RESEARCH ARTICLE

Editorial Process: Submission:02/11/2025 Acceptance:10/31/2025 Published:11/21/2025

DN200434: An Inverse Agonist of Estrogen Related Receptor Gamma Enhances Na+/I- symporter Function through Mitogen-Activated Protein Kinase Signaling in Radioiodine-Refractory Papillary Thyroid Cancer Cells

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Abstract

Background: Earlier, we have reported a series of inverse agonist of estrogen related receptor gamma (ERRγ), viz DN200434, GSK5182 and its derivates, that enhance sodium iodide (Na+/I-) symporter (NIS) function through mitogen-activated protein (MAP) kinase signaling in anaplastic thyroid cancer cells. But, the effect of our recently discovered DN200434, on NIS function in papillary thyroid cancer (PTC) refractory to radioactive iodine (RAI) therapy has not been reported. Herein, we studied the effects of DN200434 in RAI-resistant PTC cells on ERRγ-mediated regulation of NIS function. **Methods:** RAI-refractory BCPAP cells were exposed to lower concentrations of DN200434 and the NIS function in the PTC cells was serially assessed by radioiodine uptake assay. Immunoblot assay was performed to study the effect of DN200434 on ERRγ, NIS, the mitogen-activated protein (MAP) kinase pathway, and iodide-handling genes. To examine whether the DN200434-induced NIS functional activity can be affected by inhibition of the MAP kinase pathway, radioiodine uptake was performed after the application of U0126, a selective MEK inhibitor to DN200434-treated cells. Finally, the cytotoxic effect of ^{131}I was determined in DN200434 treated and untreated cells by clonogenic assay. **Results:** Treatment of DN200434 resulted in dose-dependent increases in iodide uptake in BCPAP cells, downregulation of ERRγ protein and the activation of extracellular signal-regulated kinase (ERK) 1/2. Treatment of the specific MEK inhibitor overturned the increased radioiodine uptake and ERK1/2 activation of BCPAP cells. DN200434 treatment enhanced the membrane localization of NIS in BCPAP cells. Clonogenic assay results revealed enhanced cytotoxic effects of ^{131}I in DN200434 pre-exposed BCPAP cells. **Conclusion:** Thus, we successfully demonstrate that our novel inverse agonist of ERRγ, DN200434, enhances the responsiveness of radioiodine therapy by modulating NIS function in RAI-refractory papillary thyroid cancer cells via the regulation of ERRγ and the MAP kinase signaling pathway.

Keywords: Estrogen-related receptor gamma- sodium iodide symporter- radioiodine- radioiodine uptake

Asian Pac J Cancer Prev, **26 (11)**, 3987-3995

Introduction

Thyroid cancer is considered as the most common endocrine disorder in the last decade [1]. Thyroid cancers are classified based on their origin, and more than 80% of thyroid cancers are well-differentiated cancer that are derived from follicular epithelial cells such as papillary thyroid carcinoma (PTC), and follicular thyroid carcinoma (FTC). Anaplastic thyroid cancer (ATC) is undifferentiated cancer that consist of less than 5% and is highly lethal. Lack of deep molecular insight studies and existing primitive diagnostic tools contribute to the failure of the clinical cure for radioiodine-refractory PTC and ATC [2]. Therefore, a better understanding of the signaling pathways during thyroid cancer development is required to identify new biomarker and therapeutic target for a novel therapeutic approach.

The sodium iodide (Na+/I-) symporter (NIS) is a transmembrane glycoprotein which are endogenously expressed in normal thyroid and cancerous cells. NIS actively transport iodide into follicular cells of the thyroid gland as a normal process for thyroid hormone synthesis [3]. Over the years, endogenous NIS expression has been exploited for diagnosis purpose as well as radioiodine

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therapy for eliminating malignant cells. However, poorly differentiated cancer cells, including ATC cells and radioiodine-refractory PTC tend to exhibit a progressive dedifferentiation that leads to a decrease in levels of NIS [4, 5]. Papillary thyroid cancers (PTCs) are often treated effectively with RAI due to higher expression of functional NIS and iodide-handling genes. Nevertheless, some cases of PTCs do not response efficiently to radionuclide therapy due to advanced dedifferentiation of the tumor. RAI-refractory PTCs are often associated with lower expression of NIS and iodide-handling genes [5, 6] leading to their resistance to radionuclide therapy with poor patient compliances. Several studies have explained the mechanism for the decrease in functional NIS expression in poorly differentiated PTCs by modifying the epigenome [7, 8]. Various chemical or agents with epigenetic modulating properties and gene delivery have been explored to restore NIS function in poorly differentiated PTC cells [9 -11], but the outcome are not satisfactory.

