AN ABSTRACT OF THE THESIS OF

Lisa Kirkemo for the degree of Honors Baccalaureate of Science in Biochemistry & Biophysics presented on May 22, 2014. Title: Permeability of Various Thyroid Hormone Derivatives and Discovery of a Novel Metabolite.

Abstract approved:

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Kevin Ahern

In this investigation, six different endogenous thyroid hormone derivatives—T_4, T_3, TA_4, TA_3, T_1-Amine, and N-Acetylated T_1-Amine—were tested to gain insight into their permeability coefficients and efflux ratios across a monolayer of Madin-Darby Canine Kidney (MDCK II) cells. The results indicate that T_3 and T_4 were unable to traverse the membrane due to lack of compatible membrane bound transporters, which supports that the transport of both T_3 and T_4 into their cellular targets relies on membrane proteins. The data also suggested that T_1-Amine and N-Acetylated T_1-Amine may cross the plasma membrane by an active transport mechanism, while the remaining derivatives express efflux ratios indicative of a passive diffusion mechanism. Incubating N-Acetylated T_1-Amine with MDCK II cells produced a novel metabolite, which was identified as a sulfated form of N-Acetylated T_1-Amine.

Key Words: Thyroid hormone derivatives, permeability coefficients, transporters

Permeability of Various Thyroid Hormone Derivatives and Discovery of a Novel Metabolite

by

Lisa Kirkemo

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Lisa Kirkemo, Author

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Introduction

Thyroid hormone is one of the key hormones in the endocrine system. Regulation of thyroid hormone is intricately controlled through a negative-feedback mechanism involving the pituitary, hypothalamus, and thyroid gland, termed the hypothalamic/pituary/thyroid (HPT) axis.[1] The thyroid gland, which is responsible for the production of thyroid hormone, utilizes stored iodine to form these important endogenous hormones. Thyrocytes, the native cells of the thyroid, produce a protein called thyroglobulin, an essential component in the production of thyroid hormone (Fig. 2).[2] Within a single thyroglobulin moiety, there are up to 120 tyrosine residues, which can be covalently modified with iodine by the enzyme thyroid peroxidase. This reaction can yield one of two products: a mono-iodinated product or di-iodinated product, monoiodotyrosine (MIT) or diiodotyrosine (DIT), respectively.[2–4] In the lumen of thyroid follicular cells, thyroid peroxidase catalyzes the covalent linkage of two DIT residues in thyroglobulin to form a tetraiodothyronine (thyroxine) residue (Fig. 1).[2] A mechanistically similar reaction involving a single MIT molecule binding to a single DIT molecule leads to the formation of triiodothyronine, although this is less frequent.[3]

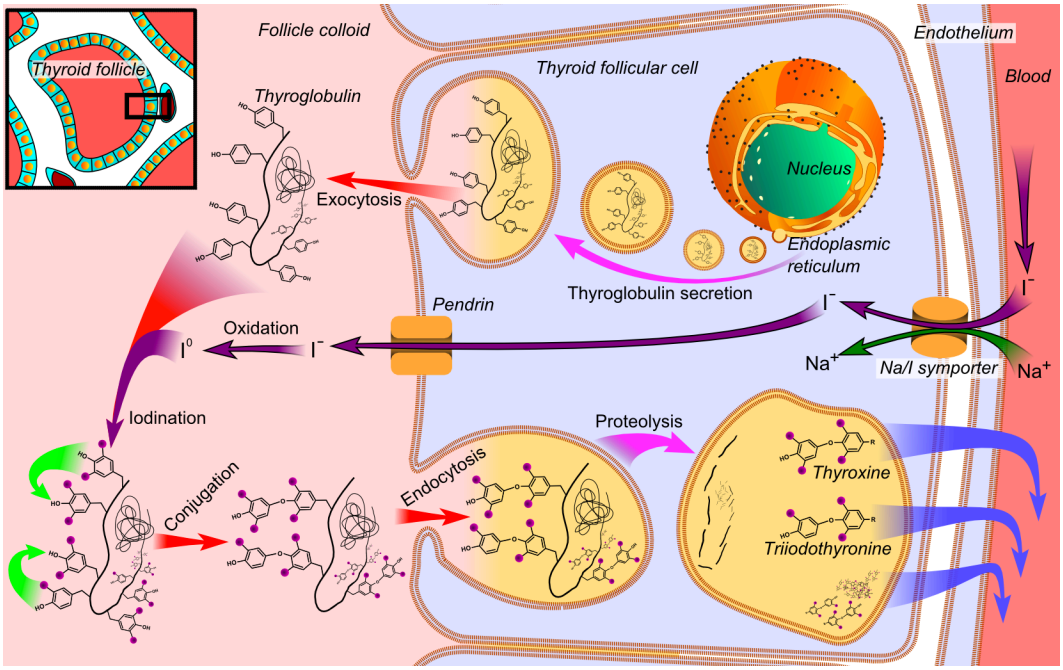


Figure 1: Mechanism by which T_4 and T_3 are formed and released into the bloodstream.[5] Mature thyroglobulin is initially secreted into the lumen of the thyroid follicular cells, where it undergoes iodination, as well as conjugation, leading to covalent linkage of either two DIT residues, or a single MIT residue with a DIT residue. The thyroglobulin is endocytosed back into the follicular cell, where it undergoes proteolysis, and secretes T_4 and T_3 into the bloodstream.

Secretion of the necessary hormones into the blood is dependent on endocytosis, whereby thyroglobulin is taken into the cells from the apical surface of the thyroid follicular cell, and digested via lysosomal proteolysis, which in turn releases T_4 and T_3 into the bloodstream (Fig. 1).[6] T_4 is a prohormone found in the blood at high concentrations—almost 40 times higher than that of T_3—but it has very little biological activity. Efficient transport of thyroid hormone is capable through the binding of T_4 and T_3 to soluble protein structures in the blood, such as thyroxine binding globulin (TBG), albumin, and thyroid binding prealbumin.[2] The levels of total unbound T_4 and T_3 in the blood are very low, estimated to be around 0.03% and 0.3% (w/v) of total serum,

respectively.[2] Presence of thyroid hormone in its inert form, T_4, allows for transport throughout the body tissues, without exerting its effects in non-specific targets. Once transported to the site of action, the prohormone, T_4, undergoes a 5'-deiodination of the outer ring to form active T_3 through the enzymatic action of either a type I or type II deiodinase as outlined in figure 2.[7] Type I deiodinases are generally found in peripheral tissues, such as the kidney and liver, and are responsible for regulating the amounts of active T_3 found in circulation, while type II deiodinases are found in the brain, pituitary, and brown adipose tissue, which cleave T_4 for intracellular use at the nuclear receptor.[2]

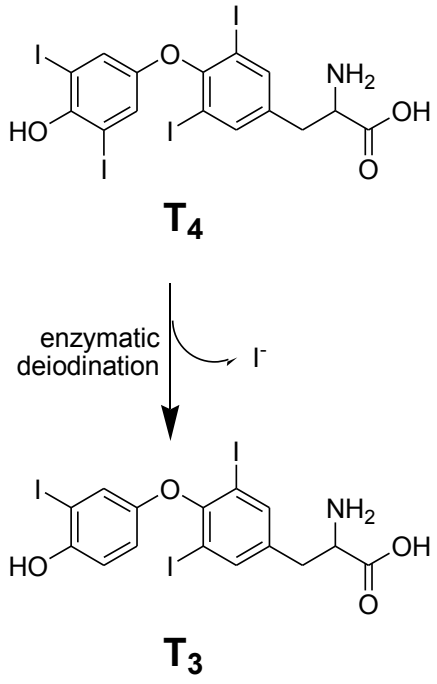


Figure 2: Mechanism by which both type I and type II deiodinases convert the majority of endogenous T_4 into active T_3. Depending on the type of tissue, as well as the presence of type I or II deiodinase, the activated T_3 will either be available to surrounding tissues through the circulatory system, or it will be used directly at the nuclear receptor.

