition of depsipeptide (Fig. 4B, hatched bars). The increase was greater after 3 days of depsipeptide and was largely inhibited by sodium perchlorate, indicating the existence of a functional iodine trapping mechanism (Fig. 4C).

Figure 5 shows the luciferase activity in FTC and ATC cells transiently transfected with a reporter plasmid containing a luciferase gene under the control of a TG promoter/enhancer element. This construct has been shown to be highly specific for cells of thyroid origin (data not shown). The activity of this reporter construct is compared with that of a constitutively active TK-Luc reporter. The latter used as the positive control was assigned a value of 100% in each cell line. As can be seen, the activity of the TG promoter/enhancer element, which ranges from 28.6% to 84.5% that of TK-Luc in the absence of depsipeptide, is increased markedly after the addition of depsipeptide. The increase in luciferase activity after depsipeptide treatment is consistent with trans-activation of the reporter plasmid. This increase is observed in all cell lines.

**Discussion**

This study describes the induction of a functional Na\(^+\)/I\(^-\) symporter in poorly differentiated thyroid carcinoma cell lines by the HDAC inhibitor, depsipeptide. The induction was observed in four independent cell lines, including two derived from anaplastic thyroid carcinomas, using very low concentrations of depsipeptide. Cellular iodine accumulation studies demonstrated marked increases in iodine accumulation in all four cell lines. While these *in vitro* studies...
must be considered preliminary, they provide the hope that iodine accumulation could potentially be increased in WDTCs that have lost the ability to trap iodine and in anaplastic thyroid carcinomas.

Although the current therapeutic modalities for WDTCs, including surgery and radioiodine therapy, are generally very effective, they fail to be curative in up to 15% of cases. A significant proportion of these therapeutic failures are due to the progressive loss of the ability of WDTCs to trap iodine or the lack of this ability at the time of initial diagnosis, as is the case with poorly differentiated FTCs and ATCs. The loss of iodine accumulation properties may also be accompanied by other features of dedifferentiation, such as loss of responsiveness of TG expression to TSH or, more rarely, complete or partial loss of baseline TG expression (26). A therapeutic approach that could redifferentiate malignant thyrocytes, even partially, could potentially lead to reconstitution of their ability to trap radioiodine, which would then be organized and retained in these cells, thus inducing cytotoxicity. In this regard, several “antitumor” agents with differentiating properties such as retinoic acid have been considered for the treatment of noniodine-avid thyroid carcinomas (18). Increased levels of mRNA for the NIS and 5’-deiodinase have been reported after the addition of retinoic acid to thyroid derived cell lines in vitro, and a study using retinoic acid reported restoration of iodine uptake in a small number of patients with poorly differentiated thyroid cancers (19–22).

Depsipeptide (FR901229) is a novel histone deacetylase inhibitor currently in phase I trials in the United States. As
with other HDAC inhibitors, it is thought to act by promoting histone acetylation and in turn gene expression. Pharmacokinetics in patients receiving depsipeptide indicate that approximately 90% of circulating drug is protein bound. However, levels exceeding 500 ng/mL have been achieved without significant toxicity, indicating that the concentrations used in the present study can be seen easily achieved in patients (unpublished observations).

In summary, we report the induction of a functional NIS in four thyroid carcinoma cell lines including two derived independently from ATCs. This was achieved with very low concentrations of depsipeptide, which should be easily achieved in patients without significant toxicity. Carefully designed clinical trials using depsipeptide or other HDAC inhibitors to modulate iodine trapping in selected patients with thyroid carcinoma refractory to radiiodine therapy are supported by these observations.

References
19. Simon D, Kohrle J, Schmutzler C, Mainz K, Reiners C, Roher HD. 1996 Rediffere-
chemistry. 29:10351–10356.
25. Lin R, Leone JW, Cook RG, Allis CD. 1989 Antibodies specific to acetylated histones document the existence of deposition- and transcription-related hi-