The Effect of Tanespimycin (17-AAG) on Radioiodine Accumulation in Sodium-Iodide Symporter Expressing Cells

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Abstract

Purpose The heat shock protein 90 inhibitor, tanespimycin, is an anticancer agent known to increase iodine accumulation in normal and cancerous thyroid cells. Iodine accumulation is regulated by membrane proteins such as sodium iodide symporter (NIS) and pendrin (PDS), and thus we attempted to characterize the effects of tanespimycin on those genes.

Methods Cells were incubated with tanespimycin in order to evaluate ^{125}I accumulation and efflux ability. Radioiodine uptake and efflux were measured by a gamma counter and normalized by protein amount. RT-PCR were performed to measure the level of gene expression.

Results After tanespimycin treatment, ^{125}I uptake was increased by ~2.5-fold in FRTL-5, hNIS-ARO, and hNIS-MDA-MB-231 cells, but no changes were detected in the hNIS-HeLa cells. Tanespimycin significantly reduced the radioiodine efflux rate only in the FRTL-5 cells. In the FRTL-5 and hNIS-ARO cells, PDS mRNA levels were markedly reduced; the only other observed alteration in the

levels of NIS mRNA after tanespimycin treatment was an observed increase in the hNIS-ARO cells.

Conclusions These results indicate that cellular responses against tanespimycin treatment differed between the normal rat thyroid cells and human cancer cells, and the reduction in the ^{125}I efflux rate by tanespimycin in the normal rat thyroid cells might be attributable to reduced PDS gene expression.

Keywords Thyroid cancer · Radioiodine therapy · Tanespimycin (17-AAG) · Sodium-iodide symporter · Pendrin

Introduction

Differentiated thyroid carcinoma is one of the most common endocrine cancers. The current preferred treatments for this cancer are the surgical removal of the thyroid gland and

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radioiodine therapy [1]. Radioactive iodine therapy has proven to be a very effective treatment for residual and recurrent thyroid cancers, and is associated with a relatively high survival rate [2].

The sodium iodide symporter (NIS) mediates active iodine uptake in normal thyroid and thyroid cancer cells, and is also known to be expressed in the lactating mammary gland; additionally, NIS has been shown to enhance radioiodine accumulation in residual and recurrent cancer tissues [3, 4]. Interest in NIS has, therefore, risen with regard to the possibility of using it in the treatment and diagnosis of breast cancer [5, 6]. However, thyroid tumors gradually lose the expression of differentiation genes, including NIS, during the dedifferentiation process [7].

Certain chemicals and drugs have been reported to enhance radioiodine accumulation in thyroid and thyroid cancer cells [5, 8]. Among them, tanespimycin—previously referred to as 17-AAG (17-allylamino-17-demethoxygeldanamycin)—is considered to be one of the promising agents. In addition to its marked anticancer effects, several investigators have noted previously that tanespimycin increases iodide accumulation in rat thyroid cells (PC-C13, FRTL-5) [9, 10]. However, tanespimycin induces a slight reduction in NIS gene expression in PC-C13 cells, and it is generally assumed that the upregulation of NIS gene expression is not the mechanism by which tanespimycin acts. Tanespimycin has also been shown to reduce the rate of iodine efflux from hNIS-FRO, PC-C13, and FRTL-5 cells [9, 10]. However, the actual mechanism of tanespimycin in cells has yet to be clearly elucidated.

Tanespimycin is a molecule that restrains the activity of heat shock protein 90 (hsp90) [11]. Heat shock proteins are generally detected in the cells; they are critically important for the proper functioning of the hsp90 chaperone, and are inhibited by the ansamycin drug, tanespimycin, which binds to the hsp90 amino-terminal nucleotide-binding pocket [12]. Hsp90 client proteins, including EGFR (epidermal growth factor receptor), HER2 (human epidermal growth factor receptor 2), Akt, Raf-1, Bcr-Abl and mutant p53 [12, 13] are members of the well-known oncogenic pathway family. Therefore, the hsp90 inhibitors are potentially effective anticancer agents, in that they are capable of encouraging apoptosis by redirecting oncoproteins in their progress toward ubiquitin-mediated proteolysis [14]. Since 1999, tanespimycin has been the subject of clinical trials and is currently being employed in a variety of phase trials against different malignancies, including melanoma, thyroid, breast, and prostate cancers [15].