Thyroid hormone is used as a means to control physiological homeostasis, early brain development, and metabolism. Through extensive studies, it was found that thyroid hormone exerts its effects is intracellularly, meaning it traverses the lipid bilayer of the cell before being utilized.[2] Using radiolabeled thyroid hormone, Oppenheimer and Samuels were able to identify specific binding sites within the nucleus of cells, by which the thyroid hormone exerted its effects. These were termed thyroid hormone receptors.[2] The cell is very highly regulated, especially in terms of what gets in and out of the cell through the lipid bilayer. Most polar molecules are halted from entering cells due to the hydrophobic nature of the plasma membrane that surrounds it. One of the many regulated molecules allowed entry to the cell through membrane bound transporters are the thyroid hormones, T_3 and T_4.[3] Thyroid hormone receptors, which interact directly with intranuclear DNA, proteins, and thyroid hormone, are members of the nuclear receptor family. Nuclear receptors are located inside the cell nucleus, and regulate the expression of certain genes, thereby controlling many functions within an organism.[2] Another term for these receptors is transcription factors, as they are directly involved in regulating transcription levels of certain gene segments into mRNA, and subsequent translation into protein. Thyroid hormone receptors are grouped under the class II nuclear receptors, meaning that regardless of the binding status of the ligand, the receptor is always found in the nucleus, bound to a conserved specific palindromic segment of DNA termed the thyroid response element (TRE), which generally lies upstream from the promoter region of target genes.[28] In the absence of a bound ligand, thyroid receptors are still present within the nucleus, attached to the TRE. In the presence of a ligand, such as T_3, a co-activator protein is recruited to the site of the receptor.[8] This complex plays a pivotal role

in the recruitment of transcription factors and ultimately the formation of the basal transcription complex, which is responsible for transcription of downstream DNA into mRNA and subsequent protein formation.[2–4,8] When there is no T_3 or other bound ligand is present, the co-activator protein complex dissociates from the receptor, signaling for the recruitment of a co-repressor complex to take its place. As such, the receptor remains in the nucleus with or without the presence of a bound ligand.

The receptor-mediated recruitment of either co-repressors or co-activators leads to important molecular changes that either increase or decrease the levels of transcription, in the case of co-activators and co-repressors, respectively. If an agonist ligand is introduced and binds to the hormone receptor, it changes the conformation of the receptor in such a way as to stimulate the recruitment of the co-activator protein complex. These, in turn, target histone amine groups, adding acetyl moieties to the histone tails through their histone acetyltransferase (HAT) activity.[9] Through addition of these acetyl groups to the histone constructs, the interaction between the negatively charged DNA and the previously positively charged amine groups on the histone complexes weakens, thus allowing for the availability of short segments of DNA for transcription.[10,11] Alternatively, if an antagonist ligand is bound to the hormone receptor, it induces a conformational change that signals to the cell a similar action as when no ligand is bound, thus allowing for the binding of co-repressor proteins to the receptor. These co-repressor proteins harbor histone deacetylase (HDAC) activity, which removes acetyl groups from the histone tails, restoring the positively charged amine groups.[9] This, in turn, strengthens the association between the histones and the DNA, reducing the likelihood for transcription to occur. In the remaining pages of this thesis, the terms agonist and

antagonist will refer to those ligands that bind to the thyroid hormone receptor and, respectively, either promote or inhibit transcription.

Although a particular thyroid hormone may successfully enter the nucleus, presence of the necessary thyroid hormone receptor for binding is not guaranteed. Recent discoveries through photoaffinity labeling of nuclear samples from various tissues found that there are different sized thyroid receptors, thus, different receptor isoforms.[2] Through further study, it was realized that there were 4 thyroid hormone receptor isoforms: alpha-1, alpha-2, beta-1, and beta-2, each of which is uniquely activated through the binding of different thyroid hormone compounds.[12–14] Each receptor isoform is distributed in different tissues in different quantities.[14] T_3, the predominant human thyroid hormone, is a non-isoform selective binding ligand, meaning that it is capable of binding to all receptor isoforms successfully and exerting its effects.[15] Each receptor includes three separate domains: (1) a transactivation domain, (2) a DNA-binding domain, and (3) a ligand-binding and dimerization domain, listed from N-terminus to C-terminus. The largest differences between the alpha (1 and 2) and beta (1 and 2) isoforms are found within the N-terminal transactivation domain, as this domain is fundamental in the activation and repression of transcription and the formation of RNA complexes.[12] Within the DNA-binding domain are two sets of four cysteine side chains, each coordinated to a zinc ion, which form a “zinc finger”. One of the zinc fingers interacts directly with the major groove of the thyroid response element, while the second zinc finger binds to the minor groove.[10] When thyroid receptors are bound to TREs without a ligand present, transcription is less likely to occur. However, once a ligand is present, the propensity for

that ligand to be either an agonist or antagonist will result in the binding of the receptor to co-activators or co-repressors, respectively, thus determining the extent of transcription.[9]

When either production of thyroid hormone or the method by which it exerts its physiological effects fails, various phenotypic manifestations can arise, including Hashimoto's thyroiditis, Graves' disease, and cretinism.[16,17] Each year, hundreds of thousands of people suffer from a wide array of thyroid related illnesses, such as those listed above. The most traditional method of treatment is to utilize the active endogenous hormone T_3 as a means to regulate thyroid hormone levels. However, because of the nonselective nature of T_3, it cannot be reliably used as a thyroid hormone supplement, except in cases where the patient is suffering from severe hypothyroidism, as it targets both TR receptors. Non-specific targeting results in negative health effects such as tachycardia, muscle wasting, as well as decreased bone density.[2] To alleviate these problems, current research is looking into how to synthesize new agonist and antagonist thyromimetic alternatives and evaluating their mechanistic action. However, less research is currently underway to more fully understand the mechanism by which thyroid hormone and its derivatives gain entry into the cell, which is a critical step in constructing a useful synthetic alternative.

