ABSTRACT

FK506 and Rapamycin Bioconjugation: A Proposal to Increase the Viability of Transplanted Pancreatic Islets

Chloë A. Sells

Director: Robert R. Kane, PhD

Type 1 Diabetes Mellitus is a widespread disease stemming from the improper functioning of pancreatic islets' ß-cells which produce insulin, a sugar regulatory hormone. There is currently no cure. If the patient has received an islet transplant, damaging blood-mediated inflammatory responses (IBMIR) can ensue, and in addition frequent dosages of immunosuppressants that ultimately can cause chronic health issues are required. Even with immunosuppression therapy, IBMIR and other immune responses result in the destruction of many of the transplanted islets. Attempts to remediate these problems have been made in the form of surface modifications of the transplanted islets. The following thesis proposes a new method to increase the viability of transplanted pancreatic islets--modification via esterification of the immunosuppressant drugs, specifically FK506 and Rapamycin, for immobilization followed by slow release by a ß-eliminative linker which results in an introduction of the uninhibited version of the immunosuppressant molecule into a localized area.

APPROVED BY DIRECTOR OF HONORS THESIS:

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Dr. Robert R. Kane, Department of Chemistry and Biochemistry

APPROVED BY THE HONORS PROGRAM:

_____

Dr. Elizabeth Corey, Director

DATE: _____

FK506 AND RAPAMYCIN BIOCONJUGATION: A PROPOSAL TO INCREASE THE VIABILITY OF TRANSPLANTED PANCREATIC ISLETS

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By

Chloë A. Sells

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TABLE OF CONTENTS

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CHAPTER ONE

Introduction

Diabetes Mellitus

Diabetes mellitus, typically known simply as diabetes, is a disease that has plagued the human population for thousands of years, dating as far back as 1500 BC Egypt.[1] It is extremely prevalent in the United States, affecting 28.9 million people, almost a tenth of the population.[2] The term 'diabetes' encompasses two forms of the disease— the first an autoimmune condition, and the second generally brought on by lifestyle.[2]

Type 1 diabetes is an autoimmune disorder regulated by T-cells that impedes the body's production of insulin, a hormone produced by pancreatic islet β-cells which regulates the Glut4 glucose transporter and is therefore responsible for the maintenance of stable blood sugar levels.[3,4] T-cells are short-range, direct-interaction immune cells activated by antigens on the surface of peripheral lymphoid organ cells such as blood or tissue fluids.[5,6] In Type 1 diabetes, T-cell lymphocyte responses are activated against pancreatic β-cells, destroying them and leaving the body without the ability to generate insulin.[3,7] This type of diabetes comprises only about 5% of the total number of cases.[8] Type 2 diabetes, on the other hand, is responsible for the prevalence of diabetes around the world. Type 2 diabetes is a metabolic disease which hinders the body's ability to use insulin effectively and is typically caused by unhealthy body fat content and unhealthy lifestyle choices.[9]

Diabetes rates, almost entirely Type 2 cases, are climbing globally due to the changes that accompany societal advancement such as increased caloric intake and decline in exercise and physical activity.[10] As higher-income countries see these trends to a greater degree than lower-income nations, diabetes rates are predictably higher in countries with a larger average income— 10.1% versus 8.6%.[10] As of 2014, diabetes was the seventh leading cause of death of American citizens, however, the World Health Organization predicts that by 2030, it will rise to the fourth position in high income countries, including the United States.[11,12] Type 2 diabetes, and therefore the magnitude of these predictions, can typically be alleviated with simple diet, exercise, and in some cases, medications; Type 1 diabetes patients, conversely, require indefinite medical treatment.[2]