In this study, we have evaluated the effects of tanespimycin on radioactive iodine accumulation and the expression of NIS and PDS in rat normal thyroid cells, hNIS-transfected human cancer cells to elucidate the differential response against tanespimycin treatment between the different cell types.

Materials and Methods

Cell Culture and Gene Transduction

FRTL-5 rat thyroid cells were cultivated in Coon's modified Ham's F-12 medium (Sigma, St. Louis, MO) supplemented with 5 % calf serum (Gibco, Grand Island, NY), 1 mM nonessential amino acids (Gibco, Grand Island, NY), and a six-hormone mixture (6 H) comprising 1 mU/ml TSH, 10 µg/ml insulin, 5 µg/ml transferrin, 10 nM hydrocortisol, 10 ng/ml glycyl-L-histidyl-L-lysine acetate, and 10 ng/ml somatostatin [16]. ARO human anaplastic thyroid cancer cells were grown in RPMI-1640 medium (WelGENE, Daegu, South Korea). HeLa human cervical cancer cells, MDA-MB-231 human breast cancer cells were grown in DMEM (Dulbecco's Modified Eagle Medium, WelGENE, Daegu, South Korea) containing 10 % fetal bovine serum (Gibco, Grand Island, NY) and 1 % penicillin/streptomycin (Gibco, Grand Island, NY) in an atmosphere of 5 % CO₂/95 % air (37 °C).

ARO cells stably expressing human NIS (hNIS-ARO) were generated by retroviral transduction. A retroviral vector pMSCV-NIS was constructed to express hNIS and transfected into 293FT packing cells. Viral supernatants and media were collected after 2 days of transfection and centrifuged. Retrovirally infected ARO cells were selected with puromycin for 2 weeks. The same procedure was also used to generate hNIS-MDA-MB-231 and hNIS-HeLa cells.

Reagents

Tanespimycin (17-allylamino-17-demethoxygeldanamycin, 17-AAG) (Sigma, St. Louis, MO) was dissolved in DMSO (Sigma, St. Louis, MO) and diluted to 0.5 µM, 1 µM, 2 µM and 4 µM with tissue culture medium immediately prior to use.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Real Time Polymerase Chain Reaction (real-time PCR)

Total RNA was isolated from the DMSO-treated or tanespimycin-treated cells using TRIZOL reagent (Invitrogen, Grand Island, NY), in accordance with the manufacturer's instructions. The concentration and purity of the RNA samples were determined with a Nanodrop Spectrophotometer (Thermo Scientific, Palm Springs, CA). First-strand cDNA was synthesized with 2 µg of each of the RNA samples primed with random hexamers via M-MLV reverse transcriptase (Invitrogen, Grand Island, NY).

Synthesized cDNA was then amplified by PCR using specific primers for 35 cycles, as follows. rPDS, 5'-CAT CAAGACACATCTCCGTTGGCCCT-3' and 5'-GGTA CTTCCGTTACCACTGGGC-3'; hPDS, 5'-CGATG