To better understand the general method by which various thyroid hormone derivatives could be transported into the body, it is important to understand how the most prominent hormones, T_3 and T_4, undergo intracellularly transport. Throughout the body, T_3 and T_4 play a variety of roles within specific tissue types. It was previously thought that the transport of these hormones into the cells of the body followed a basic diffusion model. However, in one study, researchers found an almost 55-fold higher concentration

of T_3 inside of the cell than out, which indicated that perhaps an active transport mechanism was responsible.[18] More recently, scientists have discovered that the transport is tissue dependent—meaning that different transport proteins are present in varying quantities in different tissues—and mediated by an energy dependent, specific transport mechanism through various membrane transporters, including monocarboxylate transporter 8 (MCT8) protein, organic anion-transporting polypeptide 1c1 (Oatp1c1) and fatty acid translocase (rFAT).[3] Although very little is known about the latter two, the MCT8 membrane transport protein is known to be expressed in most cell types and has been found to have a high affinity for thyroid hormone, with K_M values of 1.4 nmol/L and 61 nmol/L for T_4 and T_3, respectively.[15] Patients with mutations in the MCT8 gene present with severe neurological disabilities, suggesting that thyroid hormone is intimately involved in brain development and that MCT8 is important for distribution of T_3 into the brain.[12] While the mechanism by which T_4 and T_3 are processed and transported has been the subject of much research, little is known about transport and uptake of other thyroid hormone derivatives.

Since many of these thyroid hormone derivatives share similar structures (Fig. 3), it could be possible that they bind and interact in similar ways to T_3 and T_4. Analyzing known endogenous derivatives and gaining deeper insight into their methods of transport may elucidate possible ways by which to alleviate many illnesses stemming from abnormal thyroid interaction. Although the core structural components are retained, thyroid hormone derivatives have very specific differences in their structure, which also change the way in which they interact with receptors, carrier proteins, and transporters in the body. For the remainder of this paper, discussion will focus on the six structures

shown in figure 3. While research is well underway in understanding the mechanism by which T_4 and T_3 are transported throughout the tissues of the body, very little is known about the mechanism by which other thyroid hormone derivatives, such as TA_3 (triiodothyroacetic acid, Triac), TA_4 (Tetrac), T_1-Amine, and N-Acetylated T_1-Amine are transported into the cell. This question will be examined extensively for the remainder of this report, after a brief discussion of what is currently known of each derivative shown below.

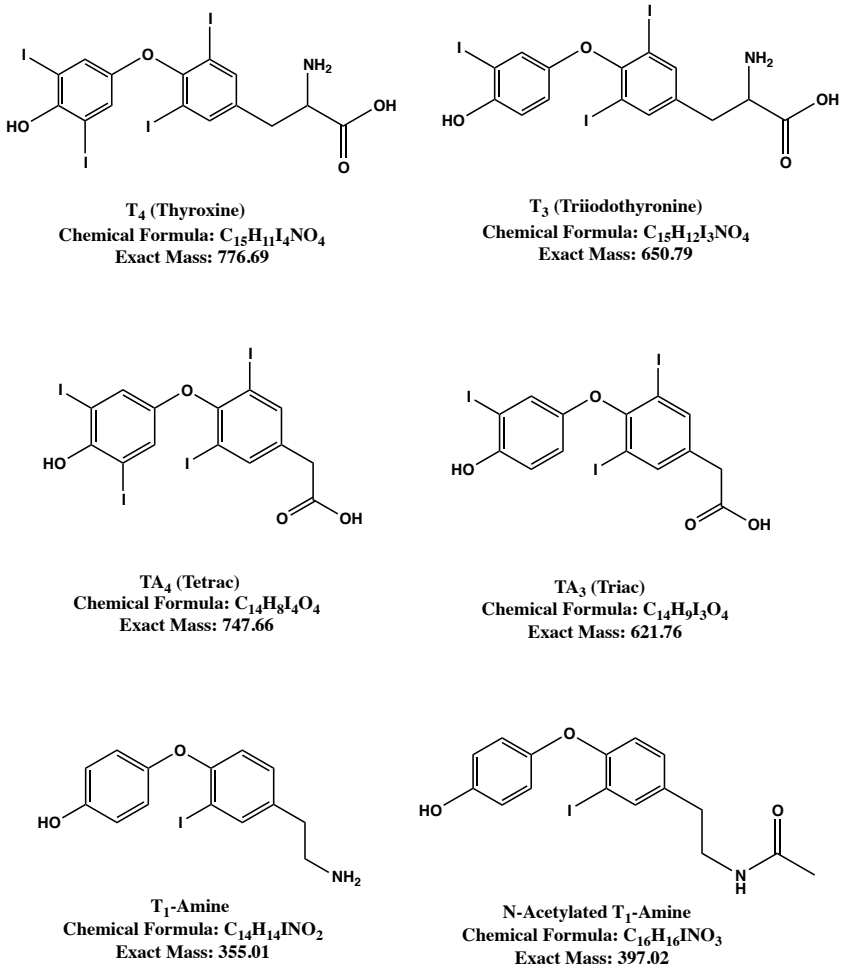


Figure 3: The structures of various thyroid hormone derivatives with their exact masses and chemical formulas.

As previously noted, recent studies have shown that patients with mutations in the gene responsible for the MCT8 transport protein suffer from severe neurological impairments, due to the lack of active T_3 present in the tissue of the brain.[15] Although the MCT8 protein serves as a transporter for T_4 and T_3 into tissues, it does not serve that same purpose for the similar structured thyronamine family.[15] When TA_3 was administered in place of T_3 in primary murine cerebellar cultures that did not contain the MCT8 transporter, development of Purkinje cells was reported, which are mature neurons that regulates motor movements.[15] This result indicates that thyronamines are able to traverse the lipid bilayer without the assistance of an MCT8 transporter, although it does not rule out the possibility of a different transporter playing a similar role for TA_3 that MCT8 does for T_3 and T_4. However, TA_3 application has its drawbacks, mainly due to its short 6-hour half-life in the body, which would mandate numerous injections throughout the day or a continuous-release administration. As a possible alternative, research is currently examining whether TA_4 could act as a prohormone to TA_3, similarly to what T_4 is to T_3. As the half-life of TA_4 in the body is 3 to 4 days, TA_4 could work as a functional option for patients with MCT8 deficiency. Recently, tests were completed on MCT8 knockout mice and wild-type animals with daily injections of TA_4, which were shown to promote neuronal differentiation in the cerebellum, striatum, and cerebral cortex during the first few postnatal weeks, highlighting the great potential of this treatment.[15]

T_1-Amine, another thyroid hormone derivative, has also been implicated in major physiological changes. In 2004, the Scanlan laboratory at OHSU isolated a novel compound that caused interesting effects in rodents, including hypothermia, hypotension, and bradycardia.[19] T_1-Amine has opposite effects from T_3, suggesting that it may be an

endogenous mechanism for modulating the action of T_3. It also has a completely different mechanism by which it exerts its effects. Whereas most hormones in the body take hours to cause any physiological changes, the actions of T_1-Amine are very fast, similar to a neurotransmitter. It has been hypothesized that since both its structure and kinetics resemble those of catecholamine and serotonin, T_1-Amine may also act as a neurotransmitter in the body. The discovery of T_1-Amine has led to the theory that endocrine homeostasis is modulated by a variety of distinct alterations made to T_4.[20] While 5'-deiodination of T_4 yields the active T_3, which exerts it effects through the slow binding to nuclear thyroid receptors and eventual alteration in gene transcription levels, the decarboxylation and de-iodination of T_4 yields T_1-Amine, a fast acting neurotransmitter-like biogenic amine.[20] These two enzymatic paths for the alteration of T_4 may give the body a bimodal method by which to maintain homeostasis in an environment that is rapidly changing.