Due to the patients' inability to produce insulin entirely, all Type-1 diabetics must undergo insulin therapy regimens to ensure insulin levels in the blood are sufficient for glucose-level management.[13] There are two types of treatments, the first of which is called physiologic insulin therapy, which simulates pancreatic β-cells' continuous insulin secretion by injecting insulin multiple times per day into the fatty tissue beneath the skin (typically abdominal region) via an insulin pump or manual injection.[14,15] The second type of insulin therapy also utilizes injections, but because it delivers insulin inconsistently, it is not suitable as a Type 1 diabetes regimen on its own and is used more for Type 2 diabetes patient—this type is called nonphysiologic insulin therapy.[14]

In extreme cases of Type 1 diabetes where the patient is unable to maintain normal glucose levels (normoglycemia) even with insulin therapy, pancreatic islet cell transplants can be performed to help keep insulin levels within the body stable.[2,16]

Islet Transplantation in Patients with Type 1 Diabetes Mellitus

Pancreatic islets are crucial to the health of an individual because they contain insulin-producing β-cells scattered throughout their structure.[17] If these islets are damaged or destroyed, the pancreas no longer has the ability to produce insulin, and therefore the body has no way to modulate the levels of glucose in the bloodstream, and Type 1 diabetes develops.[18] In patients with severe Type 1 diabetes an allogeneic islet transplant can be performed.

An allogeneic islet transplant is one where pancreatic islets are harvested from a brain-dead or cadaveric organ donor using collagenase enzymes to separate them from pancreatic exocrine tissue, purified using density gradient centrifugation, quality tested, and then implanted into the target patient.[16,19] Allogeneic transplants were shown by a study conducted by the National Institute for Healthcare Excellence to be effective in the short-term and possibly long-term, and though effective in insulin management, allogeneic transplant recipients still require immunosuppressive therapy indefinitely after the procedure.[20] In addition, the transplant of pancreatic islets does not alter the recipient's immune system so the new islets will be targeted by the T-cell lymphocytes in the same manner the old islets were.[18]

Another problem with allogeneic transplants is that the initial transplant often is insufficient to produce the desired result. Post-transplantation, a substantial portion ranging from 60% up to 80% of the islets are ultimately destroyed due to processes such as ischemia, thrombosis, and the instant blood mediated inflammatory reaction (IBMIR) within a matter of hours or days of the initial introduction into the body.[21] IBMIR is predominantly caused by inflammatory mediators which can either hinder the proper

functioning of transplanted islets or cause apoptosis of the islet cells.[22] This reaction, coupled with the existing destructive immune responses that caused the diabetes initially, prove to be major complications. It may be possible to mediate or even prevent these issues by modifying the islets prior to transplantation.

Modification of Pancreatic Islet Cells with PEG-Phospholipid Conjugates

Pancreatic islet cell surface modifications have provided an efficient means of mediating IBMIR and many other destructive inflammatory responses in allogeneic transplants. Successful modifications have been achieved using polyethylene glycol (PEG) phospholipid conjugates as anchors within the cell membrane of the islet and varying the PEG-phospholipid substituents in order for heparin or urokinase (both anticoagulents) to become linked to the islets' surfaces.[23,24] Other methods have likewise coated the islets in heparin but, instead of PEG-phospholipids, utilized tissue factor-P biotin as the membrane-embedded compound to which the heparin ultimately became bound.[25] While the attachment of molecules to the surface of islets using PEG-phospholipids or tissue factor P-biotin is an effective means of islet modifications, another, more prominent, surface modification technique is bioconjugation.

Bioconjugation

Bioconjugation is the covalent linkage of two or more individual molecules, where at least one is a biomolecule, to form a molecular complex that retains the functional properties of each of its constituent molecules.[26] Bioconjugate linkers often are of the bifunctional or trifunctional variety, which have a reactive functional group at each of two or three terminal end positions of the molecule, respectively.[26] Linkers of the bifunctional

variety then fall into one of two general categories, homobifunctional or heterobifunctional, based on the identity of their reactive functional group substituents. Homobifunctional linkers contain a single type of reactive functional group, whereas heterobifunctional linkers have two different functional group types.[27] The presence of two distinct reactive functional groups on heterobifunctional linkers allows for better control over the conjugation reaction as the distinct reactivities of two functional groups can direct the linker to react at specific locations on the molecules.[26] Figure 1 depicts the bioconjugation of two distinct biomolecules utilizing a heterobifunctional linker.