GGAACCAGGAATTCA-3' and 5'-TCTCAGGACCA CAGTCAACA-3'; rat β -actin, 5'-CCAGCAAGGATACT GAGAGCAAG-3' and 5'-TGTTATGGGGTCTGG GATGG-3'; human β -actin, 5'-GAGAAGGCTGGGGCT CATT-3' and 5'-CAGTGGGGACAGGGAAGG-3'. Amplified RNA fragments were electrophoresed in 1 % agarose gel and stained with loading star (Dyne bio, Seoul, South Korea). Signals were detected by LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Relative quantitative real-time PCR on 96-well optical plates was conducted using selected reagents and analyzed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Lincoln, CA). Reverse transcribed total RNA (100 ng) in 5 μ l, 1.25 μ l primers and probe (2.5 μ M), 12.5 μ l TaqMan PCR 2 $\times$ master mixture (Applied Biosystems, Lincoln, CA) and 6.25 μ l water were prepared. The PCR conditions used were as follows. After initial activation of uracyl-*N*-glycosylase at 50 °C for 2 min, AmpliTaq Gold was activated for 10 min at 95 °C, and the subsequent PCR condition consisted of 40 cycles of denaturation at 95 °C for 15 s and annealing extension at 60 °C for 1 min per cycle. During the PCR amplification, the amplified products were continuously measured via fluorescence emission determinations. The expression levels of the target genes were normalized to internal human 18 S and rat 18 S, and represented as relative expression levels. The PCR primer and Probe sets (Applied Biosystems, Lincoln, CA) for SLC5A5 (human NIS, Assay ID: Hs00166567_m1), Slc5a5 (Rat NIS, Assay ID: Rn00583900_m1), SLC26A4 (human PDS, Assay ID: Hs00166504_m1), and Slc26a4 (rat PDS, Assay ID: Rn00570082_m1) were used in the real-time PCR investigation.

¹²⁵I Uptake Assay

The accumulation of steady-state radioactive iodide was verified in FRTL-5, hNIS-ARO, hNIS-MDA-MB-231 and hNIS-HeLa cells incubated for 48 h with 1 μ M of tanespimycin or DMSO. Cells were seeded in 24-well plates, washed with Hank's balanced salt solution (HBSS), and incubated for 30 min at 37 °C with 500 μ l HBSS containing 0.1 μ Ci of ¹²⁵I and 10 μ M/l non-radioactive sodium iodide. The cells were then washed twice with ice-cold HBSS and lysed for 10 min with 0.2 % SDS. The cell lysates were collected and radioactivity was measured using a gamma counter (Canberra-Packard, Meriden, USA). The radioactivity was normalized by the amount of protein present at the time of the assay. Experiments were conducted in triplicate.

¹²⁵I Efflux Assay

Iodine efflux rates in FRTL-5, hNIS-ARO, hNIS-MDA-MB-231 and hNIS-HeLa cells were assessed after treatment with tanespimycin or DMSO. For the ¹²⁵I efflux assay, cells

were seeded on six-well plates, treated for 48 h with DMSO or tanespimycin (1 μ M), and then washed with HBSS and incubated for 30 min at 37 °C with 2 ml HBSS containing 0.4 μ Ci of ¹²⁵I and 10 μ M/l non-radioactive sodium iodide. After the cells were subsequently washed twice with ice-cold HBSS, the medium was collected after 3 min and replaced with 1 ml new cold HBSS. This was repeated every 3 min until the 30 min mark, and then collected every 10 min until the 60 min mark. Cells were lysed with 0.2 % SDS and radioactivity was measured with a gamma counter (Canberra-Packard, Meriden, USA). The radioiodine remaining in cells at each time-point was calculated as the sum of the radioactivity of the washed HBSS and the lysates. Radioactivity was normalized via a protein assay. Experiments were conducted in triplicate.

Statistical Analysis

The statistical contrast of ¹²⁵I uptake and efflux between groups was evaluated via a *t*-test. *P* values of < 0.05 were considered statistically significant.

Results

Treatment Time and Concentration of Tanespimycin

When the hNIS-ARO cells were incubated with various concentrations of tanespimycin for 24 h, the treatment of 1 μ M tanespimycin showed highest level of I-125 uptake (Fig. 1a). In the KClO₄-treated cells, NIS-mediated iodide uptake was specifically eliminated (*first lane*). For optimizing the treatment time, we observed the I-125 uptake for various time points for 72 h. At 48 h, iodide uptake increased to a maximum after treatment with 1 μ M tanespimycin (Fig. 1b).