The final derivative that was analyzed throughout this project was a newly discovered compound called N-Acetylated T_1-Amine. Although very little is known about the overall function of this compound, it may mediate some of the effects attributed to T_1-Amine. In previous research, N-Acetylated T_1-Amine was found in 8 week old mouse serum following a single IP injection of T_1-Amine, which strengthens the argument that N-Acetylated T_1-Amine somehow mediates the physiological effects of T_1-Amine.[21]

Although all of these drugs have been implicated in some physiological response in organisms, the mode of their transport into and out of tissues has not yet been determined. Since these compounds have all been found in human tissues, it is clear that

some mode of transport into these tissues is necessary, whether it be an active transporter, such as MCT8, or by passive diffusion. The goal of this project was to characterize the mode of transport across an epithelial monolayer of MDCKII cells for six different thyroid hormone derivatives: T_4, T_3, TA_3 (triiodothyroacetic acid, Triac), TA_4 (Tetrac), T_1-Amine, and N-Acetylated T_1-Amine. Through better understanding of the rates and efflux ratios of each derivative, it will be possible to begin making synthetic alternatives with tissue specific uptake, that are capable of crossing various epithelial barriers.

Materials and Methods

Madin-Darby Canine Kidney II (MDCK II) Cell Culture

The MDCK II cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and were allowed to incubate (37 degree Celsius and 5% CO_2) until confluent. Cells were trypsinized and diluted to 1 million cells per 1 mL of media. In a 6-well Corning Transwell polycarbonate membrane cell culture plate (24 mm, 3.0 μm, Corning, Corning, NY, USA), 2 mL of fresh DMEM was added to the basolateral fluid compartment, and 2 mL of the cell mixture was added to the apical fluid compartment. Fresh DMEM was added every day for two days to each fluid compartment. All drug dilutions were made to a final concentration of 20 μM in Hank's Balanced Salt Solution (HBSS) (25 mM HEPES, pH 7.4) with 0.5% DMSO. On the day of the experiment, wells were aspirated on both the apical and basolateral fluid compartments and washed with PBS. The cells were allowed to incubate for one hour in 2mL of HBSS (25 mM HEPES, pH 7.4) in both the apical and basolateral sides. The wells were then aspirated, and subsequently replaced with 2 mL of drug dilution (20 μM, 0.5% DMSO) to either to the apical or the basolateral fluid compartment, and fresh HBSS to the opposite region. At 30, 60, 90, 120, 180, and 240-minute time points, 1 mL samples from the chamber opposite of the initial drug treatment were collected, and replenished with fresh HBSS. A final time point (at 240 minutes) was also taken from the initial treatment chamber, to allow for calculation of percent recovery. These experiments were performed in triplicate for each derivative.

HPLC Data Collection

A sample volume of 950 µL was injected onto a C18 column (Poroshell, 4.6 x 100 mm, 2.7 micron, Agilent, Santa Clara, CA, USA) at a flow rate of 1.5 mL/min. The mobile phase (A) consisted of water with 0.5% (v/v) acetic acid and the mobile phase (B) was acetonitrile with 0.5% (v/v) acetic acid. Separation was achieved by a linear gradient, starting at 80% A/20% B, increasing to 10% A/90% B over 11 minutes, and then returning to 90% A/10% B over the remaining 4 minutes of the run. The area under the curve in the HPLC trace was reported for each derivative at each time point. Standard curves were prepared for T_1-Amine, N-acetylated T_1-Amine, TA_3, TA_4, T_3, and T_4 at 20 µM, 5 µM, 1 µM, and 200 nM. The slopes from the collected standard curves were used later in calculating the permeability coefficient (P_{app})

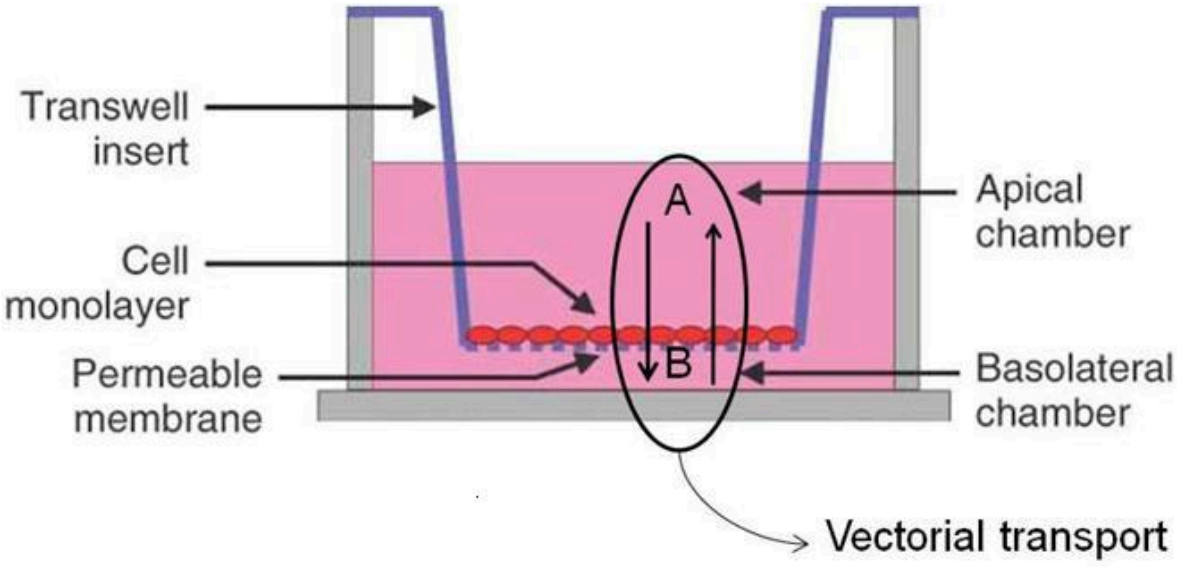


Figure 4: Outline of the experimental layout for permeability testing. The layer of MDCK II cells formed an impermeable monolayer. By transferring drug stock to either the apical or basolateral fluid compartments, the mode of action for the transport of drug can be identified. Figure adapted from Xenoblis ADMET Experts.

Data Analysis

The permeability of the substrate through the cell layer was calculated with the equation $P_{app} = (dQ/dt) / (C_0 \times A)$, where dQ/dt is the rate of permeation of the drug across the cell monolayer, C_0 is the concentration at time zero, and A is the area of the monolayer. Drug efflux ratios were measured and reported as a P_{app} (B-A) / P_{app} (A-B). Active transport is characterized by a ratio below 0.5, whereas an active efflux response is characterized by a ratio greater than two. An example calculation can be viewed in appendix A. To test for a confluent monolayer, transepithelial electrical resistance (TEER) measurement was utilized to establish the formation of tight junctions. Rates (dQ/dt) were based on areas under the curve from the HPLC traces over time, and were normalized with the slopes from the standard curves for each respective derivative.