Estrogen Related Receptors (ERRs) are recognized as the first orphan nuclear receptors based on their sequence similarity with estrogen receptors (ERα, ERβ, and ERγ) [12]. ERRs are abundantly expressed in the most of the vital organs including brain, liver, heart, pancreas, kidney, and placenta [13]. ERRs subfamily consists of three members: ERRα, ERRβ, and ERRγ, and play a central role in energy homeostasis, cellular metabolism and cancer [14]. Estrogen related receptor gamma (ERRγ) has been reported to play vital role in the development of metabolic diseases such as type 2 diabetes mellitus, liver injury, alcohol-induced oxidative stress [14]. In addition, ERRγ has been reported as potential biomarker in various cancers including liver cancer [15], breast cancer [16], prostate cancer [17], and gastric cancer [18]. Recently, based on immunohistochemistry (IHC) results from 36 normal, 96 PTC and 26 ATC thyroid tissue samples, we revealed a significantly higher expression of ERRγ in ATC and PTC tissues [19]. Earlier, for the first time, we discovered the role of ERRγ in upregulating NIS function in anaplastic thyroid cancer (ATCs) [20] and RAI-refractory PTC [21] using the selective inverse agonist of ERRγ, GSK5182 (a 4-hydroxy tamoxifen analog). Next, we discovered another novel orally active inverse agonist of ERRγ, DN200434 which can booster NIS expression in ATC bearing mice model making it susceptible to radioiodine therapy [19]. The potential of DN200434 in upregulating NIS function in PTC remains to be explored. In context to the larger number of patients with RAI-refractory PTC patient compared to ATC, it is important to study whether DN200434 can facilitates the responsiveness to radioiodine therapy by modulating sodium iodine symporter (NIS) function in RAI-refractory advanced PTCs cells via ERRγ and MAP kinase signaling pathway.

Herein, we tried to investigate the changes in endogenous ERRγ protein and MAP kinase signaling upon DN200434 treatment leading to restoration of NIS and iodide handling genes, radioiodine avidity, and finally enhancing cytotoxic effects of ^{131}I in iodine-resistant PTC cells.

Materials and Methods

Cells

BCPAP, papillary thyroid cancer cell lines, was purchased from DMSZ, German collection of cell lines. BCPAP cell lines were maintained in high-glucose Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and a 1% penicillin – streptomycin solution (Invitrogen) at 37 ºC in a 5% CO_2 atmosphere.

^{125}I uptake assay

Radioiodine ^{125}I uptake assay was performed as mentioned in our previous reports [19, 20]. Briefly, for the ^{125}I uptake assay, BCPAP cells were plated in 24-well plates, followed by the treatment of DN200434 the next day for another 24 h. (DN200434 was synthesized by Daegu-Gyeongbuk Medical Innovation Foundation (DGMIF, Daegu, Korea and prepared as a 50mM stock solution in DMSO and stored at -80°C). BCPAP cells were washed twice with HBSS (Hank's balanced salt solution), followed by incubation of 3.7 kBq carrier-free ^{125}I (Perkin-Elmer) and 10μmol/L sodium iodide (specific activity of 740MBq/mmol) prepared in 500μL of (HBSS) supplemented with 0.5% bovine serum albumin (BSA) at 37°C for half an hour. The cells were lysed with of 2% sodium dodecyl sulfate (SDS) after washing thoroughly with bHBSS. The radioactivity in the cell lysates were determined by a gamma counter (Packard Cobra II gamma-counter (PerkinElmer, MA). For radioiodine inhibition assay, cells were pre-incubated with 300 μM KClO4 (NIS inhibitor) for 30 min, followed by treatment of ^{125}I as mentioned above.

^{18}F-FDG (Fluoro-Deoxy-Glucose) uptake assay

BCPAP cells were seeded in 24-well plate and the next day, DN200434 was treated for 24 h. Before ^{18}F-FDG uptake assay, the cells were thoroughly washed with cold HBSS and incubated with HBSS containing 0.5% BSA and 74 kBq of ^{18}F-FDG / mLfor 30 min at 37°C. The cells in 2% SDS lysis solution were subjected to constant vortexing to lyse the cells. Finally, the radioactivity was measured using a gamma-counter. The radioactivity of the cells was normalized using total protein concentrations determined by a BCA kit as described [19].

Clonogenic Assay

BCPAP cells (1000 cells/well) were seeded into 6-well plates and the cells were allowed to adhere firmly for 2 days. Then, the cells were treated with 12 μM of DN200434 for 24h, followed by thorough washing with cold phosphate-buffered Saline (PBS). The BCPAP cells were further incubated for 6 h in DMEM medium with or without 50μCi ^{131}I. After washing these cells with HBSS, each well was replenished with completed culture medium and left for few days up to six doublings. Once, the colonies were visible, the cells were washed with PBS, fixed in 4% Paraformaldehyde (PFA) solution followed by 0.05% crystal violet staining. The treated and untreated colonies were counted after washing the excess crystal violet stain with tap water.