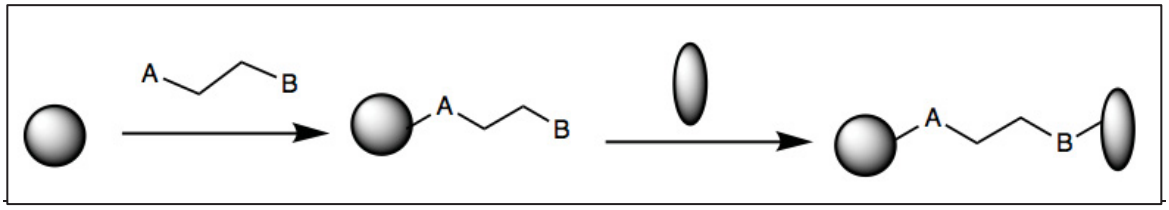

Figure 1: Basic bioconjugation with a heterobifunctional linker

Bioconjugate linker chemistry is a valuable tool in drug design and medical treatments, as the linkers' ability to effectively bind a drug and a macromolecule together into a complex has been shown to improve the pharmacokinetics (in vivo drug movement) of a given drug.[28] In some instances, however, it is the linker's ability to have this complexation reversed that makes it valuable. For instance, by generating a complex with an ester linkage from a carboxylic acid and hydroxyl, non-specific esterases will gradually cleave the ester and release the molecule from the complex.[29] This allows the molecule to move freely and function as if it were never inhibited, thereby extending the half-life (amount of time it takes for half of the drug to be used up) significantly. This is especially valuable in instances where prolonged treatment is required.

ß-Elimination

Linkers can also be tailored to self-cleave in the absence of enzyme activity while still maintaining half-lives that can reach up to several months at physiological pH.[29] This cleavage type has been termed ß-elimination. ß-elimination typically involves a carbamate linkage of the two molecules, and on the ß carbon (a carbon two carbon-carbon bonds away from a functional group) of the linker, possesses an acidic hydrogen and an electron withdrawing group called a modulator which controls the pKa of this acidic hydrogen and therefore, the rate of elimination.[28,29] Upon ß-elimination, CO_2, the original drug, and an alkene derivative of the linker are produced.[28]

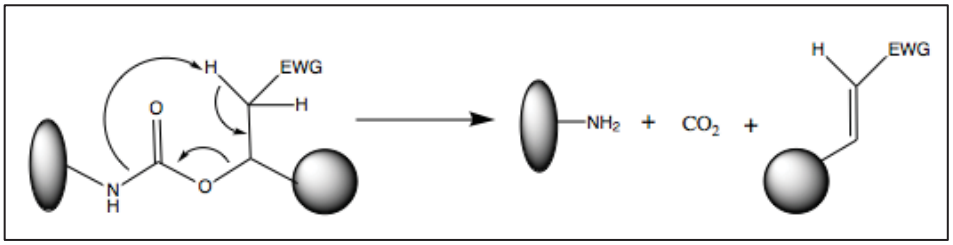

Figure 2: Simplified mechanism of B-eliminative cleavage of a linker

The stronger the electron-withdrawing group is, the shorter the half-life of the complex is, so by varying the modulator group's electron withdrawing properties, the half-life of the linkage and rate of drug release can effectively be tailored to the demand of the treatment.[29] Linkers are not limited to carbamate or ester linkages, they can be attached to the various target molecules using many types of bioconjugation and reactive functional groups.

