MODULATION OF SODIUM/IODIDE SYMPORTER EXPRESSION AND FUNCTION IN THYROID

Dissertation

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By

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Na\(^+\)/I\(^-\) Symporter (NIS) is an intrinsic membrane glycoprotein expressed on the baso-lateral membrane of thyroid follicular cells that mediates active iodide uptake into the thyroid gland. NIS-mediated iodide uptake and iodide organification is the basis for the post-operative use of radioiodide in detection and targeted ablation of differentiated thyroid cancer. However, about 20-30% patients with metastatic thyroid cancer do not benefit from radioiodine therapy due to reduced or absent NIS expression/function. Thus, it is of clinical importance to investigate the underlying mechanism of NIS modulation such that strategies to selectively upregulate NIS expression and/or functional activity can be devised.

Much effort has been made to identify reagents that increase NIS-mediated radioiodine accumulation in cultured thyroid cells, i.e. our lab has previously reported that 17-allyamino-17-demethoxygeldanamycin (17-AAG) increases NIS-mediated radioiodine accumulation by decreasing iodide efflux in NIS-expressing immortalized non-transformed and RET/PTC1 oncogene transformed cultured thyroid cells. However, the finding has not been validated using immune-competent genetically engineered mice carrying thyroid tumors surrounded by physiologically/pathologically relevant microenvironment. Non-invasive radionuclide imaging with micro-single photon
emission computed tomography (micro-SPECT) was used to examine and quantify temporal radioiodine accumulation in both thyroid and salivary glands modulated by triiodothyronine (T3), bovine TSH (bTSH) and/or 17-AAG. The extent of thyroidal radioiodine accumulation stimulated by a single dose of exogenous bTSH in T3-supplemented endogenous TSH suppressed mice was much less than that in non-treated mice, justifying the clinical use of multiple doses of recombinant human TSH or the merit of thyroxine (T4) withdrawal in thyroid cancer patients prior to radioiodine therapy. In addition, the extent and duration of radioiodine accumulation stimulated by bTSH was reduced in thyroid tumor-bearing thyroid glands of thyroid-targeted RET/PTC1 (Tg-PTC1) thyroid cancer mouse model compared to the thyroids in wild type (WT) mice. Lastly, the effect of 17-AAG on increasing thyroidal, but not salivary, radioiodine accumulation was validated in WT mice and in Tg-PTC1 thyroid cancer mouse model. This encourages the study of 17-AAG derivatives not only as novel chemotherapeutic agents to impede tumor progression but also to improve radioiodine therapy in patients with thyroid cancer.

We aimed understand the mechanism underlying regulation of NIS expression/function to identify potential pharmacological inhibitors targeting signaling nodes that would increase NIS mRNA/protein levels and/or radioiodine accumulation in cultured thyroid cells. In particular, the crosstalk between PI3K-Akt-mTORC1 and MEK signaling nodes on NIS modulation was examined. Inhibition of PI3K by LY294002 increased NIS-mediated RAIU activity through upregulation of NIS expression. In contrast, inhibition of mTORC1 by Rapamycin decreased NIS-mediated RAIU activity yet increased NIS protein levels. The differential regulation of NIS-mediated RAIU
activity by LY294002 and Rapamycin is likely due to their differential effect on pERK and pAkt levels. The effect of Akt inhibition on NIS expression/function was further examined showing that Akti-1/2 markedly increased NIS-mediated RAIU activity despite that NIS mRNA and protein levels were not increased. The possible effect of Akt inhibition on cell surface NIS levels was excluded. Akt inhibition decreased iodide efflux rate and increased iodide transport rate and iodide affinity of NIS, suggesting that Akt inhibition increased NIS functional activity. Furthermore, treatment of Akti-1/2 further increased NIS-mediated RAIU activity and decreased total NIS protein levels in LY294002, Rapamycin or PD98059 treated PC Cl3 cells suggesting that NIS modulation by Akt at the protein level as well as RAIU activity appears to prevail over PI3K, mTORC1, and MEK signaling nodes. Interestingly, Akti-1/2 had little effect on acute TSH-stimulated RAIU activity in PC Cl3 cells. The discrepancy of Akti-1/2 effect on RAIU activity between PC Cl3 cells chronically and acutely stimulated by TSH indicates that Akti-1/2 effect on increasing RAIU activity is less prominent in cells acutely stimulated by TSH. Lastly, the effect of LY294002 and Akti-1/2 on NIS protein levels and RAIU activity is not restricted to PC Cl3 cells, but also seen in FRTL-5 cells. However, LY294002 and Akti-1/2 had little effect on cell surface or total NIS protein levels yet decreased NIS-mediated RAIU activity in HEK293 human kidney cells stably expressing exogenous rat NIS. This suggests that NIS-mediated RAIU activity may be modulated by LY294002 and Akti-1/2 in a cell context dependent manner. Consequently, PI3K and Akt inhibitors may serve as potential pharmacological reagents to selectively increase thyroidal radioiodine accumulation.
Dedication

Dedicated to my mother
ACKNOWLEDGMENTS

I would like to first thank my advisor, Dr. Sissy Jhiang, for her support, guidance, encouragement and patience with my graduate studies and career development. I would also like to thank my dissertation committee members, Dr. Lawrence Kirschner, Dr. Chenglong Li and Dr. Jiayuh Lin for their advice on my scientific development.

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<tr>
<td>%ID</td>
<td>Percent of Injected Dose</td>
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<td>17-AAG</td>
<td>17-allyamino-17-demethoxygeldanamycin</td>
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<td>ATC</td>
<td>Anaplastic Thyroid Cancer</td>
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<tr>
<td>AUC</td>
<td>Area Under Time-Activity Curve</td>
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<td>bTSH</td>
<td>Bovine TSH</td>
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<td>BW</td>
<td>Body Weight</td>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>cDNA</td>
<td>Complimentary Deoxynucleic Acid</td>
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<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
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<td>Curie</td>
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<td>DC</td>
<td>Decay Control</td>
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<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<td>dox</td>
<td>Doxycyclin</td>
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<td>DTC</td>
<td>Well-Differentiated Thyroid Cancer</td>
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<td>E</td>
<td>Glutamic Acid</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
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G  Glycine
hCG  Human Chroionic Gonadotropin
HDAC  Histone deacetylases
hNIS  Human Sodium Iodide Symporter
Hsp90  Heat Shock Protein 90
IGF-1  Insulin-like Growth Factor 1
i.p.  Intraperitoneal
ISA-1  3-biphenyl-4'-yl-5,6-dihydroimidazo[2,1-b]thiazole
ITD  Iodide Transport Defect
LPS  Lipopolysaccharide
MEK/MAPK  Mitogen-activated Protein Kinase
mNIS  Mouse Sodium Iodide Symporter
mTORC1  Mammalian target of rapamycin complex 1
NIS  Sodium Iodide Symporter
NO  Nitric Oxide
NOS  Nitric Oxide Synthase
OMIM  Online Mendelian Inheritance in Man
P  Proline
Pax8  Paired box gene 8
PC Cl3  PC cell line clone 3 rat thyroid cells
PI3K  Phosphatidylinositol 3-kinases
PKA  Protein kinase A
pNIS  Porcine Sodium Iodide Symporter
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<td>Q</td>
<td>Glutamine</td>
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<td>R</td>
<td>Arginine</td>
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<td>RAIU</td>
<td>Radioiodine Uptake Assay</td>
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<td>Ras</td>
<td>Rat sarcoma</td>
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<td>RET/PTC</td>
<td>Rearranged during Transfection/Papillary Thyroid Carcinoma</td>
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<td>rhTSH</td>
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<td>ROI</td>
<td>Region of Interest</td>
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<td>S</td>
<td>Serine</td>
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<td>SGLT1/2</td>
<td>Sodium-dependent Glucose Transporter 1/2</td>
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<td>siRNA</td>
<td>Small Interference RNA</td>
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<td>SLC5A</td>
<td>Solute Carrier Family 5</td>
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<td>Smad</td>
<td>Signaling Mother against Decapentaplegic Peptide</td>
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<td>SMCT</td>
<td>Sodium-dependent monocarboxylate transporter</td>
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<td>SMIT</td>
<td>Sodium-dependent myo-inositol transporter</td>
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<tr>
<td>SMVT</td>
<td>Sodium-dependent multivitamin transporter</td>
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<tr>
<td>SPECT</td>
<td>Single Photon Emission Computed Tomography</td>
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<tr>
<td>SSSF</td>
<td>Sodium/Solute Symporter Family</td>
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<tr>
<td>T</td>
<td>Threonine</td>
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<tr>
<td>T3</td>
<td>Triiodothyronine</td>
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<td>T4</td>
<td>Thyroxine</td>
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<td>Tg</td>
<td>Thyroglobulin</td>
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<td>Tg-PTC1</td>
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<td>THW</td>
<td>Thyroid Hormone Withdrawal</td>
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<td>TPO</td>
<td>Thyroid Peroxidase</td>
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<tr>
<td>tRA</td>
<td>all trans Retinoic Acid</td>
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<td>TSH</td>
<td>Thyroid Stimulating Hormone</td>
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<td>TSHR</td>
<td>TSH Receptor</td>
</tr>
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<td>UTC</td>
<td>Undifferentiated Thyroid Cancer</td>
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<tr>
<td>V</td>
<td>Valine</td>
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<td>WT</td>
<td>Wild Type</td>
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CHAPTER 1

INTRODUCTION

Thyroid gland and NIS

Thyroid gland is the largest endocrine gland that is located on the lower anterior side of the neck region in humans and consists of two lateral lobes connected via isthmus. The basic structural unit of thyroid is composed of spheroid-shaped follicles, which are made up of a single layer of polarized epithelial follicular cells enclosing a lumen that contains colloid, and interspersed C cells. The primary function of thyroid is to synthesize thyroid hormones, triiodothyronine (T3) and thyroxine (T4), which play important roles in systemic metabolism as well as the development of the nervous system in newborns. Lack of thyroid hormone has been shown to result in severe development and metabolic abnormalities.

Iodine is an essential component of thyroid hormone yet a rare element in the environment, therefore the thyroid gland has evolved an effective way to sequester iodine 20 to 40 times against its concentration gradient from the systemic circulation. Iodide is actively transported into thyroid follicular cells by sodium iodide symporter (NIS), an intrinsic membrane glycoprotein expressed on the basolateral membrane of the thyroid follicular cells. Once iodide is transported into the thyroid gland, it is then translocated
across the apical membrane to the follicular lumen through a process called iodide efflux that is proposed to be mediated by the apical iodide transporter or chloride-iodide transporter pendrin. In the follicular lumen, iodide is then organified or incorporated into the tyrosine residues of the thyroglobulin (Tg) prohormone by thyroid peroxidase (TPO) and stored in the follicular lumen of thyroid. Iodinated Tg serves as precursors for thyroid hormones, and when there is a physiological need for thyroid hormone, the iodinated Tg is endocytosed into thyroid follicular cells and targeted to the lysosome. Hydrolysis of iodinated Tg result in release of T3 and T4 into the bloodstream. The dynamics of thyroid accumulating iodide and thyroid hormone synthesis is tightly regulated by thyroid stimulating hormone (TSH). Binding of TSH to TSH receptor (TSHR) localized on the basolateral membrane of the thyroid follicular cells activates the cyclic AMP (cAMP) signaling pathway and results in an increase of thyroid hormone synthesis (Fig 1.1) (see review in Shen et al, 2001).

The molecular characterization of NIS started with the cloning of rat NIS (rNIS) complimentary DNA (cDNA) from the FRTL-5 Fisher rat thyroid cell line derived cDNA library in *Xenopus laevis* oocytes (Dai, Levy et al. 1996). This led to subsequent cloning of human NIS (hNIS) in our laboratory by screening the human thyroid cDNA library based on the homologous sequence of rNIS (Smanik, Liu et al. 1996). rNIS transcript is 2.8 kb and encodes a protein of 618 amino acids with a predicted molecular mass of 65 kDa, whereas hNIS transcript is 3.7 kb and encodes a protein of 643 amino acids with a predicted molecular mass of 69 kDa. hNIS gene is located at chromosome 19p12-13.2 and consists of 15 exons intervened by 14 introns (Smanik, Ryu et al. 1997). Mouse (mNIS) and porcine NIS (pNIS) have also been isolated (Perron, Rodriguez et al. 2001;
Pinke, Dean et al. 2001; Selmi-Ruby, Watrin et al. 2003). Sequence analysis showed that mNIS and rNIS exhibited the highest homology among all four species of NIS proteins while hNIS and rNIS exhibit 84% identity and 92% similarity.

NIS protein was proposed to contain 13 transmembrane domains with an extracellular amino-terminus and an intracellular carboxyl-terminus (Fig 1.2). In addition, loops between transmembrane domains II and III, IV and V, VI and VII, VIII and IX, X and XI, and XII and XIII are located extracellularly while loops between transmembrane domains I and II, III and IV, V and VI, VII and VIII, IX and X, and XI and XII are located intracellularly. The difference between hNIS and rNIS is that there is an additional 5 amino acids in the extracellular loop between transmembrane domains XII and XIII and an additional 20 amino acids in the cytoplasmic COOH terminus of hNIS. There are three putative N-linked glycosylation sites on asparagines residues at position 225, 297 and 485 and are conserved among species. Position 225 is located within the extracellular loop between VI and VII while positions 297 and 485 are located within the last extracellular loop between XII and XIII. Site-directed mutagenesis showed that abolishment of all or partial N-linked glycosylation sites did not completely abolish NIS function, stability or cell surface trafficking (Levy, De la Vieja et al. 1998). In addition, NIS contains a potential leucine zipper motif that consists of four leucine residues at positions 199, 206, 213 and 220 in transmembrane domain VI. This motif might contribute to oligomerization of NIS at the cell membrane. Studies have suggested NIS to be an oligomeric protein based on the appearance of 9 nm diameter particles in freeze-fracture electron microscopy study performed in NIS-expressing Xenopus laevis oocytes, as well as detection of high molecular weight NIS of about 180 kDa in western blot...
analysis (Eskandari, Loo et al. 1997; Levy, Dai et al. 1997; Mitrofanova, Unfer et al. 2004). Other than N-linked glycosylation sites and leucine zipper motif, NIS has been suggested to be a phosphoprotein and that TSH modulates NIS phosphorylation pattern (Riedel, Levy et al. 2001). Moreover, our laboratory showed that NIS is phosphorylated at positions S43, T49, S227, T577, and S581. S43 and S581 are potential corresponding phosphorylation sites responsible for modulation of NIS iodide transport velocity as demonstrated by kinetic studies. T577 is important for NIS protein stability and T49 is critical for proper conformation of NIS (Vadysirisack, Chen et al. 2007).

NIS is a member of the solute carrier family 5 (SLC5A) or the sodium/solute symporter family (SSSF) according to the Online Mendelian Inheritance in Man [OMIM] classification. This family also includes the Na\(^+\)-dependent glucose transporters 1 and 2 (SGLT1 and SGLT2), Na\(^+\)-dependent monocarboxylate transporter (SMCT), Na\(^+\)-dependent myo-inositol transporter (SMIT) and Na\(^+\)-dependent multivitamin transporter (SMVT) and others (Wright and Turk 2004). All members in the SLC5A family utilize a secondary active transport mechanism in order to translocate its substrate i.e. NIS relies on the electrochemical gradient of Na\(^+\) as the driving force to transport iodide across the cell membrane. The electrochemical gradient of Na\(^+\) is maintained by the activity of Na\(^+\)/K\(^+\)-ATPase. Electrophysiological analysis of NIS revealed the translocation of two Na\(^+\) down their electrochemical gradient with the simultaneous translocation of one iodide against its electrochemical gradient into cells. Furthermore, it has been proposed that binding of Na\(^+\) to NIS occurs before iodide binding (Eskandari, Loo et al. 1997) and that amino acids T351, S353, T354, S356, and T357 containing hydroxyl group in transmembrane IX may play important roles in Na\(^+\) binding and translocation (De la
However, amino acids and the region of NIS that bind to substrate are still yet to be identified. Other than iodide, NIS can also transport a wide variety of other monovalent anions such as perchlorate (ClO₄⁻), astatine (At⁻), rhenium (ReO₄⁻), and technetium (TcO₄⁻) (Eskandari, Loo et al. 1997; Van Sande, Massart et al. 2003; Dadachova and Carrasco 2004; Zuckier, Dohan et al. 2004). Perchlorate can be transported into thyroid cells via NIS thus acts as a competitive inhibitor for NIS-mediated iodide transport, whereas radioactive forms of astatine, rhenium, and technetium have important implications for NIS-mediated radionuclide imaging and therapy. It has been shown that using high energy alpha particle-emitter At²¹¹ may allow more efficient tumor killing than I¹³¹ for NIS-mediated radioiodine therapy (Petrich, Quintanilla-Martinez et al. 2006).

NIS in thyroid

*Physiologically*

TSH is the main hormonal stimulator of thyroidal NIS expression/function. Up-regulation of NIS-mediated radioiodine accumulation by TSH has been demonstrated in FRTL-5 rat thyroid cells (Ajjan, Watson et al. 1998) as well as human thyroid primary cultures (Saito, Endo et al. 1997; Kogai, Curcio et al. 2000). Moreover, TSH has been used clinically to treat differentiated thyroid cancer patients. Recombinant human TSH (rhTSH) in adjunct to radioiodine therapy has been tested in 54 patients with metastatic or regional lesion thyroid cancer showed 74 % of patients having positive radioiodine accumulation (Jarzab, Handkiewicz-Junak et al. 2003). The increase in radioiodine...
accumulation by TSH is mainly regulated at the transcriptional level, which resulted in an upregulation of NIS mRNA levels (Saito, Endo et al. 1997; Ajjan, Watson et al. 1998; Trapasso, Iuliano et al. 1999; Kogai, Curcio et al. 2000) as well as NIS protein levels (Levy, Dai et al. 1997; Saito, Endo et al. 1997; Kogai, Curcio et al. 2000). Studies have also suggested that upregulation of NIS-mediated radioiodine accumulation by TSH may also occur at the post-translational level. Riedel, et al showed that 3 days of TSH withdrawal, NIS was redistributed from the plasma membrane to intracellular compartments in FRTL-5 cells (Riedel, Levy et al. 2001). This suggests that TSH may upregulate NIS activity by mediating NIS targeting to and retention at the plasma membrane. TSH activates downstream signaling pathways through binding and activation of the G-protein coupled receptor, TSHR. Indeed, TSHR agonist, human chorionic gonadotropin (hCG), has been shown to exert its thyroid stimulatory action through direct binding to the TSHR (Tomer, Huber et al. 1992; Yoshimura, Hershman et al. 1993). Hershman, et al showed that 48 hr treatment with hCG (1000U) increased radioiodine accumulation and cAMP levels in FRTL-5 cells (Hershman, Lee et al. 1988). In addition, Arturi, et al demonstrated that the increase in iodide uptake by hCG was mainly due to an increase in NIS mRNA and protein levels in FRTL-5 cells (Arturi, Presta et al. 2002).

TSH stimulates thyroidal NIS expression/function through the adenyl cyclase-cAMP-PKA signaling pathway. Forskolin, an activator of adenyl cyclase, has been shown to upregulate NIS mRNA and NIS-mediated radioiodine accumulation in PC Cl3 rat thyroid cells and primary human thyroid cultures (Saito, Endo et al. 1997; Trapasso, Iuliano et al. 1999). In addition, inhibition of PKA by H89 (10 µM) for 24 hr decreased, but did not abolish, TSH induced NIS mRNA levels in FRTL-5 cells suggests that
regulation of NIS expression by TSH is mediated through both a PKA-dependent and PKA-independent pathway (Garcia and Santisteban 2002). Interestingly, leptin, a hormone produced by adipose tissues that regulates metabolism, has been shown to decrease TSH induced NIS-mediated radioiodine uptake by downregulating NIS mRNA levels despite the increase in cAMP levels in FRTL-5 cells (Isozaki, Tsushima et al. 2004). Moreover, incubation of thyroid glands slices dissected from rats with $10^{-9}$ to $10^{-5}$ M leptin and Na$^{125}$I for 2 hr decreased thyroidal Na$^{125}$I accumulation by 33 % (de Oliveira, Teixeira Silva Fagundes et al. 2007). In contrast to the *in vitro* study, *in vivo* study showed that chronic leptin stimulation (8 µg/100 gBW/day for 6 days) increased thyroidal radioiodine accumulation at both 15 min (1.3- fold) and 2 hr (10 %) post Na$^{125}$I injection in rats (de Oliveira, Teixeira Silva Fagundes et al. 2007). This suggests that leptin may increase thyroidal iodide uptake through an unknown mechanism that surpasses its direct inhibitory effect on NIS expression/function.