Unknown Peak Characterization

The MDCK II cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, and were allowed to incubate (37 °C and 5% CO_2) until confluent. The DMEM was aspirated, whereby 10 mL of 50 μM N-Acetylated T_1-Amine in HBSS was added to the cells, and allowed to incubate overnight. The HBSS was extracted, and filtered through a 0.22 μm sterile syringe filter (Fisher Scientific, USA), to extract any contaminants or cell fragments. Samples with 950 μL of filtered experimental HBSS were injected directly onto a C18 column (Eclipse Plus, 4.6 x 250 mm, 5.0 micron, Agilent, Santa Clara, CA, USA) at a flow rate of 2 mL/min. The mobile phase (A) consisted of 2 mM ammonium acetate (pH 5.0) and the mobile phase (B) was

pure acetonitrile. Separation was achieved by a linear gradient, starting at 80% A/20% B, increasing to 10% A/90% B over 11 minutes, and then returning to 90% A/10% B over the remaining 4 minutes of the run. The samples were collected using manual fraction collection, and each fraction was sent for GC-MS analysis. The remainder of each fraction was lyophilized, dissolved in deuterated DMSO, and further analyzed via overnight NMR.

Sulfated N-Acetylated T_1-Amine Synthesis

N-Acetylated T_1-Amine (0.016 g) was dissolved in dimethylformamide (DMF) (52 μL) and added to a mixture of cold DMF (69 μL) and DCC (0.041 g). A mixture of 18 M H_2SO_4 (2.31 μL) was added to DMF (52 μL) and added to the reaction vessel. Reaction was allowed to stir at room temperature for one hour, a precipitate was formed, which was isolated in vacuo.

Results and Discussion

Determination of Permeability Coefficients

The transport of thyroid hormone across the plasma membrane has been shown to take place within the vast majority of tissues of various species. Since the predominant thyroid hormone in circulation is the inactive form, T_4, transport into the cell from the extracellular space is necessary for the subsequent action of both the 5'-iodide cleavage by intracellular type II deiodinases to form active T_3, and the binding of T_3 to its nuclear receptors.[2] Although knowledge of how the molecule exerts its effects on the body intracellularly is essential, understanding the permeability of molecules through different tissue types is the first step towards understanding how and where its physiological properties are put into action.

The epithelial monolayer used throughout this study was Madin-Darby canine kidney cells transfected with the human *MDR1* gene, encoding for the P-glycoprotein (P-gp) efflux protein.[22] As such, not only did this monolayer act as a functional cell monolayer, it also worked to determine efflux, as well as carry out the specialized function of a kidney cell in the pathway towards excretion of wastes. The most striking results were both the high rate of flow through the monolayer of T_1-Amine and N-Acetylated T_1-Amine, as well as the formation of novel metabolites from TA_3 and N-Acetylated T_1-Amine, as shown in figure 5. The role of the selected derivatives is not well understood, however, research has shown that T_1-Amine action is more like that of a neurotransmitter, as opposed to a hormone.[19,20] Analysis of the permeability coefficients illustrates that T_1-Amine and N-Acetylated T_1-Amine have more rapid transport from the

apical to basolateral fluid compartments, in comparison to the opposite direction (basolateral to apical). An efflux ratio of 0.65 and 0.59 for T_1-Amine and N-Acetylated T_1-Amine, respectively, calculated using P_{app} (B-A) / P_{app} (A-B), implies that more of the drug was transported in the apical to basolateral direction. When seeding MDCK II cells onto a growth plate, they adhere to the growth surface with an inherent polarized growth pattern.[23] Because of this phenomenon, the selective permeability of drug solution from one fluid compartment to another could indicate the presence of membrane bound protein transporters, which bind selectively to certain derivatives and may only be expressed on one side of the membrane. Although this result could signify active transport of these compounds across cellular barriers, more data is necessary to come to a definitive conclusion.

The data acquired for T_3 and T_4 have markedly lower permeability coefficients than the remaining derivatives, accompanied by very large error (Fig. 5, inset). Such small values—almost 1/5,000 that of the remaining derivatives—suggests that the transporters responsible for T_3 and T_4 are not found on the membranes of MDCK II cells, and thus, no T_3 and T_4 is seen being transported across the cell membrane. Since T_3 and T_4 both have amino acid side chains present in their structures, which are absent in the remaining derivatives, they also exhibit a heightened polarity, making passive diffusion unlikely. This information has been known and applied to many other molecules throughout the body. The structural relationship between dopamine and its amino acid counterpart, L-DOPA, is much the same as thyroid hormone derivatives and T_3, except for the fact that dopamine is incapable of passively diffusing through cell barriers to areas where it is needed. Instead, L-DOPA acts as a pro-drug by interacting with large amino

acid transporters (LATs) to be actively transported across membranes, where it is then cleaved into active dopamine.[24] Most molecules with amino acid side chains are too polar to passively diffuse through membranes. However, many amino acids, including phenylalanine, tyrosine, leucine, arginine, and tryptophan interact with large amino acid transporters (LATs) in order to become available to the rest of the body.[24] Given the data present from this report, it has been supported that T_3 and T_4 rely on membrane bound protein systems for transport across cellular lipid bilayers and are very unlikely to traverse cell membranes through a passive diffusion model. From the complete lack of T_3 and T_4 found in the chamber opposite the initial drug treatment, it has been hypothesized that MDCK II cells do not express the necessary membrane transporters for the transport of T_3 and T_4 across the lipid bilayer. The remaining derivatives, TA_4 and TA_3, exhibited similar transport across the epithelial barrier from one fluid compartment to the other, with an efflux ratio of 1.17 and 1.12, respectively. This suggests that both of the thyronamines flowed via a diffusion based mechanism from each fluid compartment.