Western Blot

The BCPAP cells treated with or without DN200434 were lysed with RIPA buffer containing protease inhibitor (PI). Plasma membrane (PM) was prepared after incubation of cell lysates with protein biotinylation kit (EZ-Link™Sulfo-NHS-Biotin, Thermo Scientific). After washing the cells with ice-cold PBS/CM (PBS with 0.1 mM calcium chloride and 1 mM magnesium chloride), the cells were incubated with EZ link NHS-Sulfo-SS-biotin (1 mg/mL) in PBS/CM for 30 min at 4 ºC. The cells were incubated in 100 mM glycine in PBS/CM at 4 ºC for 20 min. After thorough washing of cells with PBS/CM, the cells were lysed using RIPA buffer containing PI cocktail and phosphatase inhibitor and incubated in ice for 1 hour with regular vigorous shaking. Next, the supernatant (total protein) of the lysates were collected after centrifugation at 16,000g for 30 min at 4 ºC. The remaining membrane protein was further incubated in 100 µL streptavidin beads (Thermo Scientific) for 1 h. After washing the beads with RIPA buffer, and bound proteins were eluted with 50 µL of Laemmli buffer at room temperature. The total and biotinylated PM protein was resolved using gradient Bis-Tris gel. and later transferred to 0.2-µm PVDF membrane. After blocking the membrane with 1% BSA for 1 hour, the blot is incubated in primary NIS antibody (dilution, 1:1000) overnight at 4 ºC, followed by incubation with respective HRP conjugated secondary antibody for 1 h. After 3 time washing of the blots, the band were developed using ECL solution. The quantification of band densities was done using ImageJ software.

Quantitative RT-PCR

Trizol (Invitrogen) was used to extract total RNA of treated or untreated BCPAP cells and reverse transcription reaction set up using RevertAid First Strand cDNA Synthesis Kit. Next the primer sets: ERRγ (forward, 5'-CAG ACG CCA GTG GGA GCT A -3'; reverse, 5'- TGG CGA GTC AAG TCC GTT CT - 3'), NIS (forward, 5'-TCT AAC CGA TGC TCA CCT CTT CTG -3'; reverse, 5'- AGA TGA TGG CAC CTC CTT GAA CC -3'), and acidic ribosomal protein 36B4 (forward, 5'-CCA CGC TGC TGA ACA TGC T -3'; reverse, 5'- TCG AAC ACC TGC TGG ATG AC -3') were employed for quantitative RT-PCR using SYBR Green PCR master mix and Real-Time PCR System instrument. Finally, all the target genes were normalized with 36B4 gene, and the relative mRNA expression levels were determined.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) from three representative experiments. Statistical significance was determined using an unpaired Student's t-test using Graph Pad (Prism 6), and the p-values of < 0.05 were considered statistically significant.

Results

DN200434 treatment enhanced radioiodine uptake in RAI-refractory PTC cells

Recently, we have reported that the treatment of inverse agonist of ERRγ, GSK5182 could restore the ability of radioiodine uptake in RAI-refractory PTC cells by an increase of NIS protein [21]. Herein, we examined the effect of DN200434 on radioiodine uptake in PTC cells, BCPAP cells. Treatment of DN200434 significantly increased radioiodine uptake in BCPAP cells in a dose- and time-dependent manner (Figure 1A, B) even at much lower concentration 6 µM and 12 µM showing more effectiveness than GSK5182. Treatment of KClO4, a specific inhibitor of NIS completely minimized the increase in radioiodine uptake in DN200434 treated cells (Figure 1C).

DN200434 decreased ERRγ and increased iodide handing gene expression in RAI-refractory PTC cells

Treatment of DN200434 decreased the expression of ERRγ in BCPAP cells at both the mRNA and protein levels in a dose dependent manner, at lower concentration 6 µM and 12 µM (Figure 2A, B). Earlier, we reported that GSK5182 decreased ERRγ mRNA level in BCPAP cells at 25 µM, but not at 12 µM [21].