In addition to TSH-adenyl cyclase-cAMP-PKA, PI3K appears to also play an important role in regulation of NIS expression/function. Indeed, over-expression of RasV12C40 that preferentially binds to and activates PI3K decreased NIS protein levels in WRT rat thyroid cell lines (Cass and Meinkoth 2000). In addition, the inhibitory effect of insulin-like growth factor 1 (IGF-1), a hormone that collaborates with TSH in regulating thyroid proliferation and differentiation, has been demonstrated to decrease TSH induced NIS mRNA and protein levels through PI3K activation (Garcia and Santisteban 2002). Overexpression of G$\beta/\gamma$ subunit, which is dissociated from TSH activated TSHR coupled G protein (Selzer, Wilfing et al. 1993; Laugwitz, Allgeier et al. 1996), decreased NIS mRNA levels through activation of PI3K (Zaballos, Garcia et al. }
In contrast, inhibition of PI3K by LY294002 increased NIS-mediated radioiodine accumulation in a dose- (10^{-7} to 30 \mu M) and time-dependent (24 to 96 hr) manner in FRTL-5 cells (Kogai, Ohashi et al. 2008). While LY294002 also targets casein kinase 2 (Davies, Reddy et al. 2000; Davies, Mason et al. 2004), casein kinase 2 inhibitor, CK2i had minimal effect on NIS mRNA levels in FRTL-5 cells (Kogai, Sajid-Crockett et al. 2008). Akt is a major downstream signaling node of PI3K, effect of inhibition of Akt on NIS expression/function was investigated in this study and another group. Kogai, et al showed that 24 hr treatment with Akti-1/2 (30 \mu M) did not significantly increased radioiodine accumulation and modestly increased NIS mRNA levels in FRTL-5 cells (Kogai, Sajid-Crockett et al. 2008). While the result of Akti-1/2 on NIS mRNA levels is consistent between our lab and reported study, our result showed that 24 hr treatment with Akti-1/2 (10 \mu M) dramatically increased NIS-mediated radioiodine accumulation to 3 fold, yet decreased NIS protein levels in FRTL-5 cells. Moreover, we also demonstrated that 24 hr treatment with Akti-1/2 (10 \mu M) significantly increased NIS-mediated radioiodine accumulation to 3-6- fold in PC Cl3 cells with minimal effect on NIS mRNA levels and decreased NIS protein levels. Despite the contradictions in the Akti-1/2 data, the differential regulation of NIS expression/function by PI3K and Akt suggests that Akt may not be the downstream signaling node of PI3K in NIS regulation. Taken together, activated PI3K down-regulates NIS-mediated radioiodine accumulation by downregulating NIS mRNA levels through an Akt-independent pathway. In summary, TSH not only upregulates NIS expression/function through activation of adenyl cyclase-cAMP-PKA pathway, but also downregulates NIS expression/function through activation
of PI3K signaling pathway. Chapter 3 describes the detailed findings of NIS regulation by PI3K downstream, Akt1/2 and mTORC1 in thyroid cells.

Previous study showed that TSH or cAMP, on the one hand stimulates activation of Ras through the formation of PI3K-Ras complex, on the other hand decreased the formation of Raf-1-Ras complex thus resulting in inhibition of Raf-1-MEK (MAPK) signaling (Ciullo, Diez-Roux et al. 2001). Nonetheless, MEK signaling pathway appears to play a role in regulation of NIS expression/function. RasV12S35 that preferentially binds to and activates Raf-1, which consequently activates the MEK signaling pathway, has been shown to completely abolish TSH-induced NIS expression in WRT thyroid cells (Cass and Meinkoth 2000). Expression of a constitutively active MEK1 resulted in a decrease in NIS mRNA levels in the dox- inducible PC Cl3- MEK1 E217/E221 cells (Knauf, et al, 2003). It is interesting to note that inhibition of MEK by 24 hr treatment with PD98059 did not have an effect in increasing NIS mRNA or protein levels in FRTL-5 or PC Cl3 cells, yet decreased NIS-mediated radioiodine accumulation in thyroid cells (Garcia and Santisteban 2002; Knauf, Kuroda et al. 2003; Vadysirisack, Venkateswaran et al. 2007). However, further inhibition of MEK by PD98059 for 48 hr increased both NIS protein and NIS-mediated radioiodine accumulation in PC Cl3 cells (Vadysirisack, Venkateswaran et al. 2007). A summary of reported MEK, PI3K, Akt or mTORC1 signaling nodes on NIS modulation is listed in Table 1.1.

Several other signaling pathways have been shown to regulate NIS expression/function in thyroid cells. p38 mitogen-activated protein kinase (p38 MAPK) has been shown to be activated in response to TSH and cAMP stimulation, and inhibition of p38 MAPK with ML3403 for 48 hr decreased NIS-mediated radioiodine accumulation
in a dose-dependent manner ($10^{-7}$ to $5 \times 10^{-5}$ M) (Kogai, Ohashi et al. 2008). In addition, Promerance, et al showed that inhibition of p38MAPK with SB203580 (20 µM) decreased TSH- and forskolin- induced NIS mRNA expression in FRTL-5 cells (Pomerance, Abdullah et al. 2000). Nitric oxide synthase (NOS) that catalyses nitric oxide (NO) synthesis which leads to formation of cGMP was shown to also play a role in NIS regulation. NOS inhibitors, l-NAME (1 mM) increased NIS-mediated radioiodine accumulation (no perchlorate) in a time-dependent manner (24 hr: 20%; 48 hr: 30%; 72 hr: 40%) in FRTL-5 cells. Furthermore, 48 hr treatment with cGMP-dependent kinase inhibitor, KT5823 (5 µM) increased radioiodine accumulation by 2-fold in FRTL-5 cells (Fozzatti, Velez et al. 2007).

Apart from TSH, iodide itself has been shown to regulate iodide uptake in thyroid cells. Wolff and Chaikoff reported that iodide organification is blocked when plasma iodide level reached a critical threshold in the rat thyroid through a possible protective mechanism, which is later called the acute Wolff–Chaikoff effect (Wolff and Chaikoff 1948). Several studies have shown that excess iodide decreases thyroidal iodide uptake yet the mechanism remains yet to be determined. While Spitzweg, et al showed treatment with $10^{-4}$ M iodide decreased NIS mRNA levels, Eng, et al showed treatment with $10^{-3}$ M iodide had no effect on NIS mRNA levels yet decreased NIS protein halflife. A recent study showed treatment with 10 mM iodide for 48 hr decreased NIS promoter activity, NIS mRNA level and NIS protein levels in FRTL-5 cells (Eng, Cardona et al. 1999; Spitzweg, Joba et al. 1999; Eng, Cardona et al. 2001; Suzuki, Kimura et al. 2010). In summary, iodide negatively regulates NIS-mediated radioiodine accumulation in thyroid cells. In addition, Tg has also been shown to decrease NIS mRNA and protein levels and
other thyroid specific gene expression i.e. TPO, TSHR and Tg in FRTL-5 cells (Suzuki, Lavaroni et al. 1998; Kohn, Suzuki et al. 2001; Suzuki, Kimura et al. 2010). Downregulation of NIS by iodide and Tg indicates the presence of negative feedback autoregulatory mechanism on TSH induced NIS expression/function for maintaining thyroid hormone homeostasis.

Cytokines have also been shown to play a role in regulating NIS-mediated radioiodine accumulation in thyroid cells. Transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), TNF-β, interferon-γ (IFN-γ), interleukin-1α (IL-1α), IL-1β, and IL-6 all showed inhibitory effect on TSH-induced NIS protein expression and NIS-mediated radioiodine accumulation in FRTL-5 cells and human thyroid cells (Kawaguchi, Ikeda et al. 1997; Ajjan, Watson et al. 1998; Pekary and Hershman 1998). Caturegli, et al further showed that IFN-γ significantly decreased NIS expression in mice thyroid gland (Caturegli, Hejazi et al. 2000). Downregulation of NIS expression/function by TGF-β was shown to be mediated through signaling mother against decapentaplegic peptide (Smad), Smad3 binding to Pax-8 resulting in decreased Pax-8 mRNA levels and decreased Pax-8 binding to NIS promoter (Costamagna, Garcia et al. 2004).

**Pathologically**

Congenital iodide transport defect (ITD) is an autosomal recessive condition caused by mutations in NIS. The disorder is characterized by little iodide uptake, hypothyroidism, goiter and a low saliva/plasma iodide ratio. To date, twelve loss-of function NIS mutations have been reported (Levy, De la Vieja et al. 1998; Dohan, Gavrielides et al. 2002; De La Vieja, Ginter et al. 2004; Reed-Tsur, De la Vieja et al. 2004).
2008) and several were studied extensively. Among them, four mutations were demonstrated by site-mutagenesis study in COS7 monkey kidney cells expressing exogenous NIS to not have an effect on total or cell surface NIS protein levels, but decreased NIS function. Levy, et al showed that the β-hydroxyl group at amino acid T345 is essential to maintain NIS function through the study of T345P mutation (Levy, De la Vieja et al. 1998). Dohan, et al showed that the presence of an uncharged amino acid with a small side chain at position G395 is also necessary for NIS function through the study of G395R mutation (Dohan, Gavrielides et al. 2002). The Q267E mutation decreased NIS function by decreasing the NIS substrate transport rate (De La Vieja, Ginter et al. 2004). The V59E mutation abolished NIS function due to the disruption of the neutral, helix-promoting amino acid at position 59 (Reed-Tsur, De la Vieja et al. 2008). On the other hand, COS7 cells expressing exogenous NIS with G543E mutation has been reported to have decreased iodide uptake due to impairment of NIS protein maturation and cell surface trafficking. Substitution of position 543 with other small neutral amino acids restored NIS activity suggests that position 543 is in a tightly packaged region of NIS protein (De la Vieja, Ginter et al. 2005).

**NIS in thyroid cancer**

Thyroid cancer is the most common endocrine malignancy and accounts for approximately one percent of all human malignancy (Sherman 2003). There are four types of thyroid cancer with some more common than others: papillary (>70 %), follicular (15 %), medullary (4 %) and anaplastic (1 %) thyroid cancer. Most thyroid cancers derive from follicular cells and are classified as papillary and follicular thyroid
cancers as well-differentiated thyroid cancer (DTC) or anaplastic thyroid cancers as undifferentiated thyroid cancer (UTC) (Hundalh, et al, 1998). Most DTC patients can be effectively treated with near-total or total thyroidectomy in combination with $^{131}$I ablation of residual or metastatic tumor and suppressive thyroid hormone to minimize proliferation of residual thyroid tumors by TSH. Adjuvant radioiodine therapy improves clinical outcome and decreases the rate of recurrence (Mazzaferri and Jhiang 1994; Wartofsky, Sherman et al. 1998). NIS-mediated iodide uptake ability and prolonged retention of iodide by organification in thyroid has long been served as the basis for the clinical use of radioiodine therapy in the detection and targeted ablation of residual thyroid cancer in treating thyroid tumor patients underwent post-thyroidectomy (Singer, Cooper et al. 1996; Mazzaferri 1999). In order to increase functional NIS expression for effective radioiodine therapy, TSH level is elevated ($\geq 30$ mU/l) by either bTSH or rhTSH stimulation and/or thyroid hormone (T3 or T4) withdrawal. Elevated serum TSH levels not only increases NIS-mediated radioiodine uptake but also increases Tg expression which is the main way of storing iodine. The use of bTSH, started 50 years ago, was proved by Schneider, et al showing that bTSH and hTSH had comparable effect on inducing human thyroidal radioiodine accumulation (Schneider, Robbins et al. 1965), yet was abandoned due to severe allergic reactions developed by patients. Krishnamurthy, et al showed that 43% of 42 post-surgical thyroid cancer patients who underwent T4 withdrawal and received 3 doses of bTSH injection showed allergic reactions to bTSH in a 25 year follow up study (Krishnamurthy and Blahd 1977). In addition, repeated use of bTSH results in development of neutralizing antibodies that reacts to both bTSH and hTSH, thus lead to hypothyroidism and failure to respond to
stimulation to exogenous TSH. Moreover, mad cow disease limited the source and the use of bTSH purified from bovine’s pituitary gland. Current regimen for using rhTSH in treating post-surgical thyroid cancer patients is one intramuscular injection of 0.9 mg rhTSH per day for 2 consecutive days, followed by I\textsuperscript{131} on the 3rd day. Serum TSH levels can also be increased by elevating endogenous TSH levels through discontinuing thyroid hormone therapy (thyroid hormone withdrawal/THW) resulted hypothyroidism thus alleviating the negative feedback loop of supplemented thyroid hormone on the hypothalamus and the pituitary gland in post-surgical thyroid cancer patients. Generally, patients are off the T4 treatment for 3 to 5 weeks or alternatively off the T4 treatment and switch to T3 for 2 to 4 weeks followed by T3 withdrawal for 2 weeks before they are subjected to radioiodine therapy (Pacini, Schlumberger et al. 2006; Luster, Clarke et al. 2008). Several studies have compared the efficacy of thyroid remnant ablation after rhTSH administration versus THW. Even though the area under the curve of serum TSH concentration after 4 weeks of T4-withdrawal was 4.6 times higher than that after one 0.9 mg of rhTSH per day for two consecutive days (Rosario, Salles et al. 2009), numerous studies have shown the efficacy of radioiodine ablation is quite similar between THW versus rhTSH when using 30, 54 or 100 mci I\textsuperscript{131} (Lee, Yun et al.; Pacini, Ladenson et al. 2006; Chianelli, Todino et al. 2009; Elisei, Schlumberger et al. 2009). However, patients who cannot tolerate hypothyroidism or failed to elevate endogenous TSH levels due to impaired hypothalamic-pituitary-thyroid axis are administered rhTSH instead. While most thyroid cancer patients benefit from radioiodine therapy, some DTC patients and all UTC patients cannot benefit from radioiodine therapy due to reduced/absent NIS expression/function (Caillou et al. 1998; Castro et al. 2001; Oler Et Cerutti 2009; Patel et
al. 2002; Ward et al. 2003). Thus, it is of clinical importance to identify/develop pharmacological reagents that could further increase and/or restore radioiodine accumulation in advanced thyroid cancer. As radioiodine accumulation is the net outcome of iodide influx and efflux, strategies to increase radioiodine accumulation include selectively increase iodide influx and/or decrease iodide efflux in thyroid.

Thyroid cancer harbor several genetic alterations including RET/PTC rearrangements, Ras mutation, and BRAF$^{V600E}$ mutations, all of which activate the MAPK signaling pathway (Jhiang 2000; Fagin 2002; Nikiforov 2002; Santoro, Papotti et al. 2002; Xing 2005; Ciampi and Nikiforov 2007). RET/PTC oncogene is rearrangement forms of the rearranged during transfection (RET) proto-oncogene which encodes a membrane receptor tyrosine kinase. Generation of RET/PTC occurs through chromosomal recombination of the intracellular tyrosine kinase domain of RET fused to the 5’ region of unrelated partner genes that are constitutively expressed in thyroid follicular cells. Currently, there are thirteen RET/PTC rearrangements identified: RET/PTC1-9, ELKS-RET, PCM1-RET, and HOOK3-RET. Among all RET/PTC rearrangements, RET/PTC1 and RET/PTC3 are the most common types found in papillary thyroid cancers (PTC) (Grieco, Santoro et al. 1990; Bongarzone, Monzini et al. 1993; Bongarzone, Butti et al. 1994; Santoro, Dathan et al. 1994; Klugbauer, Demidchik et al. 1998; Klugbauer, Demidchik et al. 1998; Nakata, Kitamura et al. 1999; Corvi, Berger et al. 2000; Ciampi, Giordano et al. 2007; Ciampi and Nikiforov 2007). RET/PTC1 expression has been shown to decrease NIS expression and radioiodine accumulation in cultured thyroid cells as well as thyroid tumors of transgenic mouse model (Cho, Sagartz et al. 1999; Trapasso, Iuliano et al. 1999). In addition, activated
RET/PTC has been reported to decrease NIS mRNA levels through the Shc/Ras/MAPK signaling pathway and that inhibition of MAPK/ERK pathway using pharmacological inhibitors restored NIS mRNA and protein levels in PC Cl3 cells expressing RET/PTC (Knauf, Kuroda et al. 2003). BRAF^{V600E} mutation has been identified as the most prevalent genetic event in papillary thyroid cancer (Xing 2005). BRAF^{V600E} expression decreased NIS mRNA levels in PC Cl3 cells and thyroid cells of thyroid targeted BRAF expressing (Tg-BRAF) transgenic mouse model (Knauf, Ma et al. 2005; Mitsutake, Knauf et al. 2005; Mitsutake, Miyagishi et al. 2006). Whereas RET/PTC rearrangements and BRAF^{V600E} mutations are common in PTCs, Ras mutations, particularly N-Ras mutation at codon 61, occur mainly in FTCs and follicular variant of PTCs (Nikiforov 2002; Vasko, Ferrand et al. 2003; Zhu, Gandhi et al. 2003). Expression of oncogenic Ras resulted in a decrease in NIS mRNA levels in the dox-inducible PC Cl3-H-RasV12 cells and treatment with PD98059 (35 µM) restored NIS mRNA levels at 12 hr in H-RasV12 expressing PC Cl3 cells (Knauf, Kuroda et al. 2003). Interestingly, while RET/PTC rearrangement, Ras mutations and BRAF^{V600E} mutations present in almost 70 % of PTCs, the three genetic alterations are mutually exclusive (Xing 2005). Moreover, Mitsutake, et al showed that small interference RNA knockdown of BRAF inhibits RET/PTC induced downregulation of NIS mRNA levels in PC Cl3 cells (Mitsutake, Miyagishi et al. 2006). This suggests that constitutive active RET/PTC/Ras/BRAF-MEK-MAPK signaling pathway plays a central role in the pathogenesis of PTC and downregulation of NIS expression/function. Other than MAPK signaling, RET/PTC has been shown to activate PI3K and PKC signaling pathways (Miyagi, Braga-Basaria et al. 2004). Expression of RET/PTC1 resulted in constant upregulation of RET downstream signaling through its
autophosphorylated tyrosine residues activates PI3K and MEK signaling has been considered to be essential for the transforming activity of thyroid cells (Santoro, Melillo et al. 2006; Nikiforov 2008).

Pharmacological inhibitors targeting specific signaling nodes, i.e. MAPK, PI3K, PKC or receptor tyrosine kinase signaling pathway, have been extensively tested to examine its effect on increasing NIS expression/function in thyroid cells. PD98059 (35 µM) increased NIS mRNA levels at 12 hr in RET/PTC3 expressing PC Cl3 cells (Knauf, Kuroda et al. 2003). Furthermore, PD98059 (40 µM) increased NIS protein levels at 4 hr in RET/PTC1-transformed PC Cl3 cells. However, the increase in protein levels did not result in an increase, but a decrease (30%) in radioiodine accumulation at 4 hr treatment of PD98059. Further treatment with PD98059 for 48 hr increased both NIS protein and radioiodine accumulation in RET/PTC1 expressing PC Cl3 cells (Vadysirisack, Venkateswaran et al. 2007). Treatment with PI3K inhibitor, LY294002, not only increased NIS-mediated radioiodine accumulation in FRTL-5 cells, we and others showed that 24 hr treatment with LY294002 increased NIS-mediated radioiodine accumulation by 2 fold in BHP 2-7 human thyroid cancer cells stably expressing exogenous hNIS and RET/PTC1-transformed PC Cl3 cells (Kogai, Sajid-Crockett et al. 2008). Treatment with PKC stimulator, TPA, decreased NIS mRNA levels in PC Cl3 cells (Trapasso, Iuliano et al. 1999). However, treatment with PKC δ inhibitor, rotterlin did not increase, but decreased NIS-mediated RAIU activity (no perchlorate) in FRTL-5 cells. This was most likely due to the off target effect of rotterlin shown by siRNA knockdown experiment in another cell line (Kogai, Ohashi et al. 2008). Treatment with Sunitinib, a multi-targeted inhibitor of several receptor tyrosine kinases, increased NIS-
mediated radioiodine accumulation by 50% (no perchlorate) but had no effect on NIS mRNA levels in FRTL-5 cells (Salem, Fenton et al. 2008). While studies showed exciting results in the ability of increasing NIS-mediated radioiodine accumulation in normal rat thyroid cells, the findings have not been tested in physiologically/pathologically relevant cell lines, i.e. human thyroid cancer cell lines.

The loss/decreased NIS expression/function due to dedifferentiation in some thyroid cancer patients rendered them to not benefit from radioiodine therapy (Jhiang, Cho et al. 1998; Ryu, Senokozlieff et al. 1999; Ringel, Anderson et al. 2001). Numerous studies using three different categories of redifferentiating reagents have shown to increase NIS expression/function in thyroid cancer cells: 1. HDAC inhibitors; 2. demethylation agents; 3. Retinoic acids. 1. Treatment with HDAC inhibitor, depsipeptide (FR901228) (1 ng/ml) for 3 days increased NIS mRNA levels and NIS-mediated radioiodine accumulation in a human FTC cell line (RAIU fold change relative to DMSO: FTC133= 6 fold) and a human ATC cells lines (RAIU fold change relative to DMSO: SW-1736= 10 fold) (Kitazono, Robey et al. 2001). This suggests that depsipeptide induced NIS-mediated iodide uptake is mainly regulated at the transcriptional level. Similarly, Hou, et al recently showed that 30 hr treatment with SAHA (5 µM), another HDAC inhibitor, significantly increased NIS mRNA levels by real-time RT-PCR in several human thyroid cancer cells (Hou, Bojdani et al. 2010). While a recent clinical trial showed that SAHA had no inhibitory effect on the growth and progression of radioiodine-refractory metastatic thyroid cancer (Woyach, Kloos et al. 2009), SAHA or other HDAC inhibitors may still be effective when used in adjunct to radioiodine therapy in treating thyroid cancer patients. 2. Demethylation of the promoter
region and the first exon of NIS DNA by 5-azacytidine (1 µM) for 7 days increased NIS mRNA levels but not NIS-mediated radioiodine accumulation in 2-7 (poorly differentiated human thyroid cancer) thyroid cancer cells (Provenzano, Fitzgerald et al. 2007). 3. Treatment with all trans retinoic acid (tRA) and 13-Cis-RA (Isostretinoin), well known inducers of differentiation, for 28 days (100 µg/100 g BW/day) have been shown to increase thyroidal radioiodine accumulation at 15 min post Na\textsuperscript{125}I injection, yet had minimal effect on increasing thyroidal radioiodine accumulation at 2 hr post Na\textsuperscript{125}I injection in rats (Silva, Marassi et al. 2009). This suggests that tRA and 13-Cis-RA may increase NIS expression yet decrease thyroidal radioiodine accumulation through mechanisms yet to be determined. This is in contrast to Schmutzler, et al showing that treatment with tRA decreased NIS mRNA levels and NIS-mediated radioiodine accumulation in FRTL-5 cells (Schmutzler, Winzer et al. 1997). In summary, among all studies, HDAC inhibitors have been consistently shown to increase NIS-mediated radioiodine accumulation through increasing NIS mRNA levels in various cultured human thyroid cancer cells, thus making HDAC inhibitors to be promising therapeutic reagents in increasing the efficacy of radioiodine therapy in treating thyroid cancer patients.