Tanespimycin Effect on Iodine Accumulation in Normal and Cancer Cells

In FRTL-5 normal rat thyroid cells, hNIS-ARO human thyroid cancer cells, and hNIS-MDA-MB-231 human breast cancer cells, tanespimycin induced a significant increase in radioiodide accumulation (~2.5-fold) (Fig. 2a–c). However, in hNIS-HeLa human cervical cancer cells, 1 μ M tanespimycin did not alter iodide uptake (Fig. 2d).

Regulation of NIS Expression by Tanespimycin

Highly expressed NIS at transcription levels was detected in FRTL-5, hNIS-ARO, hNIS-MDA-MB-231 and hNIS-HeLa cells. After tanespimycin treatment, NIS expression was increased in hNIS-ARO, and not significantly altered in

Fig. 1 Determination of tanespimycin concentrations and treatment time. **a** ARO cells that stably express hNIS were incubated with 0.5 μM, 1 μM, 2 μM, 4 μM tanespimycin and DMSO-dissolving solution. Radioactive iodide uptake was detected at its highest levels when the cells were treated with 1 μM tanespimycin. **b** hNIS-ARO

cells were incubated with 1 μM tanespimycin for 24 h, 48 h and 72 h, and the maximum ¹²⁵I accumulation value was noted after 48 h of treatment. The statistical contrast of ¹²⁵I uptake between groups was evaluated via a *t*-test. *P* values of < 0.05 were considered statistically significant

FRTL-5 and hNIS-MDA-MB-231 cells. Decreased NIS mRNA expression was observed in hNIS-HeLa (Fig. 3).

Effect of Tanespimycin on Radioiodine Efflux Rate from Cells

After the remaining ¹²⁵I radioactivity in the cells was measured, the efflux rate of radioiodine from the cells was

calculated at various times. The efflux rate of ¹²⁵I declined significantly in tanespimycin-treated FRTL-5 cells when compared with that observed in the DMSO-treated control cells (Fig. 4a). The percent increase in intracellular radioactivity between the DMSO-treated and tanespimycin-treated cells was significant (+17 % to +649 %). In hNIS-ARO, hNIS-MDA-MB-231 and hNIS-HeLa cells, the rates of iodine efflux were unaffected by tanespimycin treatment

Fig. 2 Altered radioactive iodine accumulation ability in cells by tanespimycin. FRTL-5, hNIS-ARO, hNIS-MDA-MB-231, and hNIS-HeLa cells were incubated for 48 h with 1 μM tanespimycin, and iodide uptake capacity was estimated via an ¹²⁵I uptake assay. **a** Radioactive iodine accumulation was markedly increased in FRTL-5, **b** hNIS-ARO, and **c** hNIS-MDA-MB-231 cells; **d** however, it was reduced slightly in hNIS-HeLa cells

Fig. 3 Altered NIS expressions induced by tanespimycin treatment. Under normal conditions, the NIS gene was expressed in FRTL-5, hNIS-ARO, hNIS-MDA-MB-231 and hNIS-Hela cells at transcription levels (darker bar). Incubation with 1 μ M tanespimycin for 48 h increased NIS expression in hNIS-ARO cells, decreased it in hNIS-HeLa cells, and effected no detectable alterations in FRTL-5 and hNIS-MDA-MB-231 cells (lighter bar)

(Fig. 4b–d). The majority of radioactive iodine was eliminated from the cells 60 min after removing the radioiodine source.