Treatment of DN200434 (12 µM.) increased NIS mRNA expression in BCPAP cells (Figure 3A). Likewise, treatment of DN200434 at lower dose significantly increased NIS protein in PTC cells but not in vehicle-treated cells (Figure 3B, C). Interestingly, we observed that DN200434 markedly increased the fully functional membranous NIS and partially glycosylated protein (95 kDa and 50 kDa, respectively) (Figure 3B and D). We also observed the upregulation of iodide-handling genes such

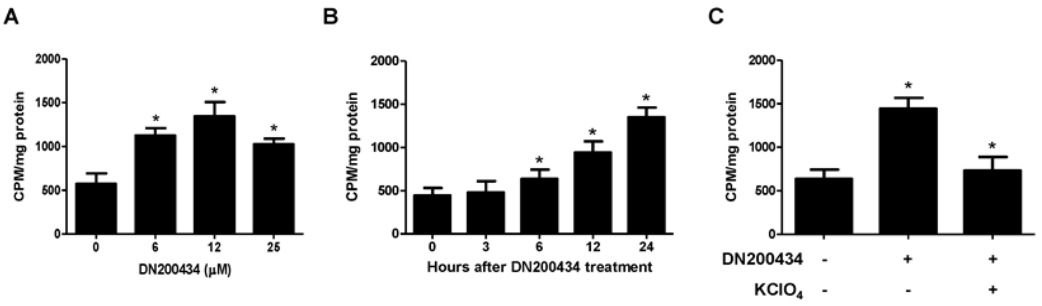

Figure 1. Effect of DN200434 on Radioiodine Uptake in BCPAP Cells. (A) Dose dependent radioiodine uptake in DN200434 treated BCPAP cells (B) DN200434-treated BCPAP cells showed time-dependent radioiodine uptake (C) NIS inhibitor inhibits radioiodine uptake in DN200434-treated BCPAP cells. *, $P<0.05$. Data are mean±SD of 3 samples per group.

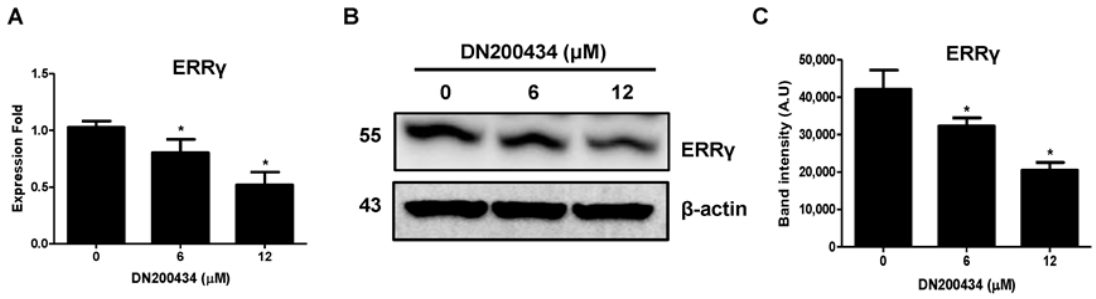

Figure 2. Effect on ERRγ Expression in DN200434-treated BCPAP Cells. (A) Dose-dependent decrease in ERRγ mRNA expression (B) Dose-dependent decrease in ERRγ protein (C) Quantification of ERRγ protein level by Image J. *, P<0.05, Data are mean±SD of 3 samples per group.

as TG, TPO, and TSHR at protein level in DN200434-treated cells compared to untreated cells (Figure 4A-D).

DN200434-induced radioiodine uptake through mitogen-activated protein (MAP) kinase signaling in RAI-refractory PTC cells

Earlier, we have reported that both the ERRγ inverse agonists, GSK5182 and DN200434 upon treatment to ATC cells significantly increased the phosphorylated MAP kinase levels, such as p44 and p42 ERK (pERK1/2) [19, 20].

In consistent with our previous findings [21], the treatment of DN200434 in BCPAP cells increase the phosphorylated MAP kinase levels, such as p44 and p42 ERK (pERK1/2), in dose dependent manner (Figure 5A, B).

In order to check whether the increased in radioiodine uptake upon DN200434 treatment in BCPAP cells is through MAP kinase pathway, we performed radioiodine uptake followed by western blotting after pre-exposure of U0126, a MEK-specific inhibitor and DN200434. The increased in radioiodine uptake after DN200434 exposure was completely inhibited by U0126 (Figure 5C). Furthermore, the immunoblotting result revealed that the upregulation of pERK1/2 after DN200434 exposure was completely blocked to basal levels by U0126 treatment (Figure 5D). Interestingly, treatment of MEK-inhibitor alone did not increase radioiodine uptake in BCPAP cells.

DN200434 effects glucose metabolism in RAI-refractory PTC cells

We observed a dose-dependent reduction in ^{18}F-FDG uptake upon DN200434 treatment in BCPAP cells (Figure 6A). Immunoblot of endogenous glucose transporter proteins like GLUT1 and GLUT4 in DN200434 treated cells further corroborate that the reduction in ^{18}F-FDG uptake is due to the decreased protein expression of GLUT1 and GLUT4 in DN200434-