Bioconjugation Types

NHS Esters

One common type of bioconjugation is between an N-hydroxysuccinimide (NHS) ester and a nucleophile. When NHS esters are reacted with nucleophiles, most commonly amines, they form a stable acylated product.[26] This reaction can be done at physiological pH and in-situ within a solution, making it a highly useful and popular bioconjugation-via-acylation technique.[26]

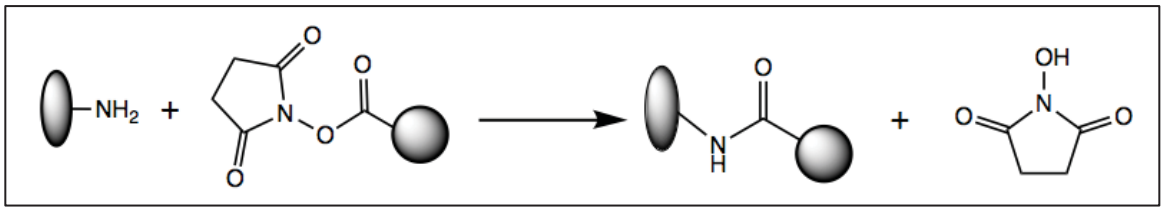

Figure 3: Bioconjugation of two different structures utilizing an NHS-amine acylation reaction

Hydrazides

Bioconjugation can also be done between hydrazides and aldehydes or ketones to form a hydrazone-bond containing product, a Schiff base.[26] The reaction is a dehydration which proceeds by a nucleophilic attack of the hydrazine amine group on the carbonyl of the aldehyde or ketone which ultimately results in the oxygen of that ketone being lost as water.[30,31] The hydrazone linkage formed is fairly stable if made from a ketone but less so if made from an aldehyde; however, with stability increase, speed is sacrificed, as aldehyde the reactions progress more quickly than ketone reactions.[26]

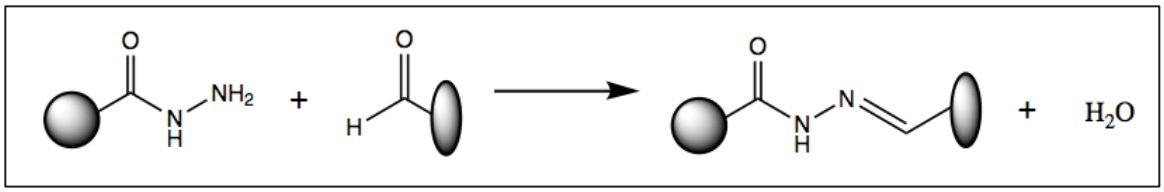

Figure 4: Bioconjugation of two structures utilizing a hydrazide-aldehyde dehydration reaction

Epoxy Rings

Epoxy ring-opening is another type of bioconjugation that has already proven to be a valuable tool. Epoxy groups are very stable and highly reactive with nucleophilic groups in alkaline conditions, therefore by fixing epoxy groups to the surface of a structure compounds with nucleophilic groups can be easily be conjugated.[32] This type of conjugation is highly favored and stable at physiological pH when coupling with sulfhydryl groups and it can take place at any pH, making it a valuable tool for protein and drug immobilizations in biological systems.[26,33]

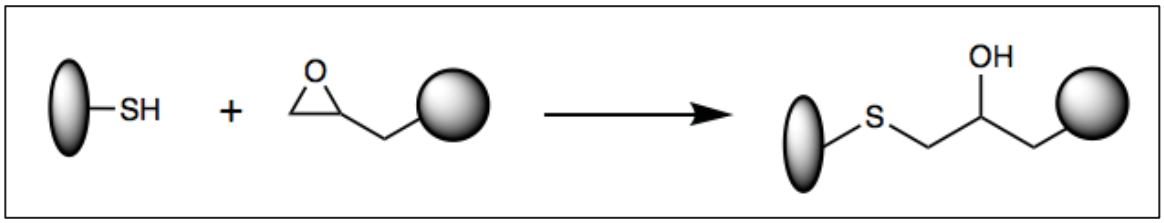

Figure 5: Bioconjugation of two different structures using an epoxy-sulfhydryl reaction