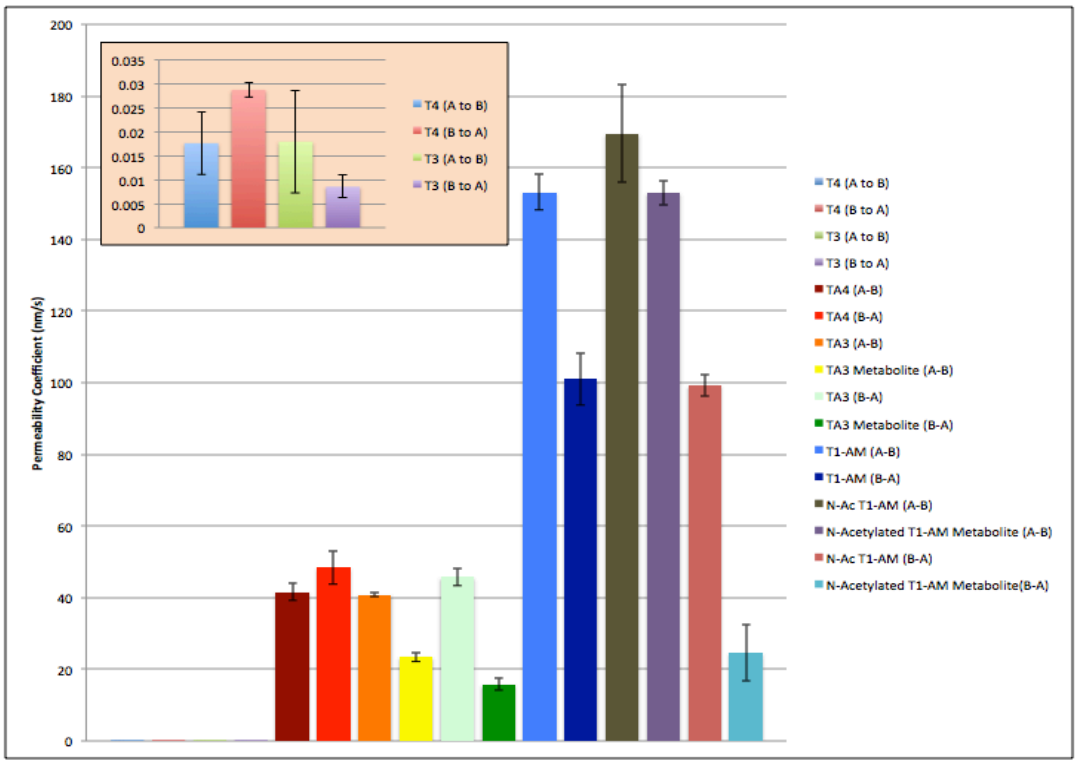


Figure 5: A comparison of permeability coefficients of different thyroid hormone derivatives through an MDCK II monolayer with T_3 and T_4 results (inset). The compounds listed on the x-axis refer to the thyroid derivative initially added to the cell layer at the start of each experiment. If a metabolite was formed throughout the experiment, a column labeled "cleaved" is directly to the right of the corresponding root-compound.

Identification of Novel Derivatives

During the experiment, an unexpected observation was made: when the cell monolayers were incubated with N-Acetylated T_1-Amine and TA_3, novel metabolites were observed. Although future studies may seek to characterize the TA_3 metabolite, this report will focus solely on the identification of the N-Acetylated T_1-Amine metabolite. The formation of the novel metabolite of N-Acetylated T_1-Amine can be visualized clearly in the time-dependent frames from the HPLC runs, shown in figure 6. As the drug was allowed more time to permeate through the cells, not only was the drug solution successfully traversing the monolayer, it was also being converted into a modified product, as observed in the growth of the second peak over time. While it was clear that the unknown product was more polar, as seen through the shorter retention time on the C18 column, it was not apparent how the structure of N-Acetylated T_1-Amine had been modified.

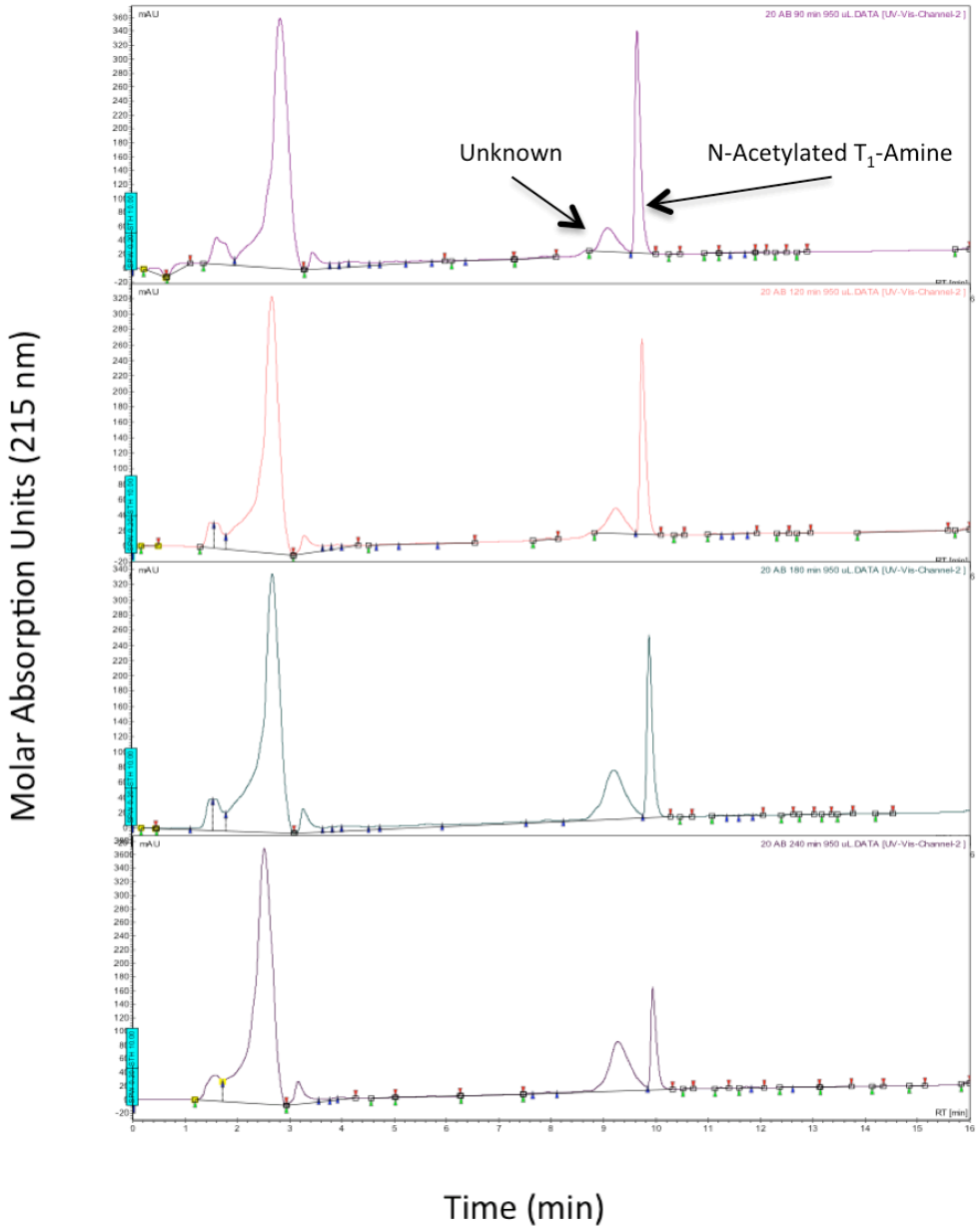


Figure 6: HPLC runs of N-Acetylated T_1-Amine after 60, 90, 120, 180, and 240 minutes showing the retention time of the unknown (9 minutes) and N-Acetylated T_1-Amine (10 minutes). The x-axis denotes time (in minutes), while the y-axis denotes molar absorbance units (215 nm). After longer periods of time the initial substrate is converted into greater amounts of the modified product, which can be visualized through the progressively larger areas of the more polar peak. As can be seen from the HPLC traces over time, more of the unknown product is being taken into the cells and processed to form the peak at 9 minutes.