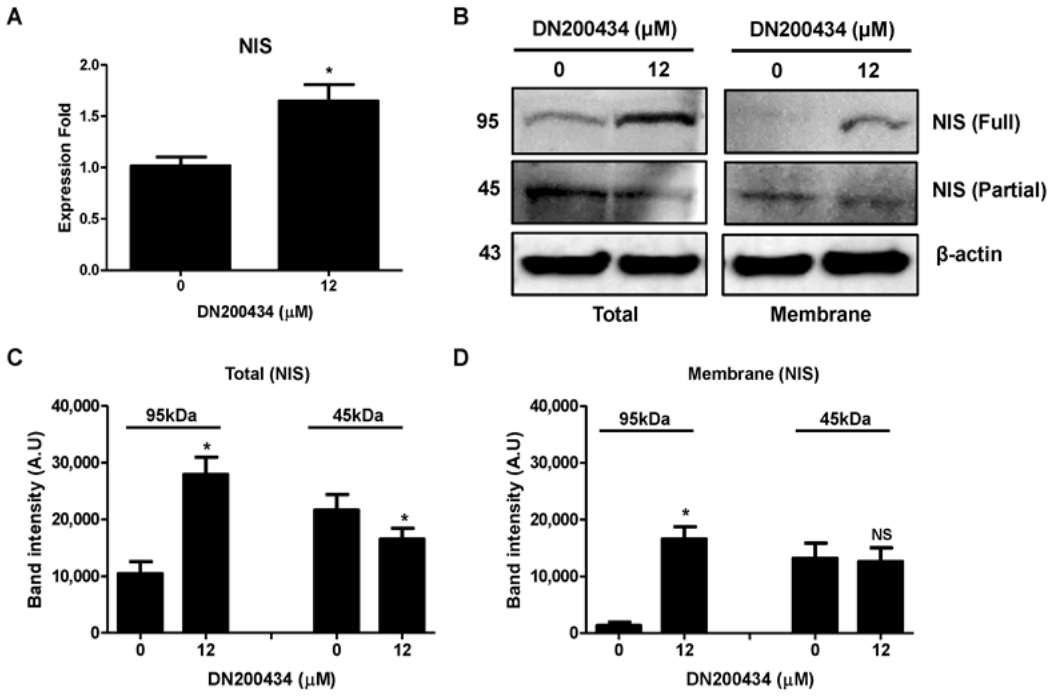

Figure 3. Effect on NIS Expression in DN200434-Treated BCPAP Cells. (A) DN200434 increased NIS mRNA expression (B) DN200434 increased membrane NIS protein expression (C and D) Quantification of total NIS and membrane NIS protein level by Image J. *, P<0.05, Data are mean±SD of 3 samples per group.

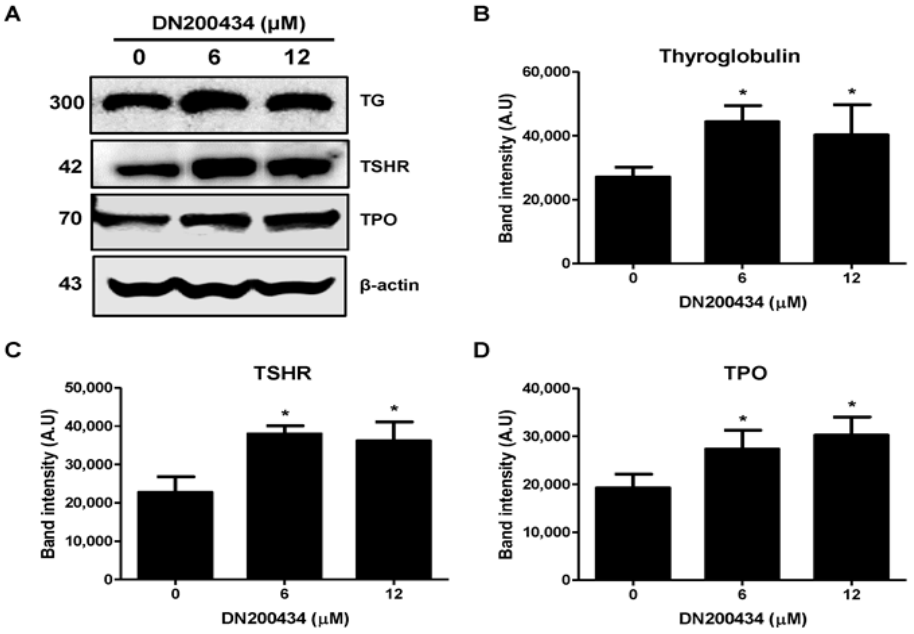

Figure 4. Changes in Iodide Handing Genes after DN200434 Treated BCPAP Cells. (A) Treatment of DN200434 increased TG, TSHR, and TPO protein expressions. (B, C, D) Quantitative analysis of TG, TSHR and TPO expression level respectively by Image J. *, P<0.05. Data are mean±SD of 3 samples per group.

treated BCPAP cells (Figure 6B and 6D).