Combination treatments with reagents have also been done to achieve an increase in radioiodine accumulation in cultured human thyroid cancer cells. Provenzano, et al showed while single treatment with 5-azacytidine (demethylation reagent) had minimal effect on radioiodine accumulation in 2-7 cells, combination treatment with 5-azacytidine and NaB (HDAC inhibitor) for 7 days significantly increased radioiodine accumulation in both cell lines (RAIU fold change relative to DMSO: 2-7 cells= 8 fold). However, NIS
mRNA levels did not correlate with radioiodine accumulation i.e. single treatment with NaB had a robust increase in NIS mRNA levels but resulted in a slight increase in radioiodine accumulation (Provenzano, Fitzgerald et al. 2007). This suggests that combination treatment with 5-azacytidine and NaB upregulates NIS-mediated radioiodine accumulation probably at the post-transcriptional level of NIS or other non-NIS factors. Hou, et al recently showed that combination treatment with HDAC inhibitor, SAHA, and pharmacological reagents targeting MAPK (RDEA119) and Akt (Perifosine), led to a significant increase in NIS mRNA levels and radioiodine accumulation in C643, K1 and KAT18 human thyroid cancer cells (RAIU fold change relative to DMSO: C643= 10 fold; K1= 3 fold; KAT18= 3 fold). Furthermore, as SAHA+ RDEA119 + Perifosine also increased TSHR mRNA levels, addition of TSH further increased radioiodine accumulation in SAHA+ RDEA119 +Perifosine treated cells (RAIU fold change relative to DMSO: C643= 12 fold; K1= 4 fold; KAT18= 4 fold) (Hou, Bojdani et al. 2010).

Numerous reagents have also been reported to increase radioiodine accumulation in thyroid cells. Adenosine, A1 receptor agonist that is supplied by ectonucleotidase digestion of ATP, has been shown to increase radioiodine accumulation mainly through an increase in NIS mRNA and protein levels in FRTL-5 cells. However, this was not accompanied with an increase in cAMP levels. Further inhibition study showed that inhibition of Gi/Go- mediated pathway by islet-activating protein blocked the increase in NIS mRNA levels in adenosine treated FRTL-5 cells suggesting that adenosine may regulate NIS expression/function through the A1-adenosine receptor- Gi/Go pathway (Harii, Endo et al. 1999). Lipopolysaccharide (LPS), a ligand of the Toll-Like receptor 4, was reported to regulate NIS expression/function in thyroid cells. 10 and 100 ng/ml LPS
were tested and showed an increase in radioiodine accumulation in a dose dependent manner in both PC Cl3 and FRTL-5 cells. Furthermore, NIS protein level was also increased in a dose-dependent manner in FRTL-5 cells. However, LPS had no effect on increasing NIS protein levels and radioiodine accumulation in TSH deprived thyroid cells suggesting that effect of LPS is dependent on TSH (Nicola, Velez et al. 2009). Resveratrol, a polyphenol found in grapes, was shown to upregulate NIS protein levels and radioiodine accumulation but did not decrease iodide efflux rate in the presence and absence of TSH in FRTL-5 cells. In addition, resveratrol did not increase cAMP levels suggesting that resveratrol may regulate NIS expression/function through a cAMP independent pathway (Sebai, Hovsepian et al.).

While many strategies have been investigated to increase radioiodine accumulation by selectively increase iodide influx, there is only a few that have been done to decrease iodide efflux in thyroid. 3-biphenyl-4'-yl-5,6-dihydroimidazo[2,1-b]thiazole (ISA-1), an organic small molecule was shown to inhibit iodide efflux and result in a 4.5 fold increase in NIS-mediated radioiodine accumulation in FRTL-5 cells (Lecat-Guillet, Merer et al. 2008). Our lab previously showed that 24 hr treatment with 3 µM 17-allylamo-17-demethoxygeldanamycin (17-AAG), an inhibitor of RET/PTC1 associated protein Hsp90, increases NIS-mediated radioiodine accumulation by decreasing iodide efflux in NIS-expressing PC Cl3 cells and RET/PTC1-transformed PC Cl3 cells (Marsee, Venkateswaran et al. 2004). This finding was subsequently confirmed by Elisei, et al showing that 17-AAG increases iodide retention time in cultured human thyroid cancer cells (Elisei, Vivaldi et al. 2006). However exciting these findings of the effect of pharmacological inhibitors on increasing radioiodine accumulation in thyroid
cells, it is still important to validate these pharmacological reagents in an immune-competent genetic engineered mouse model carrying thyroid tumors in surrounding physiological/pathological relevant microenvironment. Chapter 2 describes the use of non-invasive radionuclide imaging with microSPECT to validate the effect of 17-AAG on increasing thyroidal radioiodine accumulation in wild type FVB/N mice as well as our thyroid-targeted RET/PTC1 (Tg-PTC1) preclinical thyroid cancer mouse model.

**NIS and iodide accumulation in extra-thyroidal tissues**

In addition to thyroid gland, hNIS protein expression and hNIS-mediated radioiodine accumulation is also detected in salivary gland, gastric mucosa, lactating mammary gland, placenta and lacrimal glands (Jhiang, Cho et al. 1998; Spitzweg, Joba et al. 1999; Vayre, Sabourin et al. 1999; Cho, Leveille et al. 2000; Tazebay, Wapnir et al. 2000; Josefsson, Grunditz et al. 2002; Wapnir and Teichberg 2002). While iodide uptake activity in these extra-thyroidal tissues was inhibited by perchlorate, NIS expression/function is not responsive to TSH stimulation (Carrasco 1993). Moreover, these tissues cannot significantly organify iodide. Banerjee, et al showed that cells isolated from mouse submaxillary glands and stomach contain peroxidases that can catalyze the formation of protein bound iodothyrosine mainly comprised of monoiodothyrosine, di-iodothyrosine and an unknown iodocompound. Furthermore, while 90% of iodide accumulated in isolated salivary cells remains as free iodide, about 10% of iodide is organified in isolated salivary cells whereas more than 50% of iodide accumulated in isolated thyroid cells is organified (Banerjee, Bose et al. 1985; Banerjee and Datta 1986). In the salivary gland, hNIS is expressed in ductal epithelial cells and not
in acinar cells (Jhiang, Cho et al. 1998). In gastric mucosa, hNIS is expressed in the basolateral membrane of the mucus-secreting neck epithelial cells or in parietal cells (Jhiang, Cho et al. 1998; Spitzweg, Joba et al. 1999; Vayre, Sabourin et al. 1999). The physiological significance of NIS expression in salivary and stomach was suggested to be important for entero-thyroid circulation of iodide (Josefsson, Grunditz et al. 2002). NIS expression/function is detected in the mammary gland during late pregnancy and lactation. NIS is expressed in the basolateral membrane of alveolar epithelial cells in rat lactating mammary gland (Cho, Leveille et al. 2000; Tazebay, Wapnir et al. 2000). The physiological significance of NIS expression in lactating mammary gland is to concentrate iodide in milk for nursing newborns to synthesize their own thyroid hormones.

As thyroid is not the only tissue that has functional NIS expression, administration of radioactive iodide can also be accumulated in NIS-expressing extra-thyroidal tissues and result in damaging side effects. It has been reported that potential side effects of radioiodine therapy includes gastritis (inflammation of gastric mucosa), sialadenitis (inflammation of salivary gland), and xerostomia (dry mouth), which are mainly due to radiation-induced damage of the stomach and salivary gland (Van Nostrand, Neutze et al. 1986). Chapter 3 describes the temporal dynamics of radioiodine accumulation in salivary and correlation with thyroidal radioiodine accumulation in mice.

**Application of NIS as therapeutic gene**

Since the cloning of NIS and the successful application of radioiodine in treating thyroid cancer, many studies have shown to extend the use of NIS-mediated radioiodine
therapy to non-thyroid cancers such that non-thyroid cancer patients can also benefit from radioiodine therapy. Studies have shown successful gene transfer of functional NIS into a variety of cancer cells: colon cancer, breast cancer, glioma, melanoma, pancreatic cancer, myeloma, prostate cancer, ovarian cancer and follicular thyroid carcinoma (Mandell, Mandell et al. 1999; Boland, Ricard et al. 2000; Spitzweg, O'Connor et al. 2000; Groot-Wassink, Aboagye et al. 2002; Dingli, Russell et al. 2003; Petrich, Knapp et al. 2003; Sieger, Jiang et al. 2003; Shen, Marsee et al. 2004; Dwyer, Schatz et al. 2005; Dwyer, Bergert et al. 2006). Selective targeting NIS to specific tumor types was accomplished by the use of tissue-specific promoters to increase tissue-specific cytotoxicity and decrease the toxic side effect of radioiodine therapy in non-targeted tissues. Infection of myeloma cells with lentivirus carrying NIS cDNA driven by an immunoglobulin promoter led to myeloma-specific NIS expression that resulted in uptake of radioactive iodine and eradication of myeloma xenografts (Dingli, Russell et al. 2003). Infection of breast cancer cells with adenovirus carrying NIS cDNA driven by the mucin-1 promoter resulted in radioiodine accumulation in breast tumor xenografts (Dwyer, Schatz et al. 2005). Prostate adenocarcinoma LNCap cells stably transfected with NIS under the control of prostate-specific antigen (PSA) promoter resulted in prostate specific NIS expression and radioiodine accumulation. Moreover, administration of I$^{131}$ to mice carrying LNCap cells stably expressing NIS xenografts resulted in a reduction in the size of the xenograft (Spitzweg, O'Connor et al. 2000). As the therapeutic efficacy of radioiodine not only depends on NIS-mediated radioiodine uptake, but also radioiodine retention to exert efficient cytotoxic effect, several studies have explored ways to enhance iodide retention time by decreasing iodide efflux or co-express exogenous TPO.
and NIS. Treatment of 17-AAG or DIDS increased radioiodine retention time in NIS expressing rat thyroid cells and human thyroid cancer cells (Marsee, Venkateswaran et al. 2004; Elisei, Vivaldi et al. 2006). However, in cells that have no NIS expression, Huang, et al showed that co-expression of NIS and TPO in RH2 human non-small cell lung cancer cells increased radioiodine retention and radioiodine-induced apoptosis (Huang, et al, 2001). Another strategy to increase the therapeutic efficacy of NIS-mediated radioiodine accumulation is to use radioisotopes of higher energy and shorter physical halflife than $^{131}$I. Willhauck, et al showed that $^{188}$Re significantly enhanced the reduction of larger tumors compared to $^{131}$I in LNCaP xenograft with PSA-targeted NIS expression in mice (Willhauck, Sharif Samani et al. 2007). In summary, tissue-specific promoters driven NIS expression, the capability of NIS expression in a broad range of cell types, and prolonged radioiodine retention time confers the possibility of using NIS as a therapeutic gene allowing effective and tissue-specific radioiodine therapy in non-thyroid cancers and thyroid cancer lacking endogenous NIS.
Figure 1.1. The Na⁺/I⁻ symporter (NIS) and thyroid hormone biosynthesis. NIS is localized on the baso-lateral membrane of the thyroid follicular cells. NIS mediates active iodide uptake from the bloodstream into cells for thyroid hormone triiodothyronine (T3) and thyroxine (T4) biosynthesis. TSHR (thyroid stimulating hormone receptor); TPO (thyroid peroxidase); TG (thyroglobulin) and ATPase (Na⁺/K⁺-ATPase).
Figure 1.2. Schematic diagram shows the predicted secondary structure of rat NIS. The three putative asparagines N-linked glycosylation sites and the putative leucine zipper is depicted in the graph.
<table>
<thead>
<tr>
<th>Signal node</th>
<th>Inhibitor</th>
<th>Activator</th>
<th>Cell line</th>
<th>NIS mRNA</th>
<th>NIS protein</th>
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**Table 1.1** Summary of reported signaling nodes on NIS regulation in thyroid cells
SPECT Imaging for Temporal Dynamics of Thyroidal and Salivary Radionuclide Accumulation in 17-AAG Treated Thyroid Cancer Mouse Model

Abstract

Selective iodide uptake and prolonged iodine retention in the thyroid is the basis for targeted radioiodine therapy for thyroid cancer patients, however, salivary gland dysfunction is the most frequent non-thyroidal complications. In this study we employed non-invasive SPECT functional imaging to quantify the temporal dynamics of thyroidal and salivary radioiodine accumulation in mice. At 60 min post radionuclide injection, radionuclide accumulation in the salivary gland was generally higher than that in thyroid due to much larger volume of the salivary gland. However, radionuclide accumulation per anatomic unit in the salivary gland was lower than that in thyroid and was comparable among mice of different age and gender. Differently, radionuclide accumulation per anatomic unit in thyroid varied greatly among mice. The extent of thyroidal radioiodine accumulation stimulated by a single dose of exogenous bovine TSH (bTSH) in T3-supplemented mice was much less than that in mice received neither bTSH nor T3 (non-treated mice), suggesting that the duration of elevated serum TSH level is
important to maximize thyroidal radioiodine accumulation. Furthermore, the extent and duration of radioiodine accumulation stimulated by bTSH was less in the thyroids of the thyroid-targeted RET/PTC1 (Tg-PTC1) mice bearing thyroid tumors compared to the thyroids in wild type mice. Lastly, the effect of 17-AAG on increasing thyoidal, but not salivary, radioiodine accumulation was validated in both wild type mice and Tg-PTC1 preclinical thyroid cancer mouse model.
Introduction

The primary function of the thyroid gland is to synthesize thyroid hormones, triiodothyronine (T3) and thyroxine (T4), which play important roles in systemic metabolism. Iodine is an essential component of thyroid hormone yet it is frequently not abundant in the diet; therefore the thyroid gland has evolved an effective way to sequester iodine 20 to 40 times against its concentration gradient from the systemic circulation. Iodide is transported into thyroid follicular cells by the sodium iodide symporter (NIS), an intrinsic membrane glycoprotein expressed on the basolateral membrane of the thyroid follicular cells. Once in the thyroid cells, iodine is incorporated into thyroglobulin (Tg) through a process called iodine organification and stored in the follicular lumen of the thyroid. Effective radioiodine accumulation in thyroid tissues, mediated by both active uptake and organification, has allowed the clinical use of radioiodine to detect and/or ablate residual thyroid cancer tissue in patients who have undergone thyroidectomy (see review in (Shen, Kloos et al. 2001)).

Both iodide uptake and organification are mainly stimulated by thyrotropin (TSH). To selectively induce radioiodine accumulation in thyroid tissues for effective radioiodine therapy, TSH is elevated either by T4 withdrawal to induce endogenous hypothyroidism or by exogenous administration of recombinant human TSH (rhTSH) prior to radioiodine administration (see guidelines published by Copper et al. 2009; Luster et al. 2008). However, about 20-30% patients with metastatic thyroid cancer do not benefit from radioiodine therapy due to reduced or absent NIS expression/function (Caillou, Troalen et al. 1998; Castro, Bergert et al. 2001; Patel, Escrig et al. 2002; Ward,
Santarosa et al. 2003; Oler and Cerutti 2009). Thus, it is of clinical importance to identify/develop pharmacological reagents that could increase and/or restore radioiodine accumulation in these tumors. As radioiodine accumulation is the net outcome of iodide influx and efflux, strategies to increase radioiodine accumulation include selectively increase thyroidal iodide influx and/or decrease iodide efflux.

Much effort has been made to identify reagents that increase NIS mRNA levels and/or radioiodine accumulation in cultured thyroid cells. Among all agents tested, LY294002, a PI3K inhibitor, has been reproducibly shown to increase thyroidal NIS expression (Garcia and Santisteban 2002; Zaballos, Garcia et al. 2008)( Liu et al. manuscript in preparation) as well as NIS-mediated radioiodide uptake activity (Kogai, Sajid-Crockett et al. 2008)( Liu et al. manuscript in preparation). While thyroidal NIS expression was increased by MAPK inhibitors (Knauf, Kuroda et al. 2003; Vadysirisack, Venkateswaran et al. 2007), it was not accompanied with comparable degree of increase in NIS-mediated radioiodide uptake activity (Vadysirisack, Venkateswaran et al. 2007)(Liu et al. manuscript in preparation). H-DAC inhibitor depsipeptide appeared to modestly increase NIS mRNA level as well as radioiodine accumulation in both thyroid and non-thyroid cancer cells (Kitazono, Robey et al. 2001). A recent study showed that combination treatment of inhibitors for H-DAC, MAPK, and PI3K increased radioiodine accumulation in several human thyroid cancer cell lines (Hou, Bojdani et al. 2010), but this finding is yet to be confirmed by others. Most importantly, to our knowledge, none of these findings have been validated using immune-competent genetically engineered mice carrying thyroid tumors surrounded by physiologically/pathologically relevant microenvironment.
Our lab has previously reported that 17-allyamino-17-demethoxygeldanamycin (17-AAG) increases NIS-mediated radioiodine accumulation by decreasing iodide efflux in NIS-expressing PC Cl3 rat thyroid cells and RET/PTC1-transformed PC Cl3 cells (Marsee, Venkateswaran et al. 2004). This finding was subsequently confirmed by another study showing that 17-AAG increases iodide retention in cultured human anaplastic thyroid cancer cells stably expressing hNIS (Elisei, Vivaldi et al. 2006). In our current study, we used non-invasive radionuclide imaging with micro-single photon emission computed tomography (micro-SPECT) to validate the effect of 17-AAG on increasing thyroidal, but not salivary, radioiodine accumulation in wild type (WT) mice and in our thyroid-targeted RET/PTC1 (Tg-PTC1) thyroid cancer mouse model. Our findings encourage the investigation of 17-AAG derivative pharmacological agents to increase the efficacy of radioiodine therapy in thyroid cancer patients, in addition to their potential roles as novel chemotherapeutic agents.
Material and Methods

Animals and Treatment

The study protocol (2009A0118) was approved by our Institutional Animal Care and Use Committee that oversees the responsible use of animals in university research and instructional activities. All research activities were conformed to the statutes of the Animal Welfare Act and the guidelines of the Public Health Service as issued in the Guide for the Care and Use of Laboratory Animals (revised 1996). Mice received Harlan Teklad LM-485 Mouse/Rat sterilizable diet (Harlan Teklad Diets, Madison, Wis.) containing 2.61 mg iodine per kilogram diet. Tg-PTC1 mice were generated in the FVB/N background, and all Tg-PTC1 mice developed thyroid tumors by one month of age (Jhiang, Sagartz et al. 1996; Cho, Sagartz et al. 1999). For T3-bTSH experiments, mice were supplemented with 12 µg/ml T3 in their drinking water daily. On day 5, mice received a single intraperitoneal (i.p.) injection of bTSH in 200 µl 1% BSA/PBS and after 16 hr the mice were subjected to SPECT imaging, or sacrificed for ex vivo gamma counting, as described below. The bTSH dosages utilized are reported at the result section.

Two different experimental plans were employed for 17-AAG (National Cancer Institute: NCI 330507) experiments. We started with the protocol of using T3-bTSH treated mice i.p. injected with a single dose of 40 mg/kg 17-AAG (the highest dose used in mice reported in NCI Investigator brochure, 2004) and performed SPECT imaging at 2 hr post 17-AAG injection. We later modified the protocol using mice neither treated with T3 nor
bTSH (non-treated mice) i.p. injected with a single dose of 30 mg/kg 17-AAG and performed SPECT imaging at 3 hr post 17-AAG injection.

**In vivo multi-pinhole SPECT imaging**

Small animal SPECT imaging was performed using the X-SPECT preclinical platform (Gamma Medica, CA, USA) equipped with 2 gamma cameras, each mounted with a multiple pinhole collimator with 1 mm aperture. After i.p. injection of 100-150 μCi Na$^{123}$I ($t_{1/2}$= 13.3 hr) or Na$^{99m}$TcO$_4$ ($t_{1/2}$= 6.01 hr) in saline, mice were anesthetized using isoflurane inhalation and immobilized on the animal bed. For subsequent calibration of radioactivity in target tissues at various times, a 0.25 ml eppendorf tube containing a known amount of the same radionuclide was included as a decay control (DC). SPECT imaging was acquired with 32 projections, each for 50 sec on a 30 mm radius of rotation. SPECT images were acquired at 1, 6, and 24 hr post Na$^{123}$I injection or 1 hr post Na$^{99m}$TcO$_4$ injection.

**Ex vivo gamma counting**

Following T3-bTSH treatment, animals were i.p. injected with 20-40 μCi Na$^{123}$I in saline. Animals were sacrificed at 6 hr post Na$^{123}$I injection, and thyroid gland, salivary gland and liver were harvested and their radioactivity measured with a gamma counter (Perkin Elmer, MA, USA) along with a known activity of Na$^{123}$I as a standard for quantification.
Calculation of thyroïdal and salivary radioiodine accumulation

The total injected dose was calculated by subtracting the post-injected syringe counts from the pre-injected syringes counts. Using the Amira 3.1 software (Gamma Medica, CA, USA), region of interest (ROI) was manually drawn over the thyroid gland, salivary gland, or decay control on xy-, yx-, and xz-planes, and the total radioactivity in the 3D region defined by the three selected planes was automatically determined. Thyroidal and salivary radioiodine accumulation were calibrated by decay control counts at each time point and reported as the percentage of injected dose (%ID).