When viewing the structure of N-Acetylated T_1-Amine, the two most likely sites with high reactivity were the acetyl group and the phenol hydroxyl. The initial hypothesis was that N-Acetylated T_1-Amine was undergoing an enzymatically-catalyzed deacetylation in the cell. However, that was quickly ruled out as the mode of action after direct comparison of HPLC traces of T_1-Amine, N-Acetylated T_1-Amine, and that of the unknown product, shown in figure 7. The next most viable site for enzymatically driven structural modifications was the phenol hydroxyl group. Isolation and characterization of the peak through LC-MS was necessary before any further conclusions were made.

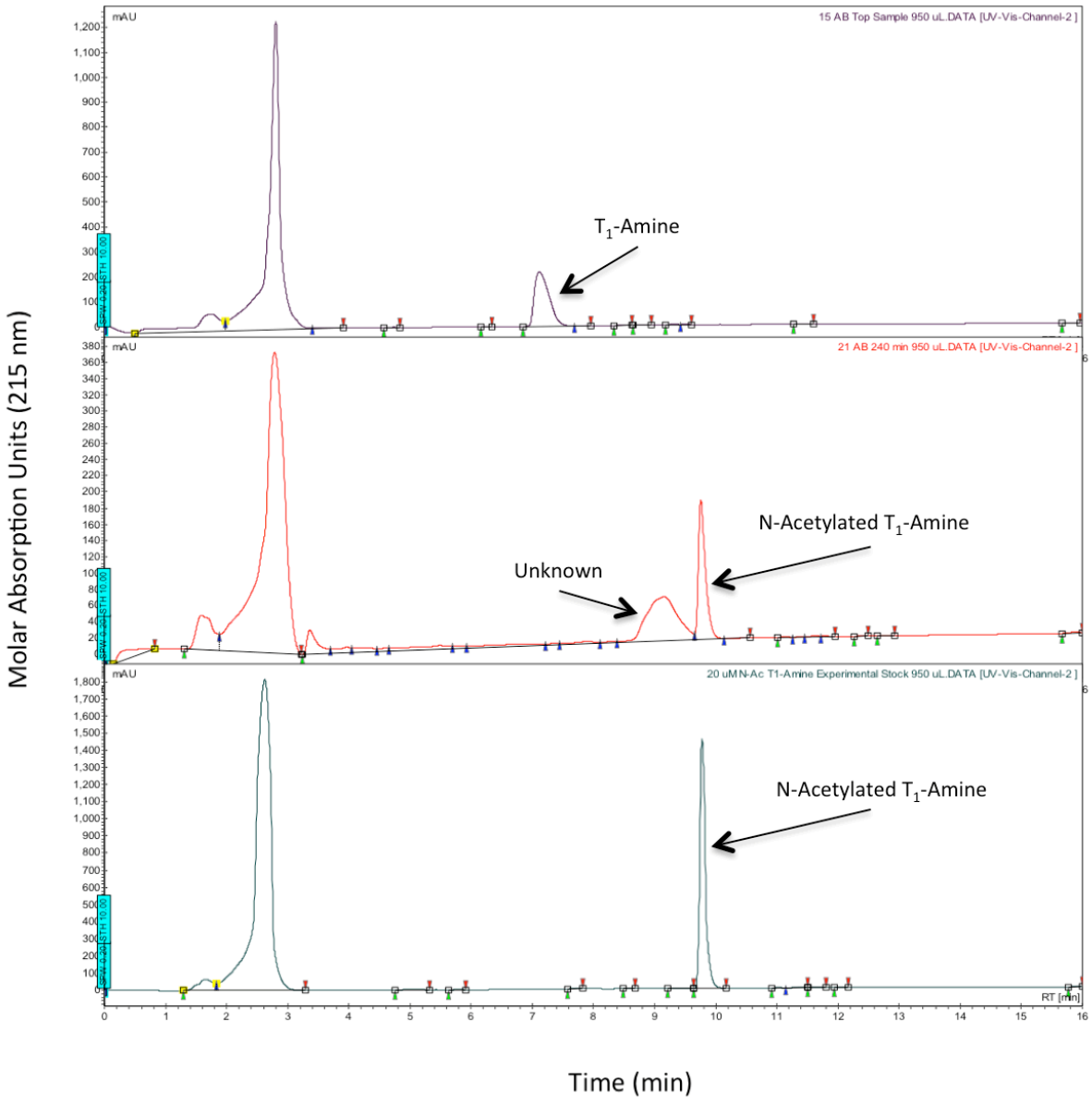


Figure 7: Comparison of HPLC Retention Times from T_1-Amine (top, 7 minutes), N-Acetylated T_1-Amine with Metabolite (middle, 9 minutes and 10 minutes, respectively), and pure N-Acetylated T_1-Amine (bottom, 10 minutes). These results show that the peak produced by the unknown metabolite is not T_1-Amine as previously predicted.

As can be visualized in figure 7, the peak corresponding to the unknown metabolite was not only poorly separated from its parent peak, it also underwent a fair amount of peak broadening, which is indicative of a partially charged species. Partially charged species indicate that a portion of the sample is in its protonated state, while another portion is in its deprotonated state. Each species will interact with the solvent slightly differently, resulting in peak broadening. One of the most important aspects of the process of isolating each peak was the development of a new solvent system, which utilized 2 mM ammonium acetate (pH 5.0) and pure acetonitrile, instead of the previous water with 0.5% (v/v) acetic acid (pH 3.4) and acetonitrile with 0.5% (v/v) acetic acid (pH 3.4). Since the pK_a of the sulfate side group is 2.99, the previous solvent system, at a pH of 3.4, allowed for partial deprotonation of the species, which led to peak broadening. By changing the solvent system (2 mM ammonium acetate, pH 5), the negatively charged side group remained deprotonated completely in all species, as the pH was more than 2 units greater than the pK_a. This enabled peak separation and loss of peak broadening, which allowed for accurate manual fraction collection.

Through utilization of LC-MS, the peak shown in figure 8 was collected and analyzed. Although it was hypothesized that the phenol was the site of enzymatic modification in the conversion to the unknown product, it was still not clear what reaction was occurring in vitro. LC-MS analysis demonstrated that the mass of the unknown product was 475.9 g/mol, which indicated that the unknown product has an addition mass of 78.8 g/mol as compared to N-Acetylated T_1-Amine. Two possible structures were proposed: a sulfated product, with a final molecular weight of 475.9 g/mol or a phosphorylated product, with a final molecular weight of 474.9 g/mol. An

overnight ^{1}H-NMR was taken, but the peaks were not clearly differentiable as a phosphorylated or sulfated product (not shown). Chemical synthesis of the sulfated product, in conjunction with an LC-MS co-spotting procedure finally elucidated the product. As there was only a single peak produced in the co-spot experiment, and the LC-MS results were similar to those from previously collected fractions of experimental product, it was concluded that N-Acetylated T_1-Amine was being sulfated in vitro, which ended any further search into a phosphorylated product. The final sulfated product is depicted in figure 9.