Increase of ^{131}I*-mediated cytotoxicity in in RAI-refractory PTC cells by DN200434*

The successful application of radioiodine therapy largely depends on the ability and susceptibility of radioiodine uptake in thyroid cancer. Therefore, the increased in radioiodine uptake upon DN200434 exposure may enhanced cytotoxic effects of ^{131}I against BCPAP cells.

Accordingly, a clonogenic assay with ^{131}I confirmed minimal cytotoxic effects in the BCPAP cells treated with either DN200434 or ^{131}I alone (Figure 7A). The relative colony-forming ability in the DN200434- and ^{131}I-treated cells is 96.6 ±9% and 92.4± 14%, respectively (Figure 7B). However, the combination of DN200434 with ^{131}I resulted

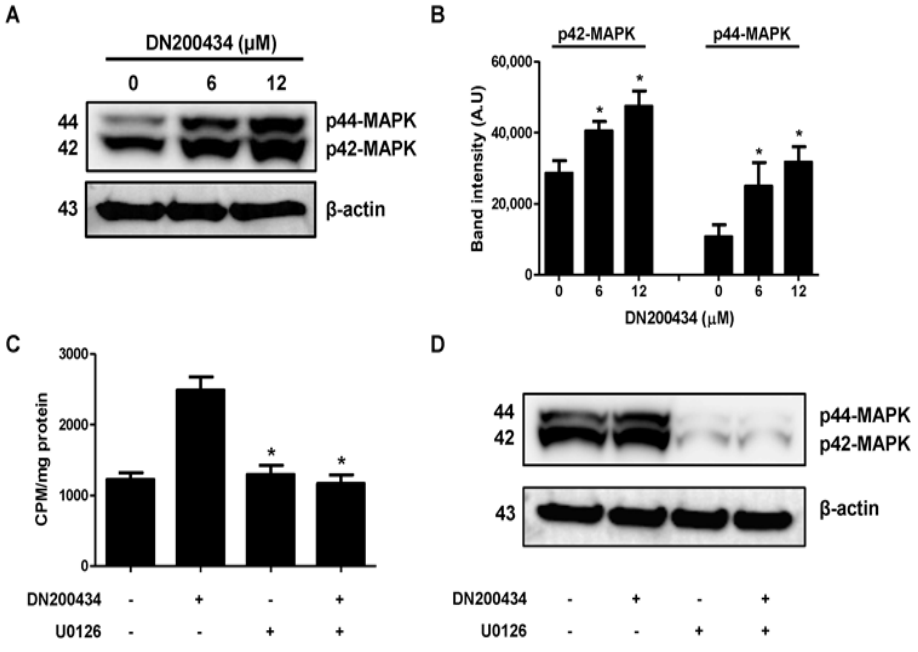

Figure 5. DN200434 Activated MAP Kinase Activity in BCPAP Cells. (A) Increased expression of pERK1/2 protein and (B) Quantification of pERK1/2 band intensity (C) Inhibition of increased radioiodine uptake by U0126 (D) Reversal of activated MAK kinase signaling by U0126 *, P<0.05. NS, not significant. Data are mean±SD of 3 samples per group.