Statistical Analysis

As many of the experiments measured thyroïdal radioiodine accumulation in mice over time, linear mixed models were used to take account of the dependency of observations within the same animals. To ensure unbiased hypothesis tests, we used a covariance structure estimation method for small samples that avoids underestimation of experimental error. Two sample t-tests were used for simple two group comparisons and Holm’s step down procedure (Holm 1979) was used to adjust for multiple comparisons. P-values <.05 for single comparisons or after adjustment for multiple comparisons were considered as significant.
Results

**Temporal dynamics of radioiodine accumulation in thyroid and salivary glands evaluated by 3D SPECT functional imaging**

Thyroidal iodide accumulation is largely the sum of NIS-mediated iodide uptake along with iodide retention by iodide organification. In comparison, salivary glands accumulate iodide through NIS-mediated iodide uptake but they lack iodide organification. The temporal dynamics of $^{123}$I accumulation in thyroid and salivary glands in WT mice was examined by non-invasive SPECT nuclear imaging (Fig. 2.1). At 1 hr post Na$^{123}$I injection (t1), $^{123}$I accumulation was evident in both thyroid and salivary glands. At 6 hr post Na$^{123}$I injection (t6), $^{123}$I accumulated in the thyroid gland was further increased due to continued iodide uptake and organification. However, $^{123}$I accumulated in the salivary gland was decreased due to lack of iodide organification and decreased $^{123}$I in the blood circulation. At 24 hr post Na$^{123}$I injection (t24), $^{123}$I accumulation in the thyroid gland continued to increase when normalized by physical decay. Conversely, no $^{123}$I accumulation was detected in the salivary gland at t24, suggesting that $^{123}$I was completely cleared from the blood circulation by excretion or tissue uptake in other tissues such as the thyroid. Consequently, thyroidal radioiodine accumulation at t24 was mainly contributed by iodide retention.

Several mice were subjected to SPECT imaging in the same experimental settings as stated above. The amount of $^{123}$I accumulation in the thyroid (%ID) varied extensively among mice and the degree of $^{123}$I accumulation at t6 was not always near maximum, i.e. to the similar extent seen at t24 (Fig. 2.2). This variability may be contributed by various
sizes of follicular lumen, as radioactivity equilibrium is reached faster in follicular lumen with smaller radius (Cantraine and Dewandre 1975). Thyroidal %ID at t1, t6, and t24 for each mice studied are shown in Table 1 (t1: 0.63-6.45%, mean= 2.07%; t6: 1.59-23.21%, mean= 8.88%; and t24: 1.48-24.66%, mean= 11.55%). Albeit our small sample number, mice of 14-15 months of age tended to have lower iodide influx as indicated by thyroidal %ID at t1. Mice of 2-3 months old appeared to have lower iodide retention ability as indicated by lower ratios of t6 or t24 over t1 thyroidal %ID. This may be at least in part contributed by their faster renal clearance rate as shown by a sharper decrease in $^{123}\text{I}$ accumulation in salivary gland at t6 versus t1 (t6/t1 %ID: 2-3 month: 0.1-0.3, mean= 0.22, N=3; > 6 month: 0.73-1.01, mean= 0.86, N=4). These findings warrant further study using a larger cohort.

The extent of single dose bTSH induced thyroidal radioiodine accumulation in T3-supplemented mice is less than that induced by continuous stimulation of endogenous mouse TSH in non-treated mice

To examine the effect of exogenous bTSH on temporal dynamics of thyroidal radioiodine accumulation in the absence of endogenous mouse TSH (mTSH), endogenous mTSH was suppressed by T3 supplementation in WT mice. As shown in Fig. 2.2, thyroidal radioiodine accumulation was diminished in all mice that underwent T3 supplementation (t1: 0.18-1.06%, mean= 0.49%; t6: 0.26-0.59%, mean= 0.41%; t24: 0-0.2%, mean= 0.06%, N=4), despite extensive variability of thyroidal radioiodine accumulation seen in non-treated mice (N=11). This data confirms that our T3
supplementation protocol is sufficient to suppress endogenous mTSH induced thyroidal radioiodine accumulation, regardless of age and gender.

In mice treated with 6, 30, or 60 μg of bTSH at day 5 of T3-supplementation, thyroidal radioiodine accumulation was increased in a dose-dependent manner (data not shown). Further increase of bTSH to 90 μg did not further increase (p-value=0.71) thyroidal radioiodine accumulation as determined by \textit{ex vivo} gamma counting of thyroids at 6 hr post Na$^{123}$I injection (Fig. 2.3A). As shown in Fig. 2.2, thyroidal radioiodine accumulation averaged across time was significantly increased (p-value= 0.0007) by a single dose of 60 μg bTSH in T3 supplemented mice (t1: 0.34-1.99%, mean= 0.93%; t6: 1.22-2.84%, mean= 2.01%; t24: 1.97-3.36%, mean= 2.46%, N=5). However, thyroidal %ID in T3-bTSH treated mice was much lower (p-value= 0.027) than that in non-treated mice. This is most likely due to the short half-life of bTSH (< 3hr, (Guyot, Sulon et al. 2007)), such that a single dose of 60 μg bTSH in T3 supplemented mice is not sufficient to induce thyroidal radioiodine accumulation comparable to non-treated mice which had continuous low-level stimulation of endogenous mTSH.

The extent and duration of thyroidal radioiodine accumulation induced by bTSH is less in Tg-PTC1 mice than that in wild type mice

Tg-PTC1 mice can serve as a preclinical animal model to examine factors that modulate radioiodine accumulation in thyroid tumors. We previously reported that thyroidal radioiodine accumulation in Tg-PTC1 mice is decreased compared to WT mice at 6 hr post Na$^{125}$I injection when normalized with tissue weight despite of elevated serum TSH level in Tg-PTC1 mice (Cho, Sagartz et al. 1999). This is consistent with
clinical findings that malignant thyroid tumors are typically “cold” nodules. Our pilot study showed that thyroidal radioiodine accumulation was also suppressed by T3 supplementation in Tg-PTC1 mice (data not shown). To compare the responsiveness of WT and Tg-PTC1 mice to exogenous bTSH toward induction of thyroidal radioiodine accumulation, $^{123}$I accumulation at t6 was evaluated ex vivo in T3 supplemented mice injected with a single dose of bTSH. As shown in Fig. 2.3 A, the extent of bTSH induced thyroidal radioiodine accumulation at t6 in Tg-PTC1 mice (1.6-2.5%, mean= 2.04%, N=4) was significantly lower (p-value= 0.001) than that in WT mice (8.9-16.3%, mean= 12.44%, N=4) without normalization for thyroid gland volume or weight. However, the size of thyroid glands in Tg-PTC1 mice was consistently larger than that in WT mice.

To compare the duration of bTSH-induced NIS activity between WT mice and Tg-PTC1 mice, Na$^{99m}$TcO$_4$, which cannot be organified, was used to evaluate NIS-mediated radionuclide influx at 17 hr or 41 hr post bTSH administration. As shown in Fig. 2.3B, thyroidal %ID of Na$^{99m}$TcO$_4$ was modestly decreased (p-value= 0.28) at 41 hr compared to 17 hr post bTSH administration in WT mice (17 hr: 1.27-1.54%, mean= 1.41%; 41 hr: 0.23-1.50%, mean= 0.93%, N=3). In comparison, thyroidal %ID of Na$^{99m}$TcO$_4$ was significantly decreased (p-value= 0.0076) at 41 hr in all three Tg-PTC1 mice examined (17 hr: 1.7-2.60%, mean= 2.01%; 41 hr: 0.00-0.57%, mean= 0.32%, N=3). Indeed, statistical analysis by linear mixed model showed that the difference of NIS activity between 17 hr and 41 hr post bTSH administration for Tg-PTC1 mice was much more evident (p-value= 0.014) than that for WT mice. Taken together, thyroid tumor bearing thyroid glands of Tg-PTC1 mice not only were less responsive to bTSH in the induction of radioiodine accumulation; the duration of NIS induction was also much
shorter than normal thyroids in WT mice. It is interesting to note that thyroidal %ID of Na$^{99m}$TcO$_4$ at 17 hr post bTSH injection in Tg-PTC1 mice was higher compared to WT mice, suggesting that the number of thyroid follicular cells responsive to bTSH at an early time point in Tg-PTC1 mice is much more than that in WT mice. This phenomenon can be explained by increased proliferation of thyroid follicular cells (Cho, Sagartz et al. 1999) and the much larger size of thyroids observed in Tg-PTC1 mice.

The area under time-activity curve of thyroid in wild type mice is markedly increased by 30 mg/kg 17-AAG

None of the T3-bTSH mice injected with a single dose of 40 mg/kg 17-AAG survived during SPECT imaging acquisition (N=3, data not shown), indicating the experimental condition was not tolerable by the mice. We then examined the effect of 30 mg/kg 17-AAG in non-treated mice which had Na$^{123}$I injection at 3 hr post 17-AAG injection (see the top panel of Fig. 2.4). Three experimental trials with identical design were performed to compare temporal radioiodine accumulation between DMSO and 17-AAG treated mice. As shown in Fig 2.4, thyroidal radioiodine accumulation at t24 in 17-AAG treated mice was much higher (p-value= 0.15) than DMSO-treated mice in each experimental trial (DMSO vs. 17-AAG: 5.42% vs. 9.22%; 20.66% vs. 65.88%; 8.07% vs. 55.05%), despite extensive variations of thyroidal %ID among different experimental trials. Conversely, thyroidal radioiodine accumulation at t1 was not increased (p-value= 0.98) by 17-AAG treatment (DMSO vs. 17-AAG: 3.58% vs. 4.02%; 1.18% vs. 0.63%; 3.24% vs. 3.44%). These results indicate that 17-AAG markedly increases iodide retention although it has little effect on NIS-mediated iodide influx.
The average of thyroidal %ID at t1, t6, and t24 among three experimental trials as well as the average ratio of thyroidal %ID at t6 or t24 versus t1 in each experimental trial are shown in Table 2.2. Iodide retention ability in WT mice was moderately increased by 17-AAG at t6 (~ 2 fold), and further increased at t24 (~ 4 fold) as compared to DMSO treated mice. Consequently, the exposure of the thyroid to radioiodine over time was markedly increased by 17-AAG as defined by the area under time-activity curve (AUC). Indeed, while the value of AUC varied greatly among experimental trials (DMSO: 224.4 ± 133.2, N=3; 17-AAG: 583.1 ± 362.1, N=3), the fold increase in AUC by 17-AAG calculated in each experimental trial ranged from 1.55 to 3.96. Taken together, the effect of 17-AAG on increasing radioiodine accumulation in cultured thyroid cells is validated in WT mice using temporal SPECT imaging. Furthermore, 17-AAG did not appear to have an effect on salivary %ID (data not shown).

The area under time-activity curve of thyroid tumor in Tg-PTC1 mice is markedly increased by 30 mg/kg 17-AAG

We next examined the effect of 30 mg/kg 17-AAG on thyroidal radioiodine accumulation in thyroid tumor bearing thyroid glands of Tg-PTC1 mice. While WT mice were tolerant of 30mg/kg 17-AAG treatment, two out of four Tg-PTC1 mice did not survive through SPECT imaging acquisition. However, as shown in Fig. 2.5, both of the remaining Tg-PTC1 mice treated with 17-AAG had evident increase of thyroidal radioiodine accumulation compared to mice treated with DMSO at t24 (DMSO vs. 17-AAG: 3.50% vs. 25.89%; 2.53% vs. 19.23%). Similar to WT mice, 17-AAG appeared to increase radioiodine retention rather than radioiodide influx in Tg-PTC1 mice. As shown
in Table 2.2, iodide retention was moderately increased by 17-AAG at t6 (~ 1.5 fold), yet it was further increased at t24 (~ 4 fold) as calculated by the average ratios of thyroidal %ID at t6 or t24 versus t1 in each experimental trial. The value of AUC was less variable between the two experimental trials in Tg-PTC1 mice (DMSO: 148.8 ± 44.3, N=2; 17-AAG: 442.6 ± 62.7, N=2), and the fold increase in AUC by 17-AAG in the two experimental trials were 2.21 and 4.14. Thyroidal iodide retention ability in 17-AAG treated Tg-PTC1 mice (t24/t1 %ID= 2.6 ± 0.6) was much lower than that in WT mice (t24/t1 %ID= 15.7 ± 0.9) at t24, yet the fold increase of iodide retention ability by 17-AAG was comparable between Tg-PTC1 mice and WT mice at t24. Taken together, the effect of 17-AAG on increasing thyroidal radioiodine accumulation is confirmed in a preclinical thyroid cancer animal model.
Discussion

In this study, we quantified temporal radioiodine accumulation in both thyroid and salivary glands modulated by T3, bTSH and/or 17-AAG in mice using SPECT functional imaging. By comparing temporal dynamics of radioiodine accumulation between salivary and thyroid glands, we found that administered radioiodine was almost completely cleared from blood circulation after 24 hr, and that iodide organification contributes greatly to thyroidal radioiodine accumulation over time. The extent of thyroidal radioiodine accumulation stimulated by a single dose of exogenous bTSH in T3 mice was much less than that in non-treated mice, justifying the clinical use of multiple doses of rhTSH or the merit of T4 withdrawal in thyroid cancer patients prior to radioiodine therapy. Furthermore, the extent and duration of radioiodine accumulation stimulated by bTSH was reduced in thyroid tumor bearing thyroid glands of Tg-PTC1 mice compared to the thyroids in WT mice. Lastly, 17-AAG significantly increased thyroidal exposure of radioiodine over time in our Tg-PTC1 preclinical thyroid cancer mouse model, which encourages the study of 17-AAG derivatives not only as novel chemotherapeutic agents to impede tumor progression but also to improve radioiodine therapy in patients with thyroid cancer. Optimally, 17-AAG derivatives could permit those tumors with low radioiodine accumulation to become amenable to radioiodine treatment and allow for those tumors with normal radioiodine accumulation to be affectively treated with lower radioiodine activities.

Salivary gland dysfunction is the most frequent non-thyroidal complications of radioiodine therapy for patients with thyroid cancer (Kloos 2009). A better understanding
of the temporal dynamics of radioiodine accumulation in the thyroid and salivary glands may lead to strategies to mitigate radioiodine-induced salivary gland damage without compromising the efficacy of thyroid cancer radioiodine therapy. A recent study showed that the time required to reach maximal accumulation of $^{99}$mTcO$_4$ uptake in thyroid glands is shorter than that in salivary glands of C57 BL6/6J mice (Franken, Guglielmi et al.). This difference could be explained by higher NIS transport activity and/or smaller NIS-expressing epithelial cell-delimiting compartments of the thyroid, i.e. thyroid colloid versus salivary acini and duct lumens. Similarly, the rate of decrease in $^{99}$mTcO$_4$ accumulation is faster in the thyroid than in the salivary gland after administration of competitive inhibitors (Franken, Guglielmi et al.). In the current study, we showed that salivary gland %ID was 1.02-5.75 fold higher than thyroid %ID at t1 (see Table 2.1), which has been reported by others (Franken, Guglielmi et al.; Zuckier, Dohan et al. 2004) and is anticipated by the larger size of the salivary gland. Indeed, voxel counts in the selected ROI of salivary were 1.93-9.38 fold higher those of the thyroid at t1 (data not shown), although functional voxel counts in selected ROI may not exactly reflect anatomic volume of the target tissue. Interestingly, the normalized means (total signal intensity counts divided by voxel counts) of salivary glands at t1 were lower than those of the thyroid (salivary/thyroid= 0.47-0.94 with a mean of 0.68), indicating that NIS expression/activity per anatomic unit is lower in salivary glands compared to the thyroid gland. Salivary normalized means at t1 were quite comparable among mice (62-80 with a mean of 72), yet thyroid normalized means at t1 varied greatly among mice (66-163 with a mean of 113). Thus, NIS activity per anatomic unit in the salivary gland is not changed by gender or age among different mice.
While rhTSH administration has been shown to be equally effective compared to thyroid hormone withdrawal in preparation of low-risk patients for post-operative thyroid remnant ablation (Pacini, Schlumberger et al. 2006; Chianelli, Todino et al. 2009; Elisei, Schlumberger et al. 2009), it remains uncertain whether rhTSH is equally effective in treating high-risk patients with known residual cancer. The level and duration of serum TSH levels required for effective ablation of thyroid remnant could be very different from that for residual cancer and may vary depending on various somatic tumor mutations that affect radioiodine accumulation. Indeed, Potzi and colleagues reported that a higher radioactivity of $^{131}$I is required to achieve comparable radioiodine accumulation in thyroid cancer metastases in patients administered radioiodine after rhTSH versus thyroid hormone withdrawal preparation (Potzi, Moameni et al. 2006). Our current study showed that thyroidal radioiodine accumulation in T3-bTSH mice was much lower than that in non-treated mice suggesting that the duration of exposure to TSH is important to promote desirable thyroidal radioiodine accumulation. We also showed that thyroid tumor bearing thyroid glands in Tg-PTC1 mice were less responsive to bTSH induced thyroidal radioiodine accumulation than normal thyroids in WT mice. Taken together, thyroid cancer foci may require higher $^{131}$I activity for treatment as opposed to normal thyroid remnants and longer duration of elevated TSH level may be required to achieve the desirable $^{131}$I activity in thyroid cancer foci. Accordingly, various thyroid cancer mouse models (Knostman, Jhiang et al. 2007; Burniat, Jin et al. 2008) can be invaluable to examine the concepts of magnitude and duration of serum TSH elevation required to confer sufficient radioiodine accumulation in thyroid tumors of different histologies that are initiated by various genetic events.
Findings using cultured cells do not always translate into preclinical animal models or patients. For example, leptin decreases radioiodine uptake activity in FRTL-5 immortalized rat thyroid cells (Isozaki, Tsushima et al. 2004) and rat thyroid slices (de Oliveira, Teixeira Silva Fagundes et al. 2007), yet it increases thyroidal radioiodine uptake activity in live rats (de Oliveira, Teixeira Silva Fagundes et al. 2007). The discordance is most likely explained by tissue microenvironments and/or systemic effects that do not exist in isolated cultured cell system. In this study, we confirmed that 17-AAG increases thyroid exposure to radioiodine over time in WT mice as well as Tg-PTC1 transgenic mice carrying thyroid tumors. The fact that 17-AAG did not increase thyroidal radioiodine accumulation at t1, but did at t6 and t24, indicates that 17-AAG has little effect on NIS-mediated radioiodine influx but mainly acts on iodide retention/organification. This result is consistent with our previous finding that 17-AAG increases radioiodine accumulation by decreasing iodide efflux in cultured cells (Marsee, Venkateswaran et al. 2004) and that 17-AAG did not increase thyroidal $^{99m}$TcO$_4$ accumulation at either 4 hr or 27 hr post-treatment (data not shown). The mechanism underlying the increase of thyroidal iodide retention by 17-AAG is currently unknown. Since Hsp90 is known for its role as molecule chaperone, it would be interesting to investigate whether 17-AAG acts to impair the secretion of iodinated thyroglobulin derivatives.

The following issues need to be addressed before our findings can be translated into clinical trials. Is 17-AAG’s effect on thyroidal radioiodine accumulation mainly mediated by Hsp90 inhibition? Do all Hsp90 inhibitors increase thyroidal radioiodine accumulation? Which Hsp90 inhibitor has the desired pharmacokinetic and
pharmacodynamic profiles? What are the optimal dose, duration, and timing of Hsp90 inhibitor treatment to increase the efficacy of radioiodine therapy? Is the magnitude of increased radioiodine accumulation by Hsp90 inhibitors sufficient to meaningfully improve the efficacy of radioiodine therapy? Are there any differences in the effects of Hsp90 inhibitors on thyroid tumors derived from various genetic abnormalities, such as B-RAF<sup>V600E</sup> mutation? Finally, while not immediately needed for Hsp90 inhibitor clinical application, what are the molecular targets of this treatment in thyroid tumors, and can they be more optimally modulated by other approaches?
Supplementary data

Interestingly to note that while the current and previous studies both showed that thyroidal radioiodine accumulation is decreased in Tg-PTC1 mice compared to wild type mice, yet there is a big difference in the fold change in thyroidal radioiodine accumulation between WT versus Tg-PTC1 mice (WT/Tg-PTC1 %ID at t6: previous study: 30 fold; current study: 6 fold). This is most likely due to the difference in chronic high endogenous TSH stimulation versus one dose of exogenous bTSH stimulation. However, there is a possibility that copy numbers of the transgene decreased over time in our Tg-PTC1 transgenic mice as a limitation in transgenic animal models (Kong, Wu et al. 2009). Moreover, there is also a possibility that cells accumulating iodide in Tg-PTC1 mice may not be tumor cells as RET/PTC1 induced dedifferentiation of thyroid cells would eventually lead to loss of TSH receptor expression thus resulting in decreased RET/PTC1 expression. While keeping that in mind, our data showed that Tg-PTC1 mice responded less to bTSH stimulation in inducing thyroidal radioiodine accumulation and decreased bTSH induced temporal duration of NIS activity compared to WT mice. Moreover, DMSO had an effect on decreasing thyroidal radioiodine accumulation in Tg-PTC1 mice at t24 but not in WT mice (Fig 2.7 versus 2.6) suggesting that cells accumulating iodide in Tg-PTC1 mice while may not be tumor cells, are different than the normal iodide accumulating thyroid cells in WT mice.