Maleimides

Maleimides are another type of compound that has been shown to have useful bioconjugation properties with sulfhydryl groups. While maleimides can be bioconjugated with most nucleophilic functional groups, sulfhydryls are typically used since their reaction proceeds up to 1000X faster at physiological pH than a reaction done with amines or other nucleophiles.[26] This large discrepancy in reactivity is due to the susceptibility of the π-

bond in the maleimide to alkylate and form a stable thioether bond to link the two structures.[26]

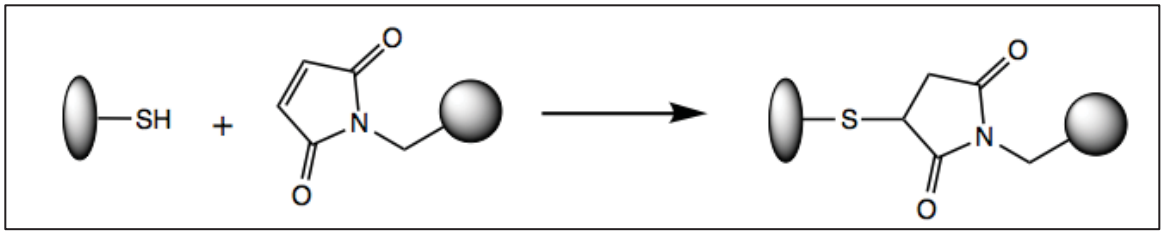

Figure 6: Bioconjugation of two different structures using a Maleimide-sulfhydryl alkylation

Click Chemistry

A very specific category of bioconjugation is click-chemistry. "Click-chemistry" is a descriptive term coined by H.C. Kolb, M.G. Finn, and K.B. Sharpless to describe any reaction that has a broad scope, is high-yielding, produces no reactive byproducts, is stereospecific, uses readily available materials, utilizes either no solvent or a benign one, and produces a product that can be isolated with relative simplicity and without chromatography.[34] Furthermore, click chemistry reactions are typically kinetically-controlled and irreversible with highly energetic reactants that often form a covalent carbon-heteroatom bond.[36] These characteristics allow click-chemistry to be utilized in biological systems and therefore, in the preparation of bioconjugates.

Copper-Catalyzed Cycloadditions

Cycloadditions are a specific type of click-chemistry bioconjugation that relies on the reactivity of π-bonds for covalent-bond formation. When an azide is reacted with a simple unactivated alkyne, a catalyst is needed to drive the reaction, which generates a five-membered, three-nitrogen containing ring with substituents on N_1 and C_4 or C_5, or

both, if the second π-system is central rather than terminal.[35] One specific example is the copper(I)-catalyzed 1,3 dipolar cycloaddition, which reacts an azide with a terminal alkyne to regioselectively generate a triazole ring.[35] If the alkyne is central, the reaction loses its regioselectivity as the substituents on either side of the alkyne will show no preference for the 3 or 4 positions on the five-membered ring.

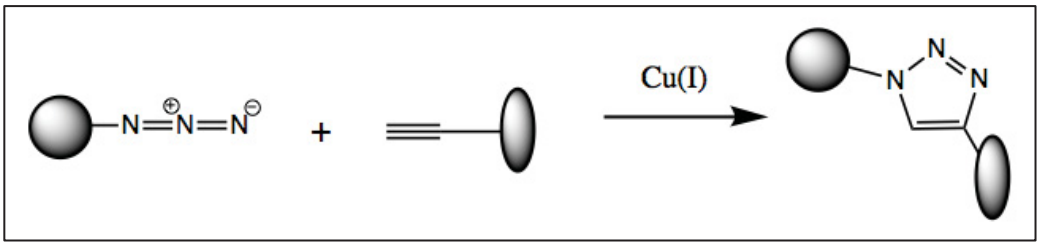

Figure 7: Bioconjugation of two different structures using a copper(I)-catalyzed 1,3 dipolar cycloaddition

Copper-Free Cycloadditions.