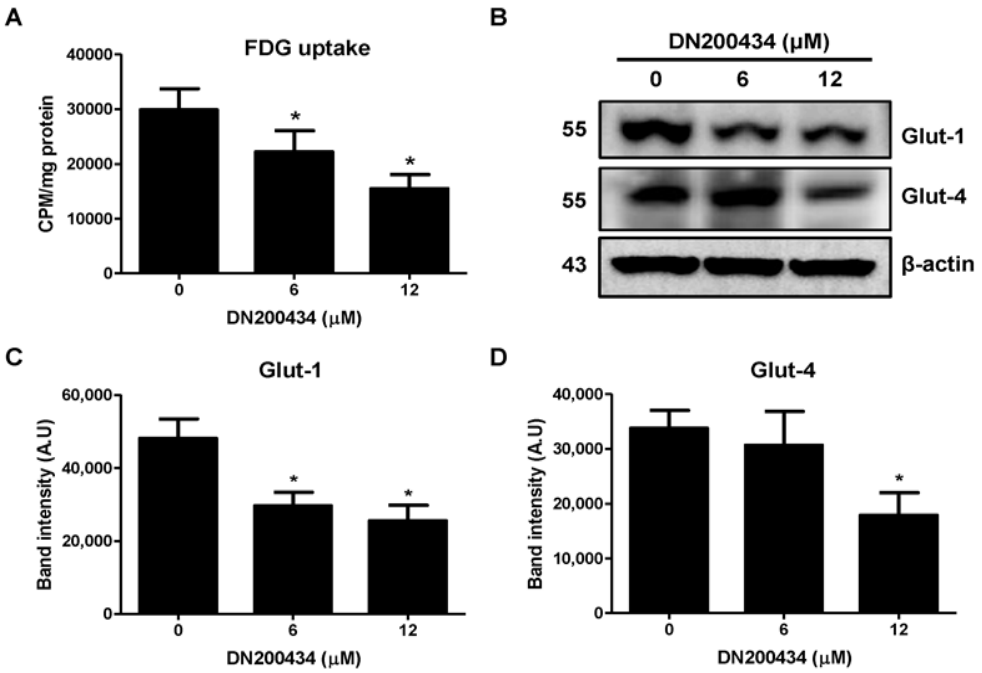

Figure 6. DN200434 Decreased Glucose Uptake in BCPAP Cells. (A) Decreased 18F-FDG uptake in BCPAP (B) Decreased of Glut 1 and 4 protein level (C and D) Quantification of Glut-1 and Glut-4 band intensities by Image J. *, P<0.05. Data are mean±SD of 3 samples per group.

in the marked reduction of colony-forming ability to 55.1 ±13% approximately in BCPAP cells (P<0.05).

Discussion

PTC accounts for more than 75% of all thyroid cancers and its incidence is increasing in the last 25 years [1, 2, 22]. Most PTCs are successfully treated by conventional therapeutic approaches [23], and radioactive iodine ^{131}I therapy has been the backbone for treating high-risk differentiated thyroid cancer (DTC) [24]. Dedifferentiated PTCs are often considered more aggressive that can metastasis to distant organs. Dedifferentiation PTCs are associated with lower expression of functional Na/I symporter (NIS) [5] and hence become resistance to radioiodine therapy [25, 26]. Patients with RAI-refractory (RAI-R) metastatic thyroid cancer have very poor outcome with less than 10% of 10-year survival rate [27]. Therefore, it is utmost importance to identify a novel and more effective therapeutic approaches to treat RAI-refractory PTC. Patients with RAI-refractory (RAI-R) have been treated with different tyrosine kinase inhibitors to re-sensitize RAI-R tumors before radio iodine therapy [28–30]. Herein, we investigated if the targeting of ERRγ

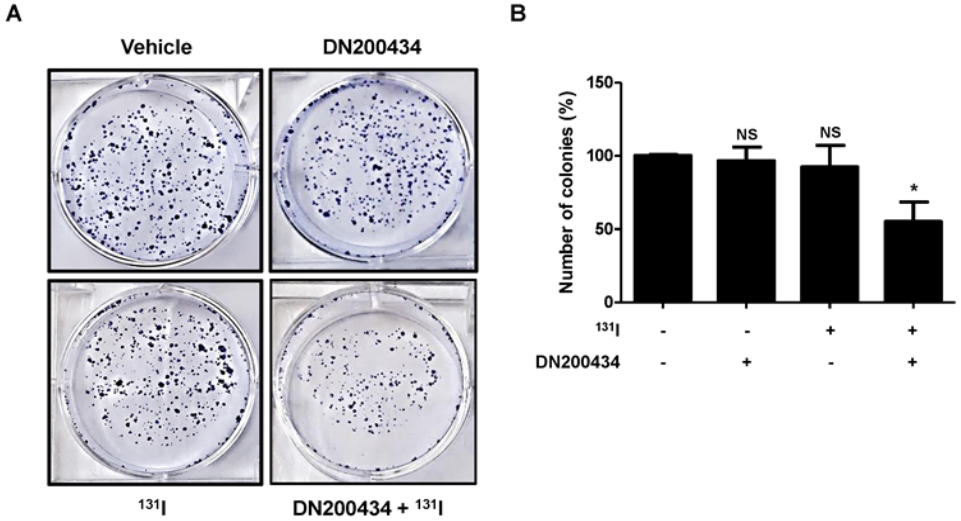

Figure 7. Increased Cytotoxicity of ^{131}I by DN200434 in BCPAP Cells. (A) Representative images of crystal violet staining after DN200434 or 131I alone, and their combination. (B) Quantification of colony number. *, P<0.05. NS, not significant. Data are mean±SD of 3 samples per group.