Heat shock protein (Hsp90) is a highly abundant protein that accounts for 1 to 2 % of total cellular proteins. Hsp90 together with several co-chaperones form the multichaperone complex that is responsible for the proper folding, stability and activity of a range of client proteins including kinases (i.e. Raf, Akt) and transcription factors (i.e.
Upon ATP hydrolysis of Hsp90, the Hsp90 multichaperone complex would assist the maturation and stabilization of the client proteins in an active state. Disruption of Hsp90 leads to ubiquitin-proteasomal degradation of client proteins, thus down-regulation of the signaling transduction. Since there is an overactivation of these signaling pathways in cancer cells, Hsp90 inhibitors have been of great interest as it targets essentially all of the oncogenic signaling pathways that regulates tumor progression and survival. The first identified Hsp90 inhibitors were N-terminal ATPase domain binders i.e. GA, 17-AAG and 17-DMAG. Among these inhibitors, 17-AAG was the first to enter clinical trials (Workman 2004; Zhang and Burrows 2004). We and others previously reported that treatment of Hsp90 inhibitor, 17-AAG increases NIS-mediated radioiodine accumulation by decreasing iodide efflux in parental and RET/PTC1-expressing cultured PCCl3 rat thyroid cells (Marsee, et al, 2004). This finding was later confirmed by another investigator showing that 17-AAG increases iodide retention time in cultured human thyroid cancer cells expressing exogenous hNIS (Elisei, et al, 2006). In this study, we tried to validate the effect of 17-AAG on increasing thyroidal radioiodine accumulation in thyroid tumor of our Tg-PTC1 transgenic mouse model. While DMSO vehicle did not seem to have an effect on modulating thyroidal radioiodine accumulation in WT mice (Fig 2.6), yet significantly decreased thyroidal %ID at t24 by decreasing thyroidal radioiodine retention ability in Tg-PTC1 mice (Fig 2.7). Furthermore, our result showed that treatment of 17-AAG 3 hr prior to Na\textsuperscript{123}I injection increased thyroidal radioiodine accumulation and AUC of thyroidal radioiodine accumulation at 27 (t24) hr, but not at 4 hr (t1) post 17-AAG in both WT and Tg-PTC1 mice compared to DMSO (17-AAG/DMSO AUC: wild type: 1.55 to 3.96; Tg-PTC1: 2.21 and 4.14). The fact that
17-AAG did not increase thyroidal radioiodine accumulation at t1, but did at t24, indicates that 17-AAG has little effect on NIS-mediated radioiodine influx but mainly acts on iodide retention/organification. This result is consistent with our finding that 17-AAG did not increase thyroidal $^{99m}$TcO$_4^-$ accumulation at either 4 hr or 27 hr post-treatment (Fig 2.8 mouse number 4). We also examined thyroidal radioiodine accumulation in WT mice treated with 16 hr 17-AAG prior to Na$^{123}$I injection and did not observe an increase in thyroidal radioiodine accumulation (Fig 2.9). This could be due to that 17-AAG pharmacokinetic study showed detectable levels of 17-AAG remained in the plasma for 3 hr and 17-AAG active metabolite, 17-AG, remained in the plasma for 4 hr post single 40 mg/kg 17-AAG injection (Egorin, Zuhowski et al. 2001).

As the major side effect of targeted radioiodine therapy is salivary gland dysfunction, the effect of 17-AAG on salivary radioiodine accumulation was also examined in this study. Treatment of 30 mg/kg 17-AAG 3 hr prior to Na$^{123}$I injection had minimal effect on thyroidal radioiodine accumulation at 4 hr post 17-AAG in WT mice (Fig 2.10; N=4). However, while treatment of 30 mg/kg 17-AAG 3 hr prior to Na$^{123}$I injection did not significantly increased salivary radioiodine accumulation at 4 hr post 17-AAG in Tg-PTC1 mice (Fig 2.11; N=4), we observed that two out of four mice had increased salivary radioiodine accumulation at t1. In summary, treatment of 17-AAG selectively increased thyroidal, but not salivary radioiodine accumulation, in both WT and Tg-PTC1 mice. However, this warrants a further study of effects of 17-AAG on salivary radioiodine accumulation in using a larger cohort of T3-supplemented endogenous TSH suppressed mice.
Figure 2.1 Temporal dynamics of thyroidal and salivary radioiodine accumulation in wild type mouse. A 7 month old male wild type mouse was injected with ~150 µCi Na$^{123}$I and subjected to SPECT imaging at 1(t1), 6(t6) and 24 hr(t24) post Na$^{123}$I injection. At t1, thyroidal radioiodine accumulation was mainly contributed by NIS-mediated $^{123}$I influx from blood circulation. At t6, thyroidal radioiodine accumulation was contributed by continuous $^{123}$I influx as well as $^{123}$I organification/retention. At t24, thyroidal radioiodine accumulation was mainly contributed by $^{123}$I organification/retention as NIS-mediated $^{123}$I uptake from blood circulation in the salivary gland was observed at t1 and t6, but not at t24. Thy: thyroidal functional image; Sal: salivary functional image; DC: decay control functional image. Decay control serves to normalize counts of thyroidal radioiodine accumulation at each time-point for quantification purposes. The color bar indicates radioiodine signal intensity increases from 20 (black) to 500 (red) in arbitrary units.
Figure 2.1
The extent of induced thyroidal radioiodine accumulation by one dose of 60 μg bTSH in T3 supplemented mice is much less than that induced by continuous endogenous mTSH in non-treated mice. WT mice were given 12 μg/ml T3 in drinking water daily. On day 5, mice were injected with one dose of 60 μg bTSH (T3-60 μg bTSH; N=5). Na\textsuperscript{123}I (100-150 μCi) was administered into mice at 16 hr post bTSH injection. SPECT images were taken at 1(t1), 6(t6) and 24 hr(t24) post Na\textsuperscript{123}I injection. For comparison, T3 mice (N=4) and non-treated mice (N=11) were subjected to SPECT imaging as well. In T3 mice, thyroidal radioiodine accumulation was almost undetected (p-value= 0.0017), confirming that T3 supplementation for 5 consecutive days is sufficient to suppress endogenous TSH induced thyroidal radioiodine accumulation. While one dose of 60 μg bTSH was sufficient to induce some extent of thyroidal radioiodine accumulation, the extent of 60 μg bTSH induced radioiodine accumulation was far less (p-value= 0.027) than that of non-treated mice.
Figure 2.2
Figure 2.3 The extent of thyroidal radioiodine accumulation and temporal duration of NIS-mediated thyroidal Na$^{99m}$TcO$_4$ influx activity induced by bTSH is less in Tg-PTC1 mice than that in wild type mice. (A) 7 month old WT and 4 month old Tg-PTC1 mice were given 12 µg/ml T3 in drinking water daily. On day 5, mice were injected with either one dose of 60 or 90 µg bTSH. Na$^{123}$I (20-40 µCi) was injected into mice at 16 hr post bTSH injection. At 6 hr(t6) post Na$^{123}$I injection, mice were euthanized and thyroids were harvested and subjected for counting of activity by a gamma counter. Statistical analysis showed that WT mice had significantly higher (p-value= 0.001) thyroidal radioiodine accumulation at t6 than Tg-PTC1 mice, while there was no significant difference (p-value= 0.71) between 60 or 90 µg bTSH induced thyroidal radioiodine accumulation in both WT and Tg-PTC1 mice. (B) T3-supplemented WT and Tg-PTC1 mice were administered with one dose of 60 µg bTSH. Na$^{99m}$TcO$_4$ (100 µCi), a substrate of NIS yet could not be organified by thyroid, was first injected into mice 16 hr post bTSH injection and SPECT images were acquired at 1 hr post Na$^{99m}$TcO$_4$ injection, i.e. at 17 hr post bTSH injection. A second dose of Na$^{99m}$TcO$_4$ (100 µCi) was injected into mice 40 hr post bTSH injection and SPECT images were acquired at 1 hr post the second Na$^{99m}$TcO$_4$ injection, i.e. at 41 hr post bTSH injection. Two out of three WT mice had comparable NIS-mediated $^{99m}$TcO$_4$ influx activity at the two different time points, whereas all three Tg-PTC1 mice had decreased NIS-mediated $^{99m}$TcO$_4$ influx activity at later time point. The difference in duration of NIS-mediated thyroidal $^{99m}$TcO$_4$ influx activity induced by single dose of bTSH between Tg-PTC1 mice and WT mice was statistically significant (p-value= 0.014).
Figure 2.3

A. 

Thyroidal % ID (Na\textsuperscript{123}I at t6)

0 2 4 6 8 10 12 14 16 18 20

WT Tg-PTC1

T3-60μg bTSH T3-90μg bTSH

Continued
Continued Fig 2.3

B.

![Graph showing Thyroidal % ID (Na$^{99m}$TcO$_4$) at t1 over time post bTSH injection with comparison between WT and Tg-PTC1](#)
Figure 2.4 The area under time-activity curve (AUC) of thyroid in wild type mice is markedly increased by 30 mg/kg 17-AAG. WT mice (15-18 months) were weighed and injected with 30 mg/kg 17-AAG in 50 μl DMSO. Na$^{123}$I (100-150 μCi) was injected into mice at 3 hr post 17-AAG injection. SPECT images were acquired at t1(4), t6(9) and t24(27) post Na$^{123}$I injection (time post 17-AAG injection). At t1, the extent of thyroidal radioiodine accumulation was quite comparable between DMSO versus 17-AAG treated mice suggesting that 17-AAG had minimal effect on thyroidal iodide influx. However, at t24, the extent of thyroidal radioiodine accumulation was markedly increased (p-value= 0.15) in 17-AAG treated mice versus that in DMSO treated mice, suggesting that 17-AAG increases iodide retention ability. Consequently, the exposure of thyroid with radioiodine over time was markedly increased by 17-AAG as defined by the AUC.
Figure 2.4

17-AAG and Na$^{123}$I

SPECT t1(4)  SPECT t6(9)  SPECT t24(27)

0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75

Thyroidal % ID

hr post Na$^{123}$I injection

DMSO

30mg/kg 17-AAG

Trial 1

Trial 2

Trial 3

Age: 15mon  15-16mon  18mon

[Graph showing data points for different trials and conditions over time]
**Figure 2.5.** The area under time-activity curve (AUC) of thyroid tumor in Tg-PTC1 mice is markedly increased by 30 mg/kg 17-AAG. Tg-PTC1 mice (15-21 months) were weighed and injected with 30 mg/kg 17-AAG in 50 µl DMSO. Na$^{123}$I (100-150 µCi) was injected into mice at 3 hr post 17-AAG injection. SPECT images were acquired at t1(4), t6(9) and t24(27) post Na$^{123}$I injection (time post 17-AAG injection). At t24, the difference in the extent of increased thyroidal radioiodine accumulation was evidently enhanced in 17-AAG treated mice compared to that in DMSO treated mice at least in part due to the decreased thyroidal %ID by DMSO at t24 in Tg-PTC1 mice. As a result, AUC was evidently increased by 17-AAG in Tg-PTC1 mice.
Figure 2.5

![Graph showing thyroidal % ID over time post Na$^{123}$I injection. The x-axis represents time in hours post Na$^{123}$I injection, ranging from 0 to 25 hours. The y-axis represents thyroidal % ID, ranging from 0 to 35. The graph shows two trials: Trial 1 (DMSO) and Trial 2 (30mg/kg 17-AAG). The age groups are 15-21 months (5-7) and 18-20 months (8-10).](image-url)
Figure 2.6 DMSO did not seem to modulate thyroidal radioiodine accumulation in WT mice. WT mice were injected with 50 μl DMSO 3 hr prior to ~150 μci Na$^{123}$I injection. SPECT images were acquired at 1, 6 and 24 hr post Na$^{123}$I injection. Thyroidal %ID of each mouse was quantified and plotted against time as shown in the figure.
**Figure 2.7** DMSO significantly decreased thyroidal %ID at t24 by decreasing thyroidal radiiodine retention ability in Tg-PTC1 mice. Tg-PTC1 mice were injected with 50 μl DMSO 3 hr prior to ~150 μci Na\(^{123}\)I injection. SPECT images were acquired at 1, 6 and 24 hr post Na\(^{123}\)I injection. Thyroidal %ID of each mouse was quantified and plotted against time as shown in the top panel of the figure. Thyroidal radiiodine retention ability was calculated and shown as t6/t1 or t24/t1 thyroidal %ID.
**Figure 2.8** 17-AAG did not increase thyroidal $^{99m}$TcO$_4$ accumulation at either 4 hr or 27 hr post 17-AAG treatment in WT mice. 4 mice were injected with either DMSO (mouse 1 and 2) or 30 mg/kg 17-AAG (mouse 3 and 4) 3 hr prior to $\sim$150 $\mu$ci Na$^{99m}$TcO$_4$ and 100 $\mu$ci Na$^{125}$I injection. Subsequently, mice were subjected to SPECT imaging at 1 hr post radionuclide injection (Tc99m t1 4hr) for $^{99m}$Tc thyroidal accumulation. 26 hr post 17-AAG injection, mice were injected with $\sim$150 $\mu$ci Na$^{99m}$TcO$_4$ followed by SPECT imaging at 1 hr post radionuclide injection (Tc99m t1 27 hr) for $^{99m}$Tc thyroidal accumulation. At 31 hr post 17-AAG injection, SPECT imaging was acquired for $^{125}$I thyroidal accumulation followed by ex vivo gamma counting of thyroidal accumulation at 35 hr post 17-AAG injection. As shown in the figure, while mouse number 3 did not respond to 17-AAG, 17-AAG increased thyroidal %ID in mouse number 4 as shown by $^{125}$I thyroidal %ID (bottom panel), yet $^{99m}$Tc thyroidal accumulation is decreased by 17-AAG (top panel) confirming effect of 17-AAG on increasing thyroidal radioiodine accumulation is not due to increased iodide influx.
Figure 2.8
**Figure 2.9** 16 hr 17-AAG did not further increase thyroidal % ID, 3 hr 17-AAG has the highest thyroidal % ID in WT mice. WT mice were injected with DMSO or 30 mg/kg 17-AAG 3 hr or 16 hr prior to ~150 μci Na$^{123}$I injection. SPECT images were acquired at 1, 6 and 24 hr post Na$^{123}$I injection. Thyroidal %ID of each mouse was quantified and plotted against time as shown in the figure.
**Figure 2.10** 17-AAG did not increase salivary iodide uptake in WT mice. Statistical analysis by ANOVA showed no significant increase of salivary gland iodine accumulation with 17-AAG treatment comparing to DMSO control for WT (p-value=0.69). WT mice were injected with DMSO or 30 mg/kg 17-AAG 3 hr prior to ~150 µci Na$^{123}$I injection. SPECT images were acquired at 1 hr post Na$^{123}$I injection. Salivary %ID of each mouse was quantified and plotted.
Figure 2.11 17-AAG did not increase salivary iodide uptake in Tg-PTC1 mice. Statistical analysis by ANOVA showed no significant increase of salivary gland iodine accumulation with 17-AAG treatment comparing to DMSO control for Tg-PTC1 mice (p-value=0.69). Tg-PTC1 mice were injected with DMSO or 30 mg/kg 17-AAG 3 hr prior to ~150 μci Na$^{123}$I injection. SPECT images were acquired at 1 hr post Na$^{123}$I injection. Salivary %ID of each mouse was quantified and plotted.
Table 2.1 Temporal dynamics of thyroidal and salivary radioiodine accumulation in non-treated wild type mice

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age (mon)</th>
<th>Thyroidal %ID</th>
<th>Thyroidal iodide retention ability</th>
<th>Salivary %ID</th>
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<td></td>
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<td>Iodide retention ability</td>
<td>AUC*</td>
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<td></td>
<td>t1</td>
<td>t6</td>
<td>t24</td>
<td>6hr/1hr</td>
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<tr>
<td>WT</td>
<td>DMSO</td>
<td>2.6 ± 1.3</td>
<td>9.3 ± 4.1</td>
<td>11.2 ± 7.9</td>
<td>3.6 ± 0.2</td>
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<td>17-AAG</td>
<td>2.7 ± 1.8</td>
<td>13.0 ± 6.6</td>
<td>43.4 ± 30.1</td>
<td>6.3 ± 3.4</td>
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<td>Tg-PTC1</td>
<td>DMSO</td>
<td>7.4 ± 5.6</td>
<td>8.8 ± 5.1</td>
<td>3.0 ± 0.7</td>
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<td>16.7 ± 2.9</td>
<td>22.6 ± 4.7</td>
<td>1.9 ± 0.5</td>
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</table>

*AUC: area under time-activity curve, it is calculated by \( \int_{t=1hr}^{t=24hr} f(t) dt \); f(t): thyroidal %ID; d(t): time(hour)

Table 2.2 Effect of 17-AAG on thyroidal radioiodine accumulation in wild type and Tg-TPC1 mice
Chapter 3

Modulation of NIS expression and NIS-mediated RAIU activity by PI3K, Akt, and mTORC1 in thyroid cells

Abstract

The Na⁺/I⁻ symporter-mediated active iodide uptake and prolonged iodide retention by iodide organification is the basis for the use of radioiodine I\(^{131}\) for diagnosis and targeted ablation for thyroid cancers. However, NIS-mediated radioiodine accumulation is often reduced in thyroid cancers due to decreased/absent NIS expression/function. As PI3K and MEK signaling is over-activated in thyroid tumors, we investigated the effects of PI3K, Akt, or mTORC1 inhibition as well as the interplay between PI3K and MEK signaling on NIS modulation. We found that inhibition of PI3K, Akt and mTORC1 differentially modulate NIS expression and function at multiple levels in thyroid cells. Inhibition of PI3K by LY294002 increased NIS-mediated RAIU activity mainly through upregulation of NIS expression. In comparison, inhibition of mTORC1 by Rapamycin did not increase NIS-mediated RAIU activity despite increasing NIS protein levels. This is most likely due to the increased pERK and pAkt levels by Rapamycin as co-treatment with PD98059 or Akti-1/2 together with Rapamycin did increase NIS-mediated RAIU
activity. Akti-1/2 treatment alone markedly increased NIS-mediated RAIU activity by decreasing iodide efflux rate and increasing iodide transport rate and iodide affinity of NIS. Combination treatments indicated that NIS modulation by Akt was dominant over PI3K, mTORC1 and MEK. Finally, while both LY294002 and Akti-1/2 increased NIS-mediated RAIU activity in PC Cl3 and FRTL-5 rat thyroid cells, they both decreased RAIU activity in HEK293 or COS7 cells expressing exogenous NIS. Taken together, LY294002 and Akti-1/2 may serve as potential pharmacological reagents to selectively increase thyroidal radioiodine accumulation and decrease radioiodine accumulation in non-thyroid tissues.
Introduction

The \( \text{Na}^+/\text{I}^- \) symporter (NIS) is a thirteen transmembrane glycoprotein that is expressed on the basolateral membrane of thyroid follicular cells. NIS functional active iodide uptake from the circulating bloodstream into the thyroid follicular cells is the first step for the synthesis of iodine-containing thyroid hormone, triiodothyronine (T3) and thyroxine (T4). NIS co-transport one iodide (I\(^-\)) ion against the iodide electrochemical gradient along with two sodium (Na\(^+\)) ions down the sodium electrochemical gradient, maintained by the \( \text{Na}^+/\text{K}^+ \)-ATPase, into the thyroid follicular cells. The iodide concentrating activity of NIS serves as the molecular basis for the effective use of radioiodine I\(^{131}\) for diagnosis and targeted ablation of residual, recurrent and metastatic thyroid cancer in post-operation thyroid tumor patients. However, all thyroid tumor patients showed reduced iodide uptake activity (see review in Shen et al. 2001). Moreover, about 20% of patients with differentiated thyroid tumor and all patients with poorly differentiated thyroid tumor fail to respond to radioiodine imaging and therapy due to decreased or absent NIS expression/function (Caillou et al. 1998; Castro et al. 2001; Oler Et Cerutti 2009; Patel et al. 2002; Ward et al. 2003). Thus, it is of clinical importance to understand the underlying mechanisms that regulate NIS expression and functional activity to provide strategies to increase or restore NIS expression/function in thyroid tumor patients for higher efficacy of radioiodine therapy.

Much effort has been made to uncover the mechanisms underlying regulation of NIS expression/function such that pharmacological reagents targeting signaling nodes that increases NIS-mediated radioiodine accumulation in thyroid tissues can be identified. NIS mRNA and protein levels were decreased in thyroid cells by activation of MEK-
ERK by RasV1C35 or expression of constitutively active MEK1. Accordingly, NIS mRNA and protein levels were increased in thyroid cells by inhibition of MEK-ERK using PD98059 or U0126. However, the increase in NIS mRNA and protein levels was not always accompanied with an increase, but rather a transient decrease in NIS-mediated RAIU activity (Cass, 2000; Knauf, 2003; Vadysirisack, 2007). Similarly, activation of PI3K by overexpression of RasV1C40 or Gβγ, or treatment of IGF-1 decreased NIS mRNA and protein levels in thyroid cells (Cass and Meinkoth 2000; Garcia and Santisteban 2002; Zaballos, Garcia et al. 2008). However, limited studies showed the interaction/crosstalk between PI3K/Akt and MEK signaling pathways on modulation of NIS protein expression and function in thyroid cells. In our current study, we focused on investigating the role of PI3K/Akt signaling pathway and its interaction with MEK signaling on regulation of NIS expression and functional activity in cultured thyroid cells. We demonstrated that while effect of mTORC1 inhibition by Rapamycin on decreasing NIS-mediated RAIU activity is contributed by activation of ERK and/or Akt signaling nodes, effect of Akt inhibition by Akti-1/2 on NIS-mediated RAIU activity prevails over PI3K, mTORC1, and MEK signaling nodes. Moreover, effect of LY294002 and Akti-1/2 on increasing NIS-mediated RAIU activity in thyroid cells and decreasing RAIU activity in non-thyroid cells encourages investigation of LY294002 or Akti-1/2 derivative pharmacological agents to increase the efficacy of radioiodine therapy and decrease the untoward effects of radioiodine treatment in non-thyroid cells in thyroid cancer patients.
Material and methods

Cell Culture and Reagents

PC Cl3 rat thyroid cells were maintained in Kaighn’s medium (Sigma), 5 % calf serum, 2 mM glutamine, 1 % penicillin-streptomycin, 10 mM NaHCO₃, and 6H hormone (1 mU/mL bovine TSH, 10 μg/mL bovine insulin, 10 nM hydrocortisone, 5 μg/mL transferrin, 10 ng/mL somastatin and 2 ng/mL L-glycyl-histidyl-lysine). PC Cl3 cells with doxycycline-inducible MEK1 (E217/E221) expression (kindly provided by Drs Jeffrey A Knauf and James A Fagin) were induced with 2 μg/ml doxycycline for 72 hr. HEK293 cells were maintained in DMEM with 10 % fetal bovine serum and 1 % penicillin-streptomycin. LY294002 was purchased from Caymen, Akti-1/2 and Rapamycin were purchased from Calbiochem. shRNA targeting Akt1 and Akt2 with its vector control were a generous gift from Dr. Mingzhao Xing.