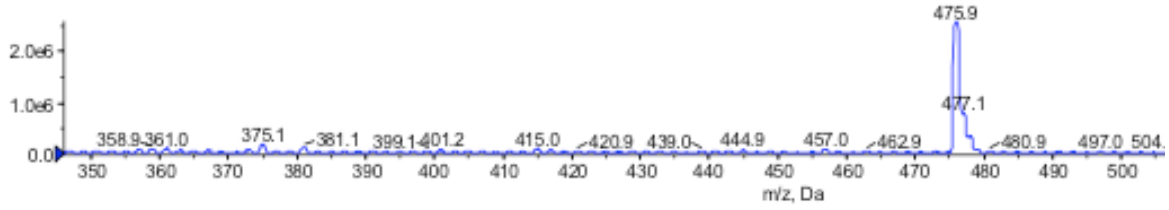
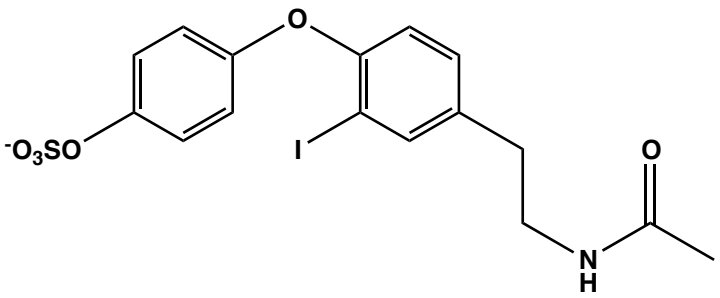


Figure 8: Results from the LC-MS of the unknown peak. A peak at 475.9 g/mol leads to an additional 78.8 g/mol, which corresponds to two possibilities: loss of an alcohol and addition of a bond between a sulfate group or a phosphate group.

The function of the enzymatic sulfation of N-Acetylated T_1-Amine is not yet understood. One hypothesis for the function of this conversion in the body is to generate a more polar, and thus more water-soluble form of N-Acetylated T_1-Amine. Although scientists have identified traces of N-Acetylated T_1-Amine in tissues, a more soluble form is an important finding, as it provides new insights into the complex metabolism of N-Acetylated T_1-Amine. Also important in this study is the choice of cell type in the permeability tests. A similar test was run with N-Acetylated T_1-Amine with Caco-2 cells

harvest from human colon. A second peak was not observed, suggesting that the modification to N-Acetylated T_1-Amine in vitro is unique to the function of MDCK II cells. MCDK II cells are harvested from kidneys, which play an important role in blood purification and excretion of impurities. A key enzyme family found in kidney cells is the sulfotransferases (SULT), which add sulfate groups to incoming molecules, increasing the polarity of the compounds for excretion purposes. One enzyme in particular, SULT1, works to sulfate hydroxyl groups, especially those attached to phenyl rings. Although the sulfated product may not serve a physiological purpose other than excretion, discovery of this product could help to explain why only trace amounts of N-Acetylated T_1-Amine are present in tissues. Instead of holding large amounts of a non-polar substance within the tissues of the body, perhaps the kidneys play a role in carrying a small supply of the sulfated counterpart, which can either be quickly excreted or allowed back into the blood supply for action elsewhere. Previous research has shown that T_4 undergoes a similar sulfation of its phenol hydroxyl group as a means by which to increase the polarity, and thus facilitate urinary and biliary clearance.[3] Only very small quantities of sulfated T_4 are present in the urine, bile, or serum, which is most likely due to the fact that the sulfation leads to rapid deiodination by type I deiodinases. This active deiodination occurs selectively on the inner tyrosyl ring, suggesting that sulfation of T_4 is an important step in the inactivation of thyroid hormone in the body.[3] Although N-Acetylated T_1-Amine is similar in structure, it may follow a different mechanism than T_4 in the way it is inactivated and excreted. More specific analysis is necessary to know whether this sulfation is also a method of inactivation for N-Acetylated T_1-Amine. Current experiments are being undertaken to isolate the sulfated product in human tissues.



Sulfated N-Acetylated T_1-Amine
Chemical Formula: $C_{16}H_{15}INO_6S^-$
Exact Mass: 475.97

Figure 9: The structure of the unknown metabolite, as concluded through NMR, LC-MS, and an HPLC co-spot with the chemically synthesized product.

Conclusion

The permeability experiment done on T_4, T_3, TA_4, TA_3, T_1-Amine, and N-Acetylated T_1-Amine yielded drastically different results than what was initially expected. Firstly, the permeability coefficients of T_4 and T_3 were significantly smaller than all of the remaining thyroid hormone derivatives. This is likely due to lack of expression of the necessary membrane transporters on the surface of MDCK II cells. The presence of the amino acid side group in the structures of T_3 and T_4 make these compounds more polar, and thus incapable of passive diffusion across the membrane. The data supports that T_3 and T_4 rely on membrane bound proteins for transport across lipid bilayers. T_1-Amine and its acetylated counterpart had the largest permeability constants at 153 nm/s and 169 nm/s, respectively, from the apical to basolateral fluid compartments, and 100 nm/s and 99 nm/s, respectively, from basolateral to apical fluid compartments. From prior experiments, T_1-Amine has been found to exert its effects in a

neurotransmitter-like manner, so a high permeability rate is surprising if it acts primarily in the neuronal synaptic cleft. More analysis will be necessary to fully understand the role T_1-Amine and its acetylated counterpart play in human physiology.

Two cleaved metabolites were discovered as a consequence of the permeability studies. The endogenous sulfated N-Acetylated T_1-Amine identified in the cell permeability tests was found to be identical to the synthetic standard through high-performance liquid chromatography (HPLC) retention time. In addition, the mass of the ion peak found through mass spectrometry (MS) at 475.9 m/z corresponded to the addition of a sulfate group to the phenol hydroxyl of N-Acetylated T_1-Amine. The second unknown metabolite is still under investigation. It is unknown whether the mechanism by which this conversion occurs is cell-type specific, or universally found in all epithelial tissue, although preliminary tests on Caco-2 cells have not yielded the modified product. As such, the conversion of N-Acetylated T_1-Amine is hypothesized to be a method by which N-Acetylated T_1-Amine is hyperpolarized by the kidneys as a strategy for increased urinary and biliary clearance.

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APPENDIX A

Example Data Calculation

TA_4 (A to B)						
Time(s)	area	Area adjusted	nmol (sample)	uM Sample	mL remaining	Total Amount in Well (nmol)
1800	11.3	11.8	293.0	293.0	2.0	585.9
3600	27	28.4	700.0	700.0	2.0	1693.0
5400	30.5	32.1	790.8	790.8	2.0	2574.5
7200	32.7	34.4	847.8	847.8	2.0	3479.4
10800	35.8	37.6	928.2	928.2	2.0	4487.9
14400	36	37.8	933.4	933.4	2.0	5426.5
Final	188.9	198.8	4897.6	4897.6	2.0	9795.2

Rate (nmol/s)	0.375
P_{app} (cm/s)	4.02×10^{-6}
P_{app} (nm/s)	40.2

$$P_{app} = (dQ/dt) / (C_0 \times A)$$