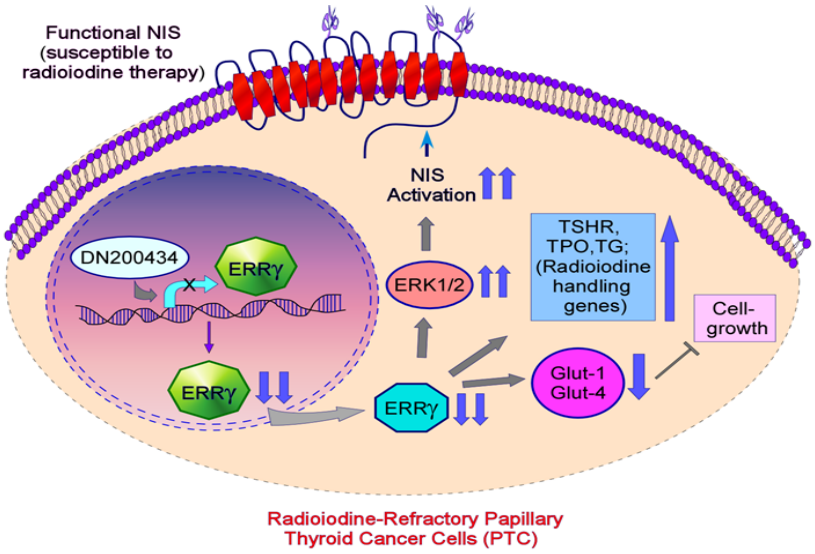

Figure 8. Enhanced Proposed Mechanism of DN200434-Induced Modulation of NIS Function in RIA Refractory PTC Cells through MAP Kinase Pathway

with our recently discovered DN200434, can overcome the mechanisms, and improve outcomes in RAI-R thyroid cancer cells (Figure 8).

The lower expression of NIS and poor radioiodine avidity in RAI-refractory PTC has been well reported [4,5]. Earlier, we have shown whether GSK5182 could restored radioiodine avidity in RAI-refractory PTC and ATC cells. Treatment of GSK5182 resulted in more radioiodine uptake due to enhanced NIS functional activity upon ERRγ modulation in ATC and PTC [20, 21]. In addition, we have shown the upregulation of functional NIS upon DN200434 treatment in ATC cells restoring the radioiodine avidity in vitro and in vivo [19]. Similarly, here we have shown that the exposure of DN200434 to RAI-refractory PTC cells could increase radioiodine uptake. DN200434 treatment could activate pERK1/2 in BCPAP cells, while pre-exposure of specific MAP kinase inhibitor U0126, inhibited the increased pERK1/2 resulting in the reduction of DN200434-induced radioiodine uptake. Thus, the targeting of ERRγ in RAI-refractory PTC cells could be a novel strategy to increase of radioiodine avidity via MAPK kinase activation.

The presence of functional NIS protein and plasma membrane-localized NIS protein is crucial for successful radioiodine therapy [3]. Herein, we evaluated whether the treatment of DN200434 in RAI-refractory PTC cells brought any changes in the total and plasma membrane NIS protein. As anticipated, DN200434 markedly induced an increase in the total NIS and fully glycosylated membrane NIS protein, that may be due to posttranslational modification [31].

Enhanced glucose metabolism is associated with poorly differentiated thyroid cancer, along with changes in iodide handling genes [32, 33]. The discovery of novel compounds that can redifferentiate the RAI-refractory thyroid cancer and restore radioiodine avidity is of great importance. In our present study, we have successfully demonstrated that targeting of ERRγ with DN200434 in BCPAP cells not only increase the expression of iodide-handling genes (NIS, TPO, TG, TSHR), but also effectively reduce glucose uptake due to lower expression of glucose transporters. Overall, our results revealed that a lower dose of DN200434 could initiate redifferentiation in BCPAP cells. Accordingly, we observed an enhanced the cytotoxic effect of ^{131}I on DN200434 treated BCPAP cells, whereas ^{131}I and DN200434 had negligible killing effect. These increased in significant cytotoxic effect of ^{131}I in DN200434 treated RAI-refractory BCPAP cells is due to the restoration of radioiodine avidity.

In conclusion, our recently discovered ERRγ inverse agonist, DN200434 could modulate the ERRγ to increase of radioiodine avidity in RAI-refractory PTC cells. We also noticed that DN200434 is more potent than GSK5182 in targeting ERRγ in RAI-resistant PTC cells in vitro. Given the fact, that DN200434 could boost functional NIS expression in in vivo model, it will be interesting to study the potential of DN200434 in in vivo RAI-refractory PTC cells. Moreover, a detailed investigation is needed to explain the mechanism of DN200434-mediated restoration of radioiodine avidity in more RAI-refractory thyroid cancer cells in vitro and in vivo model.

Author Contribution Statement

Conception and Design: T.D.S; Experiments: V.S, P.U, D.G, R.G; Analysis and Interpretation: V.S, P.U; Writer: T.D.S; Critical review: T.D.S; Funding: T.D.S; Supervision: T.D.S. All authors have read and agreed to the submitted version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

Funding

This work is supported by the Indian Council of Medical Research (ICMR), IIRP-2023-0914, Science & Engineering Research Board (SERB), India, (CRG/2023/001373), Department of Health Research (DHR), India (File No.R.11012/06/2021-GIA/HR)

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

No human samples and animal studies involved. All the cell lines experiments were performed as per applicable institutional guidelines.

Conflicts of Interest

The authors declare no conflict of interest.

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