Strain-Promoted [3+2] Azide-Alkyne Cycloaddition. Strain-promoted [3+2] azide-alkyne cycloadditions are similar in nature to copper(I)-catalyzed 1,3 dipolar cycloadditions, however, instead of a copper catalyst the reaction is driven by conformational strain. An alkyne bond, which is sp hybridized and desires a linear conformation, in a cyclooctyne ring is forced out of that conformation which makes the ring highly sterically strained. This steric strain provides a similar driving force for cycloadditions as a copper catalyst, allowing this methodology to be used for the conjugation with azides as well as with alkene π-systems.[37] By placing electron-withdrawing substituents on the carbons adjacent to the alkyne bond, the rate of reaction can be increased as the lowest unoccupied molecular orbital (LUMO) of the alkyne becomes greatly reduced in energy.[38]

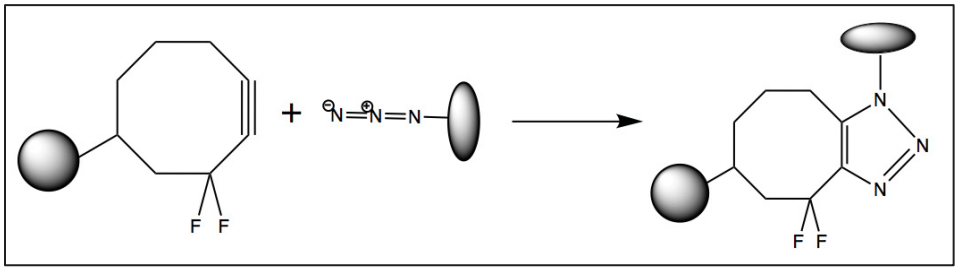

Figure 8: Bioconjugation of two different structures using a Strain-Promoted [3+2] Cycloaddition

Diels-Alder Cycloadditions. A Diels-Alder reaction is an easily achieved, common reaction that creates a cyclohexane ring and is useful in the conjugation of large, bulky molecules such as saccharides, carbohydrates, and proteins.[39] The reaction occurs because alkane sigma (σ) bonds are considerably stronger than alkene pi (π) bonds. By forming the final cyclohexene ring, the ratio of σ-bonds in relation to π-bonds increases, producing a single more stable molecule from two less-stable molecules.[40] Though heat is usually added, the Diels-Alder reaction can sometimes occur at room temperature and produces a 90% or better yield, making it useful in a variety of synthetic situations.[26]

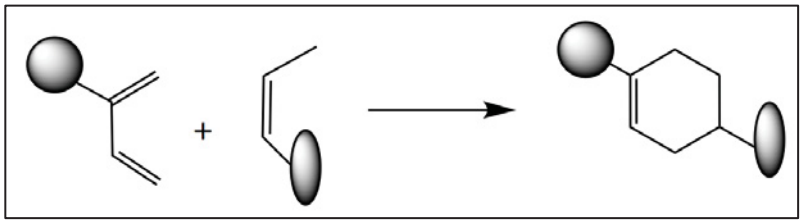

Figure 9: Bioconjugation of two different structures using a Diels Alder Cycloaddition

Traceless Staudinger Ligation

Another reaction that qualifies as click-chemistry is the traceless Staudinger ligation, a modified version of the reaction discovered in 1919 by Staudinger and Meyer, which is instigated by a nucleophilic attack.[41] Like the copper(I)-catalyzed 1,3 dipolar

cycloaddition, the traceless Staudinger ligation utilizes an azide π-system, but instead of using an alkene or alkyne as the complimentary reactant group the reaction couples using a tertiary phosphine group bound at one linkage point via an electronegative atom adjacent to a ketone.[42] This reaction is highly selective, occurs in aqueous solutions at neutral pH's and ambient temperatures, and has a rapid intramolecular acylation step making it viable for in-vivo applications.[42,38]