Regulation of PDS Expression by Tanespimycin

In FRTL-5 cells, PDS was expressed abundantly at transcription levels. Furthermore, profound reductions in the expression of PDS genes were noted in the tanespimycin-treated

FRTL-5 cells, which evidenced a significantly reduced radioiodine efflux rate by tanespimycin treatment (Fig. 5a, b). Tanespimycin treatment lowered PDS expression in hNIS-ARO and showed less effect in hNIS-MDA-MB-231 cells. The PDS gene was not expressed hNIS-HeLa cells.

Discussion

Radioiodine therapy constitutes an attractive approach to the treatment of thyroid carcinoma [17]; however, there are some limitations that should not be overlooked [18]. One is the short retention time of radioiodine in the thyroid. It was demonstrated previously that more than 90 % of radioactive iodine was removed from the cells within 30 min in the FRTL-5 cells, and ^{131}I treatment did not markedly reduce the volume of tumors generated in an in vivo study [19].

Increased accumulation of iodide in the cells is primarily attributable to increased iodide influx, but is also affected by iodide efflux [5]. Previously, some chemicals that might induce an increase in the accumulation of radioiodine in thyroid cells via enhanced iodide intake, or reduced iodine efflux from cells, have been identified. Compounds such as retinoic acid [20], valproic acid [21], and LY294002 [22] may induce increases in cellular iodide accumulation by enhancing NIS gene expressions via a variety of mechanisms. Chemicals that may reduce the efflux rates of iodine, such as lithium [23], DIDS, and tanespimycin [9, 10] have also been recognized; however, the mechanisms relevant to such activities have not yet characterized in detail.

Fig. 4 Altered efflux rate of radioactive iodine from cells by tanespimycin. ^{125}I effluxed from cells was collected every 3 min until the 30 min mark and every 10 min until the 60 min mark. **a** In FRTL-5 the efflux rate of ^{125}I from cells was significantly reduced by tanespimycin treatment. **b–d** In hNIS-ARO, hNIS-MDA-MB-231 and hNIS-Hela cells, tanespimycin did not affect the rate of ^{125}I efflux from the cells

Fig. 5 Differing PDS expressions resulting from tanespimycin treatment. Under normal conditions PDS was expressed in FRTL-5, hNIS-ARO, and hNIS-MDA-MB-231 cells at transcription levels. Notably reduced PDS expression in the FRTL-5 and hNIS-ARO cells at 48 h of incubation with 1 μ M tanespimycin was detected via **a** real-time PCR and **b** RT-PCR

Tanespimycin, a less liver toxic and more stable geldanamycin derivative [24] has been previously identified as a chemotherapeutic agent. MicroPET images showed that 4 h or 24 h of tanespimycin treatment induced significant reductions in tumor size in PC-3 tumor-bearing nude mice [25]. Though Bristol-Myers Squibb halted the development of programs for multiple myeloma at the clinical stage 3 trial because of financial issues (<http://www.myelomabeacon.com/news/2010/07/22/tanespimycin-development-halted/>), various phases of clinical trials were completed with satisfactory results in patients having melanoma, thyroid, breast, pancreatic, and prostate cancers (<http://clinicaltrialsfeeds.org/clinical-trials/results/?term=Drug+tanespimycin>).

Several investigation describing increased radioiodine accumulation in thyroid cells and thyroid cancer cells as the result of tanespimycin treatment were reported [9, 10], but the specific mechanism relevant to this effect has yet to be characterized in detail. In this study, increased iodide

uptake due to tanespimycin treatment was noted in FRTL-5 rat thyroid cancer and human NIS-expressing hNIS-ARO human thyroid cancer cells (Fig. 2). To the best of our knowledge, this study is the first to show that tanespimycin can also effect an increase in radioiodine accumulation in human NIS-expressing hNIS-MDA-MB-231 human breast cancer cells (Fig. 2c). Tanespimycin induced no detectable increases in iodide uptake in the hNIS-HeLa cells (Fig. 2d). These results demonstrate that the effects of tanespimycin on increased iodine accumulation are related to cellular origin.