RNA extraction and Real Time RT-PCR

PC CI3 cells were seeded in 100-mm dishes. After treatment with DMSO, LY294002, Rapamycin, or Akti-1/2 for 24 hr, cells were washed with PBS twice and total RNA was extracted with a mixture of Trizol reagent (Invitrogen) and chloroform (Sigma). RNA was precipitated with RNase/DNase-free isopropanol and pelleted by centrifugate at 12,000x g for 20 min at 4°C. The RNA pellet was washed with 70 % RNase/DNase-free ethanol, centrifuged again at 7,500x g at 4°C, and resuspended in 20 μl of DEPC-treated water. Contaminating DNA was removed by DNase I (Invitrogen) digestion. One microgram of extracted RNA was used for the First Strand Synthesis reverse transcription reaction (Invitrogen) performed according to the manufacturer's instructions. Quantitative
RT-PCR was performed in a 20 μl reaction mixture volume, containing cDNA template, Power SYBR Green PCR MasterMix (Applied Biosystems), and primers. Reaction mixtures were incubated at 50°C for 2 min followed by an initial activation at 95°C for 15 min, and then subjected to 40 PCR cycles of denaturation (95°C for 15 sec), annealing (56°C for 30 sec), and extension (60°C for 1min) using the ABI 7900HT instrument (Applied Biosystems) performed by The Nucleic Acids Shared Resource for The Ohio State University Comprehensive Cancer Center. Relative amounts of rNIS cDNA or rGAPDH cDNA of various samples were measured against standard curves created by serial dilutions (10^{10} to 10^{4} copies/ml) of plasmids containing rNIS or rGAPDH, respectively. The data was presented as a fold change in NIS mRNA over control and experiments were performed in duplicates.

**Radioiodine Uptake (RAIU) Assay**

Cells were pretreated with inhibitors for 24 hr before the assay. At 24 hr, cells were incubated with 2 μci NaI^{125} in 5 μM non-radioactive NaI for 30 min at 37°C with 5 % CO₂. Cells were then washed with cold Hank’s Balanced Salt Solution (HBSS) two times and then lysed in cold 95 % ethanol for 20 min at room temperature. Cell lysate was collected and counted for radioactivity by the gamma-counter (Packard Instruments).

**Western Blot Analysis and Antibodies**

Cells were pretreated with inhibitors for 24 hr prior to the analysis. At 24 hr, cells were lysed and homogenized using 18 ½ gauge needles in lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 1 mM PMSF, 10 μg/mL
aprotinin, and 10 µg/mL leupeptin. Protein concentrations were determined by Bradford assay (BioRad) and equal amounts of protein were subjected to the 10 % SDS-PAGE gel, then transferred to the nitrocellulose membrane. The membrane was blocked in 5 % dry milk in TBST buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05 % Tween 20) and incubated with various primary antibodies for 1 hr at room temperature, followed by incubation with HRP-conjugated secondary antibodies for 1 hr at room temperature. The signal was later detected using ECL detection reagent (Amersham Pharmacia). Rabbit anti-rNIS antibody PA716 was generously provided by Dr. Bernard Rousset. Rabbit anti-Na\(^+\)/K\(^+\)-ATPase was generously provided by Dr. Beth Lee. Rabbit anti-pAkt and anti-Akt were purchased from Cell Signaling. Mouse anti-beta-actin was purchased from Abcam.

**Iodide-Dependent Kinetic Analysis**

Cells were treated with 10 µM Akti-1/2 for 24 hr prior to the iodide-dependent kinetic analysis. At 24 hr, cells were incubated with various concentration of NaI (8 concentrations ranging from 1-600 µM) containing Na\(^{125}\)I of a specific activity of 80 mCi/mmol for 2 minutes. The amount of accumulated radioactive iodide was measured as described for RAIU assay. The \(V_{\text{max}}\) and \(K_m\) values are derived from the fitted Michaelis-Menten equation according to the Eadie-Hofstee plot. Experiments were performed in triplicates.

**Iodide Efflux Assay**

\(^{125}\)I efflux assay were performed as described in Marsee *et al*. 2004. Cells were pre-treated with pharmacological inhibitors for 24 hr, and then incubated in media containing
Na$^{125}$I as described for RAIU assay. Cells were washed twice and incubated with cold 1 ml HBSS for 2 minutes. HBSS was then collected and replaced with new medium. This was repeated every 2 minutes for a total of 10 minutes. Cells were then lysed with 95 % ethanol for 20 minutes. The total uptake was calculated as the sum of total efflux washes and the lysates. Experiments were performed in triplicates.

**Cell Surface Biotinylation**

Cell surface biotinylation were performed as described in Vadysirisack *et al.* 2007. Cells were pre-treated with pharmacological inhibitors for 24 hr prior to the assay. Cells were then washed with cold PBS containing 1 mM MgCl$_2$ and 0.1 mM CaCl$_2$ (PBS-Ca/Mg). Cells were incubated with 1 mg/ml sulfo-NHS-LC-biotin (Pierce) in PBS-Ca/Mg for 1 hr at 4°C. The reaction was then quenched using 100 mM glycine in PBS-Ca/Mg for 20 minutes. Cells were then lysed in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) for 30 minutes at 4°C and homogenized using 18½ gauge needles. Lysates were centrifuged at 14,000x g for 20 minutes at 4°C and supernatant containing the whole cell lysate were collected. Bradford assay (Bio-Rad) was used to determine the protein concentration. To separate the biotinylated cell surface proteins from non-biotinylated proteins, equal amounts of whole cell extract were incubated with avidin-coated agarose beads (Pierce) overnight at 4°C. The beads were then washed with lysis buffer and eluted with 2X Laemmli sample buffer at 95°C for 5 minutes.

**Flow cytometric analysis for cell surface NIS**
HEK293 cells were seeded in 100-mm dishes for 24 hr, and transfection was performed using FuGene 6 with 5 µg of various NIS constructs. Cells were harvested by 0.05 % trypsin at 48 hr after transfection. To detect cell surface NIS protein levels, flow cytometric analysis was performed using non-permeabilized cells with 1:50 diluted mouse anti-FLAG monoclonal antibody M2 (Sigma-Aldrich Corp., St. Louis, MO) that recognizes extracellular FLAG peptide, followed by 1:100 diluted fluorescein isothiocyanate-conjugated goat antimouse IgG (Sigma-Aldrich Corp.). Cells were then washed and resuspended in fixative buffer (1% paraformaldehyde in PBS) for analysis by a FACS Calibur flow cytofluorometer (BD Biosciences, Eerenbodegem, Belgium). Secondary antibody alone was used as a negative control for nonspecific fluorescence. For quantification, a positive events region was gated at a fluorescence density that had less than 1% of cells with nonspecific fluorescence.
Results

Both LY294002 and Rapamycin increased NIS mRNA and protein levels in PC Cl3 cells

It has been reported that several signaling pathways that lead to PI3K activation decreased NIS protein levels in thyroid cells (Cass and Meinkoth, 2000; Zaballos, 2008). Consequently, PI3K inhibition was found to increase NIS promoter activity, mRNA and protein levels in FRTL-5 rat thyroid cells (Garcia and Santisteban, 2002; Kogai, 2008). In agreement with the reported data, we showed that PI3K inhibition by 10 µM LY294002 for 24 hr increased NIS mRNA steady state level (left panel: 1.7-fold; N=2) and NIS protein levels (right panel: 2.1 ± 1.1-fold; N=5) compared to DMSO vehicle treated PC Cl3 rat thyroid cells (Fig 3.1A). Furthermore, we showed that mTORC1 inhibition by 20 nM Rapamycin for 24 hr also increased NIS mRNA steady state level (left panel: 1.8-fold; N=2) and NIS protein level (right panel: 1.9 ± 0.5 fold; N=7) (Fig 3.1B). Taken together, PI3K modulation of NIS expression levels may be mediated by mTORC1.

Differential effects of LY294002 and Rapamycin on NIS-mediated RAIU activity may be contributed by their differential perturbation of ERK and/or Akt signaling nodes

Since increased NIS protein levels does not always confer to increased NIS-mediated RAIU activity (Vadysirisack, 2007), we further examined NIS expression and NIS-mediated RAIU activity in LY294002 or Rapamycin treated PC Cl3 cells. While NIS protein levels were increased by both LY294002 and Rapamycin, NIS-mediated RAIU
activity was only increased by LY2940002 (Fig 3.2A: 1.6 ± 0.5- fold; N=8) but not by Rapamycin (Fig 2B: 0.7 ± 0.1- fold; N=9). This finding indicates that NIS activity was modulated at post-translational level in Rapamycin treated PC Cl3 cell. Akt and Erk signaling nodes appeared to be differentially modulated by LY294002 versus Rapamycin. LY294002 decreased, yet Rapamycin increased, pAkt levels and pERK levels (left panel of Fig 3.2A and 3.2B). Interestingly, NIS-mediated RAIU activity was slightly increased in PC Cl3 cells treated with both PD98059 and Rapamycin (1.01- fold) compared to PC Cl3 cells treated with Rapamycin (0.77- fold) alone. Similarly, NIS-mediated RAIU activity was markedly increased in PC Cl3 cells treated with both Akti-1/2 and Rapamycin (Akti-1/2+ Rapamycin versus Rapamycin: 2.96- fold versus 0.96- fold). It is also of interest to note that the extent of increase in NIS-mediated RAIU activity by LY294002 seems to correlate with the extent of decreased pERK levels (data not shown). Taken together, differential effects of LY294002 and Rapamycin on NIS-mediated RAIU activity may be contributed by their differential perturbation of ERK and/or Akt signaling nodes. The increase in pAkt and pERK levels by Rapamycin is most likely due to its inhibition of S6K1 which exerts negative regulation on PI3K-mediated Akt or ERK activation (see review by Grant, 2008).

**Akti-1/2 significantly increased NIS-mediated RAIU activity without increased NIS expression**

As the effect of pERK on NIS expression and function has been extensively studied (Knauf,2004; Vadysirisack, 2007), we focused on the effect of Akt on NIS expression and function in PC Cl3 cells. NIS mRNA steady state levels in PC Cl3 cells
was not affected by 10 µM Akti-1/2 for 24 hr (Fig 3.3A, N=2). However, NIS protein levels was decreased by Akti-1/2 to variable extent (0.13- to 0.98- fold) most likely due to variable potency of Akti-1/2 applied in various experiments (Fig 3.3B, mean= 0.5 ± 0.3- fold; N=10). Thus, unlike LY294002 and Rapamycin, Akt1/2 appears to modulate NIS at the protein level. Indeed, the extent of NIS protein levels decreased by Akti-1/2 was alleviated by co-treatment with inhibitors for lysosome and proteasome (data not shown).

However, the decrease in NIS protein levels by Akti-1/2 was not accompanied with a decrease, but rather a significant increase, in RAIU activity (Fig 3.3C, 4.1 ± 1.5-fold; N=12). Similar to Akti-1/2, treatment with 24 hr AktV, an Akt inhibitor that shares little structural similarity with Akti-1/2, also decreased NIS protein levels to 0.23- fold yet significantly increased NIS-mediated RAIU activity by 2.63- fold in PC Cl3 cells (data not shown). In addition, mixed population of PC Cl3 stable transfectants expressing shRNA knockdown Akt1 and Akt 2 (Hou, 2010) decreased NIS protein levels and increased NIS-mediated RAIU activity compared to stable transfectants expressing vector control (data not shown). Taken together, Akti-1/2 appears to modulate NIS at the post-translational level and/or non-NIS factors that contribute to increased NIS-mediated RAIU activity.

The perturbation of pERK levels by Akti-1/2 was inconsistent, sometimes increased and sometimes decreased, among different experimental trials. However, the extent of increase in RAIU activity by Akti-1/2 did not appear to correlate with pERK levels, suggesting that NIS modulation by Akt inhibition is dominant over ERK activation status in PC Cl3 cells. The interaction of ERK and Akt signaling nodes on NIS
modulation were further examined in Akti-1/2 treated doxycycline inducible MEK1 (E217/E221) PC Cl3 cells. As expected, induction of constitutively active MEK1 decreased NIS-mediated RAIU activity in the absence of Akti-1/2 (lane 1 versus 3 in Fig 3.3D). However, the extent of NIS-mediated RAIU activity increased by Akti-1/2 was comparable regardless of MEK1 induction (lane 3 versus 4 compared to lane 1 verses lane 2).

**Akti-1/2 decreased iodide efflux rate and increased NIS-mediated iodide transport rate**

Since cell surface NIS levels but not total NIS protein levels confer NIS-mediated RAIU activity, we examined whether Akti-1/2 alters the ratio of cells surface NIS levels versus total NIS protein levels. As shown in Fig 3.4A, cell surface biotinylation followed by avidin-pull down and subsequent NIS probing by western blot analysis demonstrated that cell surface NIS level was also decreased by Akti-1/2. Consequently, the ratio of cells surface NIS levels versus total NIS protein levels was comparable between DMSO (0.25) and Akti-1/2 treated cells (0.26). Immunoflourescent labeling of NIS showed that both cell surface and intracellular NIS were decreased in Akti-1/2 treated cells compared to DMSO-treated cells (data not shown). This indicates that the increase in RAIU activity by Akti-1/2 is not due to an increase in NIS cell surface levels.

As steady-state iodide accumulation in thyroid cells is the net outcome of iodide uptake and iodide efflux, we next examined the effect of Akti-1/2 on iodide efflux rate in PC Cl3 cells. The effect of 17-AAG on iodide efflux rate (Marsee et al, 2004) was performed in parallel for comparison. As shown in Fig 3.4B, iodide efflux rate was
significantly decreased by Akti-1/2 (p-value < 0.0001) and 17-AAG (p-value < 0.0001) as indicated by a decrease in the corresponding slope of curve generated by plotting log iodide remaining over time. The extent of decrease in iodide efflux rate by Akti-1/2 was less than 17-AAG (p-value= 0.0015), yet the extent of increase in RAIU activity by Akti-1/2 was greater than 17AAG (Akti-1/2 versus 17-AAG: 6- fold versus 3- fold). Thus, the decrease in iodide efflux rate may not fully account for the increase in RAIU activity in Akti-1/2 treated cells.

We next examined whether Akti-1/2 affects iodide transport rate and/or iodide affinity of NIS in PC Cl3 cells. As shown in Table 3.1, Akti-1/2 increased the maximal velocity of iodide uptake (V_{max}) by 1.3- fold, and decreased K_{m-I^-} by 0.71- fold. Since cell surface NIS level is decreased in Akti-1/2 treated cells, the increase in V_{max} by Akti-1/2 is due to increased velocity of iodide transport. In addition, Akti-1/2 slightly increased iodide affinity of NIS. Taken together, Akti-1/2 increases NIS-mediated RAIU activity by at least in part by decreasing iodide efflux rate, and increasing iodide transport rate as well as iodide affinity of NIS.

**NIS modulation by Akt prevails over PI3K, mTORC1, and MEK**

It appears that PI3K, Akt, mTORC1, and MEK differentially modulate NIS expression and function at multiple levels in PC Cl3 cells. NIS protein levels were increased by PI3K, mTORC1, or MEK inhibitors mainly by increasing NIS expression. Conversely, NIS protein level was decreased by Akt inhibitor at least in part by facilitating proteosomal and lysosomal-mediated protein degradation. Furthermore, NIS-mediated RAIU activity could be increased by Akt inhibitor by increasing NIS activity.
and decreasing iodide efflux rate yet NIS-mediated RAIU activity could be decreased by mTORC1 and MEK inhibitors. However, the interplay among these signaling nodes in NIS modulation has not been studied.

As shown in Fig 3.5A, RAIU activity were increased by both Akti-1/2 and LY294002 treatment alone, however, co-treatment with Akti-1/2 and LY294002 did not further increase RAIU activity compared to Akti-1/2 treatment alone. Furthermore, the extent of increase in NIS protein level by LY294002 was diminished by co-treatment with Akti-1/2. Surprisingly, while RAIU activity was modestly decreased by Rapamycin, co-treatment with Akti-1/2 further increased RAIU activity compared to Akti-1/2 treatment alone. However, similar to LY294002, the extent of increase in NIS protein level by Rapamycin was also diminished by co-treatment with Akti-1/2 (Fig 3.5B). In comparison, co-treatment of PD98059 with Akti-1/2 had decreased RAIU activity compared to Akti-1/2 treatment alone. Once more, the extent of increase in NIS protein level by PD98059 was diminished by co-treatment with Akti-1/2 (Fig 3.5C). Taken together, NIS modulation by Akt at the protein level as well as RAIU activity appears to prevail over PI3K, mTORC1, and MEK.

**Akti-1/2 plays a less prominent role in increasing RAIU activity in** **PC Cl3 cells acutely stimulated by TSH**

The dynamics of signaling network is known to be different in thyroid cells under chronic versus acute TSH stimulation (Ciullo, Diez-Roux et al. 2001; Iacovelli, Capobianco et al. 2001). IGF-1 has been reported to inhibit TSH-induced NIS expression through PI3K activation in FRTL-5 cells deprived of TSH (Garcia, 2002). As shown in
Fig 3.6A, TSH-stimulated RAIU activity was decreased by co-treatment with IGF-1, increased by LY294002, but had little effect by co-treatment with Akti-1/2 or Rapamycin. The discrepancy of Akti-1/2 effect on RAIU activity between PC Cl3 cells chronically and acutely stimulated by TSH indicates that Akti-1/2 effect on increasing RAIU activity is less prominent in cells acutely stimulated by TSH. However, the effect of Akti-1/2 on decreasing NIS protein level remains prominent in cells acutely stimulated by TSH. In the presence of LY294002, Akti-1/2, or Rapamycin, IGF-1 failed to suppress TSH-stimulated RAIU activity despite that NIS protein levels remained decreased in cells co-treated with TSH, IGF-1 and Akti-1/2 (Fig 3.6B). Taken together, these data further support the multiple roles of Akti-1/2 on increasing RAIU activity through NIS and non-NIS factors (i.e. iodide efflux rate). Finally, co-treatment with TSH and LY294002 had increased NIS protein levels and RAIU activity compared to TSH alone suggesting that TSH may also elicit NIS downregulation by activating PI3K signaling node.

**Both LY294002 and Akti-1/2 decreased NIS-mediated RAIU activity despite no effect on cell surface NIS protein level in HEK293 expressing exogenous NIS**

The effect of LY294002 (Kogai, 2008) and Akti-1/2 (data not shown) on NIS protein levels and RAIU activity is not restricted to PC Cl3 cells, but also seen in FRTL-5 rat thyroid cells. In comparison, LY294002 and Akti-1/2 had little effect on cell surface or total NIS protein levels in both HEK293 human kidney cells (Fig 3.7B: total NIS protein levels by western blot analysis; Fig 3.7C: cell surface NIS protein levels by flow cytometry) and COS7 monkey kidney cells (data not shown) stably expressing exogenous NIS. Furthermore, LY294002 and Akti-1/2 did not increase but markedly decreased NIS-
mediated RAIU activity in both HEK293 (Fig 3.7A) and COS7 cells (data not shown) stably expressing exogenous rNIS. Since LY294002 increases RAIU activity mainly by increasing NIS expression in thyroid cells, it is not surprising that LY294002 had no effect on the expression levels of exogenous NIS driven by CMV promoter. However, LY294002 appears to decrease RAIU activity by modulating NIS activity and/or non-NIS factors in HEK293 cells but not in thyroid cells. Furthermore, Akti-1/2 facilitated lysosomal and proteosomal mediated NIS protein degradation as well as increase in RAIU activity seen in thyroid cells were not found in HEK293 cells expressing exogenous NIS. Thus, NIS-mediated RAIU activity may be modulated by LY294002 and Akti-1/2 in a cell context dependent manner. Consequently, PI3K and Akt inhibitors may serve as potential pharmacological reagents to selectively increase thyroidal radioiodine accumulation.
DISCUSSION

In this study, we showed that inhibition of PI3K, Akt and mTORC1 differentially modulate NIS expression and function at multiple levels in thyroid cells. Inhibition of PI3K by LY294002 mainly increased NIS-mediated RAIU activity through upregulation of NIS expression. In comparison, inhibition of mTORC1 by Rapamycin did not increase NIS-mediated RAIU activity despite increasing NIS protein levels. This is most likely due to activation of pERK and pAkt levels by Rapamycin as co-treatment with PD98059 or Akti-1/2 together with Rapamycin did increase NIS-mediated RAIU activity. Akti-1/2 treatment alone markedly increased NIS-mediated RAIU activity by decreasing iodide efflux rate and increasing iodide transport rate and iodide affinity of NIS. Furthermore, NIS modulation by Akti-1/2 appeared to be dominant over LY294002, Rapamycin, and PD98059. While both Akti-1/2 and LY294002 increased NIS-mediated RAIU activity in thyroid cells through differential mechanism, both of them decreased RAIU activity in HEK293 or COS7 cells expressing exogenous NIS. Taken together, LY294002 and Akti-1/2 encourages the potential use of selectively increasing thyroidal radioiodine accumulation and decreasing radioiodine accumulation in non-thyroid cells to improve the efficacy of radioiodine therapy and decrease unwanted side-effects.