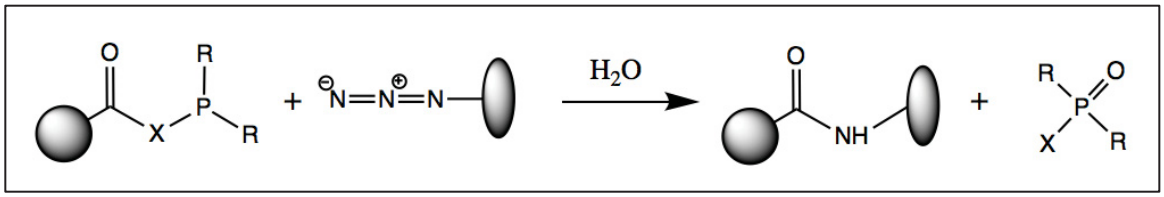

Figure 10: Bioconjugation of different structures using Traceless Staudinger Ligation. X is an electronegative atom

Current Progress in Islet Modifications

Baylor University research teams have made significant progress in the modification of human, porcine, and murine pancreatic islets utilizing acylation, oxidation, and reduction methods.[43] Each of their islet-modification methodologies utilized bioconjugation techniques, including some of those mentioned above. Their acylation, oxidation, and reduction methods utilized the bioconjugation techniques of NHS esters with primary amines, hydrazides with aldehydes, and maleimides with sulfhydryls, respectively, and each of the three modifications were shown to yield islets which had similar viability and potency as compared to control islets.[43] The success of these surface-modification experiments is promising for future study of islet-based prevention of IBMIR and overall pancreatic-islet transplant success.

Toll-Like Receptors

As mentioned previously, IBMIR is caused by inflammatory mediators. One type of inflammatory mediator is the toll like receptor (TLR) which is an integral membrane-receptor with an extracellular N-terminal ligand recognition domain, a transmembrane helix, and an intracellular C-terminal signaling domain within the cytoplasm of the cell.[44] They can be found on the surface or intracellular components of a cell and function by recognizing pathogen-associated molecular patterns (PAMPs) and instigating the proper immune responses.[27] Unfortunately, these TLR's can also initiate immune responses in instances where they are undesirable. This is the case with allogenic pancreatic-islet transplants.

A recent study of pro-inflammatory and pro-oxidant mediators in transplanted islets has found a strong correlation between TLR4 and the inflammatory responses responsible for islet damage.[45] In addition, TLR2 and TLR4 have been indicated to be involved in sensing damage to pancreatic islets and therefore, are likely key contributors to the conditions that lead to pancreatic islet cell death and unsuccessful transplants.[46] Using bioconjugation techniques, molecular inhibitors of TLR2 and TLR4 that are docked to the islets' surfaces could potentially be generated to help to mitigate inflammation and damage to transplanted pancreatic islets.

The improvement of the viability of transplanted islet cells can be expanded past simply islet surface modifications. Islet viability can also potentially be improved by blocking the immune system functions that are responsible for the damage to the islets initially. Two major components in the immune response pathways relevant to pancreatic-islet loss are Calcineurin and Mechanistic Target of Rapamycin (mTOR).

Calcineurin

A key component of an immune response pathway that ultimately damages or entirely destroys pancreatic islets is Calcineurin. Calcineurin is a phosphatase, a phosphate-group removing enzyme, of the amino acids threonine and serine and is responsible for the conversion of Nuclear Factor of Activated T-Cell (NFAT) into the form needed to assist in the transcription of cytokines such as interleukin-2.[47] Interleukin-2 and other cytokines produced from this pathway activate cytotoxic T-cell lymphocytes that kill β-cells in the pancreatic islets.[48] Calcineurin is a target of interest because it