Iodine transport across the thyroid cell membranes is a critically important mechanism for the production of thyroid hormones. Two previously characterized genes aside from NIS may be responsible for this phenomenon [26]. The first of these is SLC26A4, which encodes for the pendrin (PDS) expressed in apical membranes and has been suggested to transfer iodide into the colloid from the cells. It has

previously shown that PDS expression was detected in thyroid (B-CPAP), thyroid cancer (WRO, FRO, ARO) [27] and breast cells (MCF-7) [28]. The other is SLC5A8 (human apical iodide transporter, hAIT), which was suggested to charge the release of iodide into the colloid across the apical membrane as an iodide channel [29, 30]. However, this gene was found to serve as a sodium monocarboxylate transporter and does not mediate iodide uptake or efflux [31]. The actual actions of the PDS gene remain to be clearly demonstrated; however, PDS is primarily thought to mediate iodide efflux from cells [32].

Tanespimycin increased the mRNA expression of NIS only in hNIS-ARO cells, and not in FRTL-5 or hNIS-MDA-MB-231 cells (Fig. 3); however, it induced a significant increase in iodide uptake in FRTL-5, hNIS-ARO and hNIS-MDA-MB-231 cells (Fig. 2). Though the increased expression of NIS by tanespimycin was only observed in hNIS-ARO, the mRNA expression of PDS was markedly reduced by tanespimycin treatment in FRTL-5 and hNIS-ARO. This finding indicates that the tanespimycin-induced reduction in PDS expression may be a more important key factor than the tanespimycin-induced effect on NIS gene expression in thyroid cells.

Breast cancer cells showed tanespimycin induced radioiodine uptake without inducing NIS expression, or reducing PDS expression for decreasing the rate of radioiodine efflux. This suggests that tanespimycin might also affect other genes that have yet to be discovered, but are related to iodine accumulation. However, the iodine accumulation in cells is the result of the relation between influx and efflux of the ions, which are also dependent on the electrochemical gradient, ATP production, other anion concentrations, and the absolute amount of NIS or intracellular fixation. Also other effects are possible, like effects of tanespimycin through HSP-90, that works synergistically to stabilize the cytoskeletal anchorage of Na,K-ATPase. Therefore, the factors such as Na-K ATPase or other gene products that may effect on radioiodine uptake should be clarified by further experiments. Different cell types should be considered for understanding the mechanism of iodine uptake because a variety of proteins involving iodine uptake occurred in each cell type, indicating that their amount and distribution may be critical in determining iodine accumulation.

All trans-retinoic acids (RA) have been shown to induce diametrically opposite responses in non-transformed and carcinoma cells [33]. In FTC-133 and FTC-238 human thyroid cell lines, RA induced an increase in NIS mRNA levels; however, in FRTL-5 rat thyroid cells, RA treatment suppressed the expression of functional NIS and reduced iodine accumulation in the cells. The findings of this study also demonstrate that tanespimycin differently affects radioiodine efflux, depending on the cellular origin. Although tanespimycin increased the accumulation of radioactive

iodine under steady-state conditions in FRTL-5 and hNIS-ARO, the efflux rate of ^{125}I was reduced only in FRTL-5 normal rat thyroid cells. In the context of thyroid cancer treatment, the use of tanespimycin may generate additional outcomes by increasing radioiodine accumulation in the thyroid, which would be expected to enhance the efficacy of anti-cancer treatments. Combination therapy with other drugs should also be taken into consideration. As retinoic acid increased iodine uptake in thyroid carcinoma cells, although not in non-transformed thyroid cells [32], the combination of tanespimycin and RA may exert a synergistic effect. Tanespimycin would eliminate thyroid carcinoma with radioiodine accumulation increscent and chemotherapeutic effects; furthermore, RA may specifically increase the uptake of radioactive iodine in carcinoma tissues, thereby attenuating iodide uptake in the surrounding normal thyroid tissues [4].

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Conflicts of Interest The authors have no conflicts of interest to declare.

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