NIS modulation at transcriptional level

NIS expression and function can be selectively increased by TSH in thyroid cultured cells and tissues (Levy, 1997; Kogai, 1997; Trapasso, 1999; Saito, 1997; Aajjan, 1998). NIS induction by TSH could be mimicked by treatment of either adenylate cyclase
activator forskolin or cAMP analog, yet was inhibited by treatment of transcription inhibitor D-ribofranosylbenzimidazole (Kogai, 1997; Saito, 1997) suggesting that TSH primarily modulates NIS at the transcriptional level through cAMP signaling. NIS induction by TSH-cAMP signaling at least in part is mediated by NIS upstream enhancer (NUE) that contains Pax8 and cAMP responsive element-like binding (CREB) sites in the promoter region of rat NIS (Ohno, 1999) and human NIS (Taki, 2002).

Malignant thyroid tumors typically show up as “cold” nodules, i.e. decreased radioiodine concentrating activity, in radioiodine scintigraphic imaging. Indeed, NIS expression and/or function are found to be decreased in thyroid cancers (Caillou et al. 1998; Castro et al. 2001; Oler Et Cerutti 2009; Patel et al. 2002; Ward et al. 2003). Interestingly, MEK and PI3K signaling pathways are found to be overly activated in malignant thyroid tumors (Leboeuf, 2008; Xing M, 2010). Accordingly, over-expression of TSHR coupled G protein G\(_{\beta\gamma}\) subunit or RasV12C40 that preferentially activates PI3K decreased NIS protein levels in rat thyroid cell lines (Cass and Meinkoth 2000). In addition, insulin-like growth factor 1 (IGF-1) appeared to decrease TSH induced NIS promoter activity, mRNA and protein levels through PI3K activation (Garcia, 2002). Computer analysis showed that NIS promoter region responsible for the IGF-1 inhibitory effect was found to contain several motifs that can be bound by PI3K downstream effector, forkhead transcription factors, FKHR protein (Garcia and Santisteban 2002). The direct effect of PI3K on NIS modulation was best demonstrated by decreased NIS expression in PC Cl3 cells infected with recombinant adenovirus carrying constitutively active PI3K p110 CAAX (supplementary data).
MAPK activation has also been reported to decrease NIS expression. RasV12S35 that preferentially binds to and activates Raf-1, which consequently activates the MEK signaling pathway, has been shown to completely abolish TSH-induced NIS expression in WRT thyroid cells (Cass and Meinkoth 2000). NIS mRNA levels was decreased by expressing constitutively active MEK1 in the doxycycline-inducible PC Cl3- MEK1 E217/E221 cells (Knauf, et al, 2003). It is currently unknown whether the same transcription factors are modulated by TSH/cAMP, PI3K and MEK signaling. In this study, we showed that combination treatment with PD98059 and LY294002 had additive effect on increasing NIS protein levels suggesting that MEK and PI3K signaling may not modulate the same transcription factors (supplementary data).

NIS modulation at protein level

It has been reported that TSH deprivation decreased NIS protein half-life from 5 to 3 days (Riedel, 2001), however, the underlying molecular mechanism is not known. In this study, we showed that Akt activation plays an important role in maintaining NIS protein stability. While treatment with Akti-1/2 for 24 hr had minimal effect on NIS mRNA levels, it decreased NIS protein levels by 0.5 ± 0.3- fold (N=10) in PC Cl3 cells. The pAkt level was abolished by Akti-1/2 treatment by 30 min, yet NIS protein levels was not decreased until later hours suggesting Akti-1/2 mediated NIS degradation may require de novo protein synthesis (supplementary data). Akti-1/2 appears to facilitate lysosome- and proteasome- mediated NIS protein degradation as the extent of NIS protein reduction is alleviated by co-treatment with lysosome or proteosome inhibitor (supplementary data). Interestingly, a recent publication reported increased phosphorylation of several vesicle
transport proteins upon treatment with Akt1/2 inhibitor. Among the identified proteins, vacuolar protein sorting-associated protein 13D (VPS13D) is involved in trafficking membrane proteins between the trans-Golgi network and the prevacuolar compartment, which could retrograde transport of membrane protein for degradation (Andersen, 2010; Zouhar, 2010). Akti-1/2 modulates NIS at protein levels is further supported by our data showing that increased NIS protein levels by LY294002, Rapamycin, or PD98059 were reversed by co-treatment with Akti-1/2. Interestingly, the extent of decrease in NIS protein levels was greater in co-treatment with Akti-1/2 together with LY294002 or Rapamycin compared to PD98059. Thus, PD98059 may have an Akt-independent mechanism to maintain NIS protein levels.

NIS modulation at activity level

NIS-mediated RAIU activity does not always correlate with cell surface NIS protein levels. We have previously showed that MEK inhibition by PD98059 transiently decreased NIS-mediated RAIU activity despite increased NIS protein level in thyroid cells (Vadysirisack, 2007). In current study, we showed that Akti-1/2 decreased NIS protein levels yet increased NIS-mediated RAIU activity in thyroid cells. PD98059 decreased NIS-mediated RAIU activity at 30 min of treatment by decreasing NIS iodide transport rate. In comparison, NIS-mediated RAIU activity was increased by Akti-1/2 at later hours (> 8 hr), suggesting that Akti-1/2 may act indirectly on modulating NIS activity. For example, Akti-1/2 may elicit de novo synthesis of proteins that could increase NIS activity by altering NIS phosphorylation status or NIS complex formation. Our lab have previously reported that phosphorylation status of Ser43 and Ser581
modulates iodide transport rate of NIS and these two phosphorylation sites are not modulated by MEK signaling (Vadysirisack, 2007). It may be interesting to note that Rapamycin increased both pERK and pAkt levels which at least in part accounts for the decreased NIS-mediated RAIU activity despite the increase in NIS protein levels. It would be interesting to further examine and compare NIS phosphorylation status or NIS complex formation upon pERK and pAkt inhibition in thyroid cells to identify candidate factors that modulate NIS functional activity.

Modulation of iodide efflux rate

Our lab has previously reported that Hsp90 inhibitor, 17-allyamino-17-demethoxygeldanamycin (17-AAG) increases NIS-mediated radioiodine accumulation by decreasing iodide efflux in PC Cl3 cells and RET/PTC1-transformed PC Cl3 cells (Marsee et al. 2004). This finding was subsequently confirmed by another study showing that 17-AAG increases iodide retention in cultured human anaplastic thyroid cancer cells stably expressing hNIS (Elisei et al. 2006). In current study, we demonstrated that Akti-1/2 increased NIS-mediated RAIU activity not only due to increased NIS iodide transport rate and NIS iodide affinity, but also due to a decrease in iodide influx rate. While 17-AAG inhibits a huge range of signaling nodes including Akt, the extent of 17-AAG on decreasing iodide efflux rate is greater than that in Akti-1/2 treated PC Cl3 cells, suggesting that signaling factors other than Akt may also contribute to decrease in iodide efflux rate. It would be of great interest to examine what are the targets of 17-AAG and Akti-1/2 and how do they modulate iodide efflux rate.
NIS modulation by the interplay of various signaling nodes

The dynamics of signaling network is known to be different in thyroid cells under chronic versus acute TSH stimulation (Al-Alawai, 1995; Ciullo, 2001; Iacovelli, 2001). We showed that while Akti-1/2 increased NIS-mediated RAIU activity in chronic TSH-stimulated PC Cl3 cells, Akti-1/2 had little effect on acute TSH-stimulated RAIU activity. Indeed, Kogai et al reported treatment with 30 μM Akti-1/2 for 24 hr had modest increase in NIS mRNA levels but did not increase NIS-mediated RAIU activity in acute TSH-stimulated FRTL-5 cells (Kogai, 2008). Furthermore, while our current study showed that treatment with 20 nM Rapamycin for 24 hr decreased NIS-mediated RAIU activity in chronic TSH-stimulated PC Cl3 cells, a very recent paper reported that treatment with 100 nM Rapamycin for 48 hr modestly increased NIS-mediated RAIU activity in acute TSH-stimulated PC Cl3 cells (de Souza, 2010). These data suggests that the interplay of various signaling nodes is dynamic in modulating NIS expression and activity, and thus, NIS modulation by various pharmacological reagents may vary depending on cell context of different genetics perturbation.

NIS modulation in non-thyroid cells

As RET/PTC1 oncoprotein has been shown to activate PI3K/Akt signaling pathway (Besset, 2000) as well as downregulate NIS expression and function in thyroid cells (Trapasso, 1999 and Venkatesaran, 2004), we also examined the effects of LY294002, Rapamycin or Akti-1/2 on NIS-mediated RAIU activity in PC Cl3 cells stably expressing RET/PTC1 oncogene. Consistent with the results in parental PC Cl3 cells, LY294002 increased NIS protein level and NIS-mediated RAIU activity, Akti-1/2 decreased NIS
protein levels yet increased NIS-mediated RAIU activity, Rapamycin increased NIS protein levels but decreased NIS-mediated RAIU activity in RET/PTC1-expressing PC Cl3 cells (supplementary data). However, the extent of increased RAIU activity by Akti-1/2 in RET/PTC1-expressing cells was less than parental PC Cl3 cells. Nevertheless, the similar effect of LY294002, Rapamycin, or Akti-1/2 on NIS protein levels and NIS-mediated RAIU activity seen in parental and RET/PTC1 expressing cells indicates that signaling modulating NIS expression/function in PC Cl3 cells was not dramatically altered by RET/PTC1. In contrast, both LY294002 and Akti-1/2 decreased NIS-mediated RAIU activity in HEK-293 or COS7 cells expressing exogenously rNIS. Indeed, NIS modulation in thyroid cells appears to be very different from non-thyroid cells (Beyer et al, 2010). Accordingly, LY294002 and Akti-1/2 may be used to selectively increase thyroidal radioiodine accumulation but decrease radioiodine accumulation in non-thyroid tissues. If these findings are validated in preclinical animal models carrying thyroid tumor, not only these reagents may be used to increase effectiveness of radioiodine therapy for thyroid cancer but also to minimize unwanted side-effects in non-thyroid tissues.
Figure 3.1 Both LY294002 and Rapamycin increased NIS mRNA and protein levels in PC Cl3 cells. (A) PI3K inhibition by 10 µM LY294002 for 24 hr increased NIS mRNA steady state levels (left panel: real-time RT-PCR analysis; 1.7-fold; N=2), and increased total NIS protein levels (right panel: Western blot analysis; 2.1 ± 1.1-fold; N=5) in PC Cl3 cells. (B) mTORC1 inhibition by 20 nM Rapamycin for 24 hr increased NIS mRNA steady state level (left panel: real-time RT-PCR analysis; 1.8-fold; N=2), and increased total NIS protein levels (right panel: Western blot analysis; 1.9 ± 0.5 fold; N=7) in PC Cl3 cells. Real-Time RT-PCR results are average of two individual experiments. Western blot analysis showed equal loading of proteins normalized with β-actin and the results are representative of seven individual experiments.
Figure 3.1

A.

B.
**Figure 3.2** Differential effects of LY294002 and Rapamycin on NIS-mediated RAIU activity may be contributed by their differential perturbation of ERK and/or Akt signaling nodes. (A) Treatment with 10 μM LY294002 increased NIS protein levels (left panel: Western blot analysis; 2.1 ± 1.1- fold; N=5) and NIS-mediated RAIU activity (right panel: RAIU assay; 1.6 ± 0.5- fold; N=8) in PC Cl3 cells. In this representative western blot analysis, LY294002 decreased both pAkt and pERK levels in PC Cl3 cells. Equal loading of proteins was normalized with beta-actin and the data is representative of five individual experiments for each western blot analysis. NIS-mediated radioiodine uptake was confirmed by performing parallel experiments in the presence of 3 mM perchlorate (ClO$_4^-$), a competitive inhibitor of iodide uptake by NIS. RAIU results were representative of eight individual experiments performed in triplicates. (B) In contrast to LY294002, treatment with Rapamycin increased NIS protein levels (left panel: Western blot analysis; 1.9 ± 0.5 fold; N=7) but decreased NIS-mediated RAIU activity (right panel: RAIU assay; 0.7 ± 0.1- fold; N=9) suggesting NIS activity was modulated at post-translational level in Rapamycin treated PC Cl3 cell. Moreover, Rapamycin increased both pAkt and pERK levels in PC Cl3 cells. Equal loading of proteins was normalized with beta-actin and the data is representative of seven individual experiments for each western blot analysis. NIS-mediated radioiodine uptake is confirmed by performing parallel experiments in the presence of 3 mM perchlorate (ClO$_4^-$), a competitive inhibitor of iodide uptake by NIS. RAIU results were representative of nine individual experiments performed in triplicates. (C) Co-treatment with PD98059 and Rapamycin for 24 hr slightly increased NIS-mediated RAIU activity compared to treatment with Rapamycin alone (PD98059+ Rapamycin versus Rapamycin: 1.01- fold versus 0.77- fold). Similarly,
co-treatment with Akti-1/2 and Rapamycin markedly increased NIS-mediated RAIU activity was compared to treatment with Rapamycin alone (Akti-1/2+ Rapamycin versus Rapamycin: 2.96-fold versus 0.96-fold). This suggests that the effect of Rapamycin on NIS-mediated RAIU activity may be contributed by perturbing ERK and/or Akt signaling nodes. Equal loading of proteins was normalized with beta-actin and the data is representative of five individual experiments for each western blot analysis. NIS-mediated radioiodine uptake was confirmed by performing parallel experiments in the presence of 3 mM perchlorate (ClO$_4^-$), a competitive inhibitor of iodide uptake by NIS. RAIU results were representative of two individual experiments performed in triplicates.
Figure 3.2

A.

**LY294002**

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rNIS, pAkt, pERK, B-actin

B.

**Rapamycin**

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rNIS, pAkt, pERK, pp70S6K1, B-actin

Continued
Fig 3.2 continued

C.
Figure 3.3 Akti-1/2 significantly increased NIS-mediated RAIU activity without increasing NIS expression. (A) Akt1 and Akt2 inhibition by 10 µM Akti-1/2 for 24 hr had minimal effect on NIS mRNA steady state levels as shown by real-time RT-PCR analysis (N=2) (B) Treatment with Akit-1/2 decreased total NIS protein levels as shown by western blot analysis (0.5 ± 0.3- fold; N=10) in PC Cl3 cells. Equal loading of proteins was normalized with beta-actin and the data is representative of ten individual experiments for each western blot analysis. (C) Treatment with Akit-1/2 increased NIS-mediated RAIU activity as shown by RAIU assay (4.1 ± 1.5- fold; N=12) despite NIS mRNA and protein levels was not increased in PC Cl3 cells. NIS-mediated radioiodine uptake was confirmed by performing parallel experiments in the presence of 3 mM perchlorate (ClO$_4^-$), a competitive inhibitor of iodide uptake by NIS. RAIU results are representative of twelve individual experiments performed in triplicates. (D) PC Cl3 cells with doxycycline inducible MEK1 (E217/E227) expression were treated with 2 µg/ml doxycycline for 72 hr. Cells were then treated with or without 10 µM Akti-1/2 for 24 hr. While NIS-mediated RAIU activity was decreased upon 72 hr doxycycline induction (lane 1 vs. 3), Akti-1/2 increased NIS-mediated RAIU activity in both non-induced and doxycycline-induced MEK1 expressing cells (lane 1 vs 2; lane 3 vs. 4). NIS-mediated radioiodine uptake was confirmed by performing parallel experiments in the presence of 3 mM perchlorate (ClO$_4^-$), a competitive inhibitor of iodide uptake by NIS. RAIU results are representative of two individual experiments performed in triplicates.
Figure 3.3

A.

B.

Continued
Fig 3.3 continued

C.

D.
Figure 3.4. Akti-1/2 decreased iodide efflux rate and increased NIS-mediated iodide transport rate. Cell surface biotinylation showed treatment with 10 µM Akti-1/2 for 24 hr decreased cell surface NIS protein levels (A) whereas iodide efflux assay showed treatment with 10 µM Akti-1/2 for 24 hr decreased iodide efflux rate (B) compared to DMSO vehicle in PC Cl3 cells. (A) Biotinylated cell surface NIS were pulled down by avidin-coated beads and then subjected to SDS-PAGE gel for analysis. Equal loading of cell surface NIS protein were normalized with Na⁺/K⁺-ATPase. The results are representative of two independent experiments. (B) Cells were treated with 10 µM Akti-1/2 or 3 mM 17-AAG for 24 hr prior to iodide efflux studies (left panel) and RAIU assay (right panel). Treatment with 3 mM 17-AAG for 24 hr has been shown to decrease iodide efflux in PC Cl3 cells and was included as a positive control. Iodide retention was calculated and the log(iodide retention) was plotted at 2 min intervals. The rate of iodide efflux rate was decreased by treatment with Akti-1/2 (solid gray line; p-value < 0.0001) and 17-AAG (solid black line; p-value < 0.0001) compared to DMSO vehicle-treated cells (dotted black line). 24 hr treatment with Akti-1/2 or 17-AAG both increased NIS-mediated RAIU activity to about 6- fold and 3- fold, respectively in PC Cl3 cells. While 17-AAG increases radioiodide accumulation mainly through decreasing iodide efflux rate, the data suggest that the effect of Akti-1/2 on increasing radioiodide accumulation is only in part due to the change in iodide efflux rate. RAIU results are representative of two individual experiments performed in triplicates.
Figure 3.4

A.

**Akti-1/2**

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B.

![Graph](image7.png)

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**Figure 3.5.** NIS modulation by Akt prevails over PI3K, mTORC1, and MEK. PC Cl3 cells were treated with LY294002 (A), Rapamycin (B), or PD98059 (C) with or without Akti-1/2 for 24 hr prior to western blot analysis or RAIU assay. Co-treatment with Akti-1/2 and LY294002 did not further increase RAIU activity compared to Akti-1/2 treatment alone; co-treatment with Akti-1/2 and Rapamycin further increased RAIU activity compared to Akti-1/2 treatment alone; co-treatment with PD98059 and Akti-1/2 had decreased RAIU activity compared to Akti-1/2 treatment alone. However, the extent of increase in NIS protein level by LY294002, Rapamycin or PD98059 was diminished by co-treatment with Akti-1/2 as shown by western blot analysis. These data suggest that NIS modulation by Akt at the protein level as well as RAIU activity appears to prevail over PI3K, mTORC1, and MEK. Each NIS-mediated radioiodine uptake was confirmed by performing parallel experiments in the presence of 3 mM perchlorate (ClO₄⁻), a competitive inhibitor of iodide uptake by NIS. RAIU results are representative of two individual experiments performed in triplicates. Equal loading of proteins was normalized with beta-actin and the data is representative of two individual experiments for each western blot analysis.
Figure 3.5

A.

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RAU (cpm/ug DNA):

B.

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RAU (cpm/ug DNA):

Continued
C.

Fig 3.5 continued
**Figure 3.6.** Akti-1/2 plays a less prominent role in increasing RAIU activity in PC Cl3 cells acutely stimulated by TSH. PC Cl3 cells were TSH starved for 3 days prior to the experiment. TSH (1 mU/ml), IGF-1(100 ng/ml), LY294002 (10 μM), rapamycin (20 nM), and/or Akti-1/2 (10 μM) were administered to cells for 24 hr followed by RAIU assay (A) and western blot analysis (B). (A) TSH-stimulated RAIU activity was decreased by co-treatment with IGF-1 (black bars: lane 1 vs. 2), increased by LY294002 (black bars: lane 1 vs. 3), but had little effect by co-treatment with Akti-1/2 (black bars: lane 1 vs. 4) or Rapamycin (black bars: lane 1 vs. 5). This suggests that effect of Akti-1/2 on increasing RAIU activity is less prominent in cells acutely stimulated by TSH. NIS-mediated radioiodine uptake is confirmed by performing parallel experiments in the presence of 3 mM perchlorate (ClO₄⁻), a competitive inhibitor of iodide uptake by NIS. RAIU results are representative of two individual experiments performed in triplicates. (B) In contrast, western blot analysis showed that effect of Akti-1/2 on decreasing NIS protein level remains prominent in cells acutely stimulated by TSH (lane 1 vs. 4). Equal loading of proteins was normalized with beta-actin and the data is representative of two individual experiments for each western blot analysis.
Figure 3.6

A.

![Graph showing RAU (cpm/μg DNA) for different treatments]

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B.

![Image showing molecular weight and bands for rNIS, pAkt, and B-actin]

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<th>Treatment</th>
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(kDa) 82 63 60 47
**Figure 3.7.** Both LY294002 and Akti-1/2 decreased NIS-mediated RAIU activity despite no effect on cell surface NIS protein level in HEK293 expressing exogenous NIS. HEK293 cells transiently expressing rat NIS were treated with 10 μM LY294002, 20 nM Rapamycin, or 10 μM Akti-1/2 for 24 hr prior to RAIU assay (A), western blot analysis (B) or flow cytometry (C). (A) Treatment with LY294002, Rapamycin, or Akti-1/2 all decreased NIS-mediated RAIU activity in HEK293 cells transiently expressing rat NIS. The results are representative of two individual experiments performed in triplicates. (B) Treatment with LY294002, Rapamycin or Akti-1/2 had no effect on total NIS protein levels in HEK293 cells expressing exogenous NIS. Equal loading of proteins was normalized with beta-actin and the data is representative of two individual experiments for each western blot analysis. (C) Flow cytometric analysis showed that treatment with LY294002, Rapamycin or Akti-1/2 had minimal effect on cell surface NIS levels compared to DMSO vehicle control. Cell surface NIS level was detected in non-permeabilized HEK293 cells expressing exogenous FlagrNIS by flow cytometric analysis. Cells were incubated with fluorescein isothiocyanate-labeled goat anti-mouse antibody alone for nonspecific fluorescence (pink dashed line) or with mouse M2 anti-FLAG monoclonal antibody, followed by fluorescein isothiocyanate-labeled goat antimouse antibody (green solid line). For quantification, the positive events region was gated at a fluorescence density that had less than 1% of cells with nonspecific fluorescence (shown as a horizontal line). The data is representative of two individual experiments.
Figure 3.7

A.

![Bar chart showing RALU (cpm) for DMSO, LY294002, Rapamycin, and Akti-1/2.]

B.

**HEK 293-rNIS**

![Western blot image showing rNIS and β-actin for DMSO, LY294002, Rapamycin, and Akti-1/2.]

C.

![Flow cytometry histograms for DMSO, LY294002, Rapamycin, and Akti-1/2.]

(kDa) 82 — 64 — 47 —

rNIS

β-actin
Table 3.1. NIS kinetics in DMSO versus Akti-1/2 treated PC Cl3 cells

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<tr>
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*: cpm/2 min/μg DNA
Supplementary data

**Figure 3.8** NIS protein levels was decreased by activated PI3K in PC Cl3 cells infected with adenovirus carrying constitutively active PI3Kp110 CAAX for 48 hr.
Figure 3.9 Combination treatment with PD98059 and LY294002 had additive effect on increasing NIS protein levels in PC Cl3 cells (lane 2 and 3 versus lane 5).
Figure 3.10 Temporal effect of Rapamycin on radioiodide accumulation and total NIS protein in PC Cl3 cells.
Figure 3.11 Inhibition of lysosomal and proteasomal degradation blocked and reversed decreased NIS protein levels (A) yet did not further increase NIS-mediated RAIU activity (B) in Akti-1/2 treated PCCl3 cells. PCCl3 cells were co-treated with Akti-1/2 and leupeptin (lysosome inhibitor) for 24 hr or Akti-1/2 for 16 hr followed by Akti-1/2 + MG-132 (proteasome inhibitor) for another 8 hr prior to cell harvesting or RAIU assay.
Figure 3.12 Treatment with AktV (A) or mixed population of PC Cl3 stable transfectants expressing shRNA knockdown Akt1 and Akt 2 (B) decreased NIS protein levels and increased NIS-mediated RAIU activity. PC Cl3 cells treated with AktV, another Akt inhibitor that shares little structural similarity with Akti-1/2, decreased NIS protein levels yet increased NIS-mediated RAIU activity at 24 hr. Similarly, mixed population of PC Cl3 stable transfectants expressing shRNA knockdown Akt1 and Akt 2 decreased NIS protein levels and increased NIS-mediated RAIU activity. These data confirm the effect of Akt1 and Akt2 on maintaining NIS protein stability and decreasing NIS-mediated RAIU activity.
Figure 3.12

A.

![Graph showing AktV and RAIU activity](image)

B.

![Graph showing shCtrl and shAkt1/2 with RAIU and bands](image)
Figure 3.13 Temporal effect of Akti-1/2 on NIS expression/function. NIS-mediated RAIU activity (top panel) and NIS protein levels (lower panel) was examined at 30 min, 4 hr, 8 hr, and 24 hr of Akit-1/2 treatment in PC Cl3 cells. pAkt levels was inhibited at 30 min of Akti-1/2 treatment, yet the effect of Akti-1/2 on increasing NIS-mediated RAIU activity and decreasing NIS protein levels did not start to show till later hours (>8 hr).
Figure 3.14 LY294002 and Akti-1/2 both increased whereas Rapamycin decreased NIS-mediated RAIU activity in RET/PTC1-expressing PC Cl3 cells. RET/PTC1-expressing PC Cl3 cells were treated with 10 μM LY294002, 20 or 40 nM Rapamycin, or 10 or 25 μM Akti-1/2 for 24 hr prior to cell harvesting or RAIU assay. Similar to parental PC Cl3 cells, LY294002 increased NIS-mediated RAIU activity mainly due to increased NIS protein levels. Rapamycin increased NIS protein levels yet slightly decreased NIS-mediated RAIU activity. Akti-1/2 decreased NIS protein levels yet increased NIS-mediated RAIU activity.
**Figure 3.15** Akti-1/2 decreased total and cell surface NIS levels in RET/PTC1-expressing PC Cl3 cells by immunofluorescent labeling of NIS observed by confocal microscope.
Fig 3.16 Effect of Akti-1/2 or 17-AAG on iodide efflux rate versus NIS-mediated RAIU activity in RET/PTC1-expressing PC Cl3 cells.
Chapter 4

Summary and Future Directions

NIS-mediated radioiodine accumulation and prolonged radioiodine retention due to iodide organification in thyroid cells has long been served as the basis for radioiodine therapy in diagnostic imaging and targeted ablation of remnant thyroid cancer in treating post-surgical thyroid cancer patients. To selectively induce functional NIS expression in the thyroid gland for effective radioiodine therapy, TSH is elevated by thyroid hormone withdrawal or by administration of exogenous recombinant hTSH. While the majority of patients benefit from the treatment, some patients are unresponsive to radioiodine therapy due to reduced NIS expression. Thus, it is of clinical significance to discover and further validate novel reagents that would further increase radioiodine accumulation in thyroid tumor through the study of understanding the underlying mechanism of NIS regulation.

Chapter 2

In chapter 2, we employed non-invasive SPECT functional imaging to examine and quantify the temporal dynamics of thyroidal and salivary radioiodine accumulation in mice. At 60 min post radionuclide injection, radionuclide accumulation in the salivary gland was generally higher than that in thyroid due to much larger volume of the salivary gland. $^{123}$I accumulation was not detectable in the salivary gland at t24 which is
consistent with the report that radioiodine in blood circulation was completely excreted by 19 hr post Na\textsuperscript{125}I injection in CD1 mice (Zuckier et al. 2004). Furthermore, modulation of iodide accumulation in the thyroid of Tg-PTC1 mice seems comparable to that in thyroid cancer patients in that Tg-PTC1 mice are less responsive to exogenous bTSH stimulation in inducing thyroidal iodide accumulation. Lastly, we validated the effect of 17-AAG on increasing thyroidal radioiodine accumulation in the thyroid tumors of Tg-PTC1 mouse model. Interestingly, while thyroidal iodide retention ability in 17-AAG treated Tg-PTC1 mice (t24/t1 %ID= 2.6 ± 0.6) was much lower than that in WT mice (t24/t1 %ID= 15.7 ± 0.9) at t24, yet the fold increase of iodide retention ability by 17-AAG was comparable between Tg-PTC1 mice and WT mice at t24. This may be due to the fact that DMSO decreased thyroidal radioiodine accumulation dramatically at t24 in Tg-PTC1, but not in WT mice. However, the underlying mechanism of DMSO on decreasing thyroidal radioiodine accumulation in Tg-PTC1 mice remains unclear.

**Effect of gender and age on thyroidal radioiodine accumulation**

It has been shown that normal women had slightly higher thyroidal uptake than men at both 2 hr and 24 hr post \textsuperscript{131}I administration. Furthermore, thyroidal uptake decreased slightly when subjects were aged more than 40 years old compared to those younger than 40 years at both 2 hr and 24 hr post \textsuperscript{131}I administration (González E et al. 2008). In another study, thyroidal uptake was shown to increase with age to a maximum at about 40 years old, then decreased afterwards at both 4 hr and 24 hr post \textsuperscript{131}I administration (Schober and Hunt 1976). Despite our small sample number, we did observe that female mice had higher thyroidal radioiodine accumulation compared to that
of male mice at t1 (female: 0.63-6.45%, mean= 2.74%; male: 0.90-2.94%, mean= 1.77%), t6 (female: 1.59-23.21%, mean= 11.39%; male: 2.85-18.45%, mean= 6.79%) and t24 (female: 1.48-24.66%, mean= 13.27%; male: 3.62-21.26%, mean= 10.41%).

While this phenomenon is yet to be confirmed using a larger cohort of mice, taken together, it would be interesting to investigate whether the minimal radioactivity needed to ablate thyroid remnant in low-risk patients could be stratified by gender and age based on their differences in the magnitude of thyroidal radioiodine accumulation.

**Effect of bTSH on thyroidal radioiodine accumulation in T3-supplemented mice**

The effect of exogenous bTSH on temporal dynamics of thyroidal radioiodine accumulation in the absence of endogenous mTSH was examined and showed that the extent of thyroidal radioiodine accumulation stimulated by a single dose of bTSH in T3-supplemented mice was much less than that in mice received neither bTSH nor T3 (non-treated mice), suggesting that the duration of elevated serum TSH level is important to maximize thyroidal radioiodine accumulation as therapeutic scan is usually performed 8 days after $^{131}$I is administered. This may be due to the fact that bTSH has a short halflife of < 3hr or due to the difference in the potency between bTSH versus mTSH. While it has been reported that bTSH is six to ten times more potent than hTSH (Pierce, et al, 1981), less is known about the difference in potency between bTSH versus mTSH. However, it would still be interesting to see whether the extent of thyroidal radioiodine accumulation will be further increased by multiple doses of bTSH in T3-supplemented mice.

In addition to the short halflife of bTSH, iodide from the high concentration of T3 in drinking water decreased the ratio of radioiodine over iodide in the blood (12 µg/ml
T3: 6.97 µg/ml iodine; 150 µci $^{123}$I: 0.0078 µg iodine) may also result in the lower extent of accumulation in thyroidal radioiodine accumulation in T3-supplemented mice. Indeed, while Hernan, et al recently reported that body iodine content may be irrelevant to successful ablation rate, it is still important to avoid iodide supplementation or iodide contamination prior to radioiodine ablation (Hernan, et al, 2010). It would be interestingly to see if decreasing circulating iodide pool by including low iodide diet in our protocol would further enhance thyroidal radioiodine accumulation in our mouse model.

**Possible mechanisms underlying the effect of 17-AAG on increasing thyroidal radioiodine accumulation**

In this study, we validated the effect of 17-AAG on increasing thyroidal radioiodine accumulation in thyroid glands of WT mice and in thyroid tumor of our Tg-PTC1 transgenic mouse model. Interestingly, we observed that 17-AAG had no effect on thyroidal radioiodine accumulation at 4 hr and 9 hr, but significantly increased thyroidal radioiodine accumulation at 27 hr post 17-AAG in WT mice. On the other hand, 17-AAG treatment markedly increased thyroidal radioiodine accumulation at both 9 hr and 27 hr post 17-AAG compared to DMSO in Tg-PTC1 mice. The difference between the temporal effect of 17-AAG in WT versus Tg-PTC1 mice may be due to that Hsp90 in tumor has 100 fold higher binding affinity to 17-AAG compared to Hsp90 in normal cells as tumor Hsp90s are all present in multi-chaperone complexes with high ATPase activity (Kamal, Thao et al. 2003).
While we do not know the signaling pathways affected by 17-AAG in downregulating iodide efflux in thyroid cells, there are several signaling nodes that worth discussing. It has been reported that Hsp90 co-immunoprecipitated with Akt and disruption of Hsp90 binding to Akt leads to dephosphorylation of Akt by protein phosphotase 2A and inactivation of Akt (Sato, Fujita et al. 2000). Interestingly, our data showed that inhibition of Akt by pharmacological inhibitor, Akti-1/2, slightly decreased iodide efflux in PC Cl3 cells. In addition, disruption of Hsp90 function leads to depletion of Raf-1 and consequently leads to downregulation of MEK-ERK signaling (Schulte, Blagosklonny et al. 1996; Piatelli, Doughty et al. 2002). However, we previously showed that inhibition of MEK pathway by PD98059 did not have an effect on iodide efflux rate (Vadysirisack, et al, 2007). While it is tempting to suggest that Akt and not Raf signaling might be involved in 17-AAG regulation of iodide efflux, it is still too premature to jump to such conclusions. It would be interesting to identify potential 17-AAG targets that may be involved in increasing thyroidal iodine accumulation by applying PC Cl3 cell lysates to beads chemically crosslinked with 17-AAG followed by mass spectrometry to identify 17-AAG associated proteins.

While our lab and others have previously reported that 17-AAG increases NIS-mediated radioiodine accumulation by decreasing iodide efflux in NIS-expressing PC Cl3 rat thyroid cells and RET/PTC1-transformed PC Cl3 cells (Marsee et al. 2004, Elisei et al, 2007), the mechanism underlying the increase of thyroidal iodide retention by 17-AAG is currently unknown. Since Hsp90 is known for its role as molecule chaperone, it has been reported that protein chaperones together with reactive oxygen species may increase the solubility of the insoluble multimerized Tg with high iodine content for
thyroid hormone synthesis (Delom, 1999). It would be interesting to investigate whether
17-AAG acts to impair the secretion of iodinated Tg derivatives by examining and
comparing the ratio of different soluble forms of Tg in thyroids treated with or without
17-AAG.

**Salivary radioiodine accumulation**

Table 4.2 shows the summary of salivary radioiodine accumulation in FVB/N
mice under various treatment conditions. The extensive variability of salivary radioiodine
accumulation may be due to the limitation of separating thyroid from salivary in non-
treated and T3-bTSH mice or the salivary from the background due to heterogeneous
distribution of radioiodine in the salivary gland in T3 mice when performing ROI
selection. For non-treated mice (no treat), while salivary radioiodine accumulation
ranged from 0.93 to 13.62 at t1 and 0.69 to 9.92 at t6, radioiodine retention ability
decreased with age in both female and male mice. T3-supplementation inhibited thyroidal
radioiodine accumulation, yet did not have a significant effect on salivary radioiodine
accumulation at t1 (t1:1.82 to 7.09; N=3) and slightly decreased salivary radioiodine
accumulation at t6 (t6: 0.02 to 6.49; N=3) compared to non-treated mice. Interestingly,
one dose of 60 μg bTSH in T3-supplemented mice slightly decreased salivary radioiodine
accumulation at t1 (0.85 to 4.53; N=5), yet significantly decreased radioiodine
accumulation at t6 (0.05 to 0.99; N=5) compared to non-treated mice. This phenomenon
warrants a future study using a larger cohort of mice.

It is of interest to note that the ratio of salivary %ID versus thyroidal %ID in non-
treated mice at t1 appeared to be greater in male (1.84-4.63, mean= 3.47, N=3) than in
female (1.02-2.29, mean = 1.59, N=3) mice and seemed to increase with age (2-3 month: 1.02-3.95, mean = 2.07, N=4; 6-7 month: 2.29-4.63, mean = 3.46, N=2; 14-15 month: 5.11-5.75, mean = 5.43, N=2). This finding is consistent with the reports that total accumulation of $^{131}$I uptake in submaxillary glands is greater in male than in females in several strains of mice (Llach, Tramezzani et al. 1960). The reasons underlying the increase of salivary/thyroid %ID ratio with age is unknown as the variations of salivary %ID or thyroid %ID did not correlate with age. If the dependence of the ratio of salivary %ID versus thyroidal %ID at t1 on gender and age holds true in humans, and if salivary gland damage occurs at an early stage of $^{131}$I exposure, we would anticipate that male and elder patients would experience more salivary dysfunction after radioiodine therapy. It would be interesting to investigate whether this hypothesis is true by reviewing clinical data from patients.

**Chapter 3**

In chapter 3, we aimed to understand the mechanism underlying regulation of NIS expression/function, in particular, the interplay between PI3K-Akt-mTORC1 and MEK signaling nodes on NIS modulation. Inhibition of PI3K by LY294002 increased NIS-mediated RAIU activity through upregulation of NIS expression. On the other hand, inhibition of mTORC1 by Rapamycin decreased NIS-mediated RAIU activity yet increased NIS protein levels. The discordance between NIS protein and NIS-mediated RAIU activity by Rapamycin is due to its effect on activating pERK and pAkt levels. The effect of Akt inhibition on NIS expression/function was further examined showing that Akti-1/2 markedly increased NIS-mediated RAIU activity by decreasing iodide efflux.
rate and increasing iodide transport rate and iodide affinity of NIS. The effect of Akti-1/2 on increasing NIS-mediated RAIU activity is restricted to thyroid cells encourages the use of Akt pharmacological inhibitors to selectively increase thyroidal radioiodine accumulation and decrease radioiodine accumulation in non-thyroid tissues.

**Potential mechanism that facilitates NIS degradation in Akti-1/2 treated PC Cl3 cells**

NIS has a long half-life of 3 to 5 days, yet 24 hr treatment with Akti-1/2 decreased ~50% of cell surface NIS protein levels suggesting that Akti-1/2 may induce cell surface NIS endocytosis for lysosomal- and proteasomal- degradation. Furthermore, mass spectrometry analysis of FlagrNIS immunoprecipitates from HEK293 cells showed that clathrin, a protein involved in clathrin-mediated endocytosis, is a possible NIS-associated protein (unpublished data). It would be interesting to further examine whether Akti-1/2 facilitates clathrin-dependent NIS endocytosis to proteasome and lysosome degradation pathways in hope to identify molecular targets to inhibit cell surface NIS endocytosis and further increase RAIU activity in Akti-1/2 treated cells. As NIS is reported to be a phospho-protein, NIS Thr577 is previously reported to be a potential in vivo phosphorylation site that is important for NIS stability (Vadysirisack, 2007). Furthermore, consensus motif programs predicted Thr577 can be potentially phosphorylated by Akt/PKB (Table 4.2), it would be of interest to investigate whether Akti-1/2 induced NIS degradation is mediated through Thr577 phosphorylation.
Potential mechanisms of Akti-1/2 on increasing NIS-mediated radioiodine accumulation

The discordance between NIS protein levels and NIS-mediated RAIU activity in Akti-1/2 treated cells is due to decreased iodide efflux rate and increased iodide transport rate and iodide affinity of NIS, suggesting that Akt inhibition increased NIS functional activity. Temporal effect of Akti-1/2 on NIS expression/function showed that while pAkt levels was inhibited at 30 min post Akti-1/2 treatment, effect of Akti-1/2 on increasing NIS-mediated RAIU activity and decreasing NIS protein levels did not start to show till later hours (>8 hr) suggesting that Akti-1/2 does not directly affect NIS. Taken together, Akti-1/2 appears to modulate NIS indirectly at the post-translational level and/or non-NIS factors that contribute to increased NIS-mediated RAIU activity. Phosphorylation and glycosylation are the two common post-translational modification of protein. While it has been shown that glycosylation does not affect NIS-mediated RAIU activity (O Levy, 1998), our lab and others have previously reported in vivo NIS phosphorylation sites showing that Ser43 and Ser581 modulate iodide transport rate of NIS and these two phosphorylation sites are not modulated by MEK signaling. In addition, Thr49 is important for local conformation or structure of NIS as mutation of Thr49 to alanine, aspartic acid or serine all resulted in decreased NIS-mediated RAIU activity without affecting NIS protein levels (Riedel, 2001; Vadysirisack, 2007). Based on various consensus motif programs (Table 4.2), Ser43 and Ser581 can be phosphorylated by PI3K/Akt downstream effectors S6K, IKK and PAK whereas Thr49 can be phosphorylated by PKB/Akt. It would be of interest to further investigate whether these amino acids exert its function through Akt regulation. Alternatively, it would be
important to examine NIS phosphorylation and NIS protein complex formation in cells treated in the absence or presence of Akti-1/2 to uncover mechanisms regulating NIS functional activity.

**Effect of Akti-1/2 on NIS expression/function in chronic versus acute TSH conditions**

While it was encouraging to see that among all drugs examined in this study, Akti-1/2 showed the highest extent of increase in NIS-mediated RAIU activity in PC Cl3 cells chronically stimulated by TSH, Akti-1/2 did not increase NIS-mediated RAIU activity in PC Cl3 cells acutely stimulated by TSH. This suggests that the effect of Akti-1/2 on increasing RAIU activity may be dependent on long-term treatment of TSH. It would be of interest to examine whether extending Akti-1/2 treatment from 24 hr to 48 hr or longer hours in PC Cl3 cells acutely stimulated by TSH will increase NIS-mediated RAIU activity. If indeed, longer hours treatment with Akti-1/2 increases NIS-mediated RAIU activity in cells acutely stimulated by TSH, it would be of clinical significance to further validate the *in vitro* findings *in vivo* by examining whether treatment of Akti-1/2 increases thyroidal iodide accumulation in T3-supplemented exogenous TSH stimulated thyroid tumor-bearing Tg-PTC1 mouse model.

In summary, continued elucidation of the signaling pathways to identify target signaling nodes that upregulate NIS expression and NIS-mediated RAIU activity *in vitro* along with confirming the *in vitro* findings on selectively increasing thyroidal radiiodine accumulation in an immune-competent genetically engineered mice carrying thyroid
tumors surrounded by physiologically/pathologically relevant microenvironment will hopefully improve clinical management of patients with advanced thyroid cancers.
### Table 4.1

Salivary radioiodine accumulation and radioiodine retention ability (t6/t1) in FVB/N mice under various treatment conditions.

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Table 4.2 Prediction of potential kinases phosphorylate rNIS *in vivo* phosphorylation sites using MotifScan, KinasePhos, PPSP, NetPhosK1.0, and GPS. (modified from Dr. Douangsone Vadysirisack)

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Elisei, R., A. Vivaldi, et al. (2006). "Treatment with drugs able to reduce iodine efflux significantly increases the intracellular retention time in thyroid cancer cells stably transfected with sodium iodide symporter complementary deoxyribonucleic acid." J Clin Endocrinol Metab 91(6): 2389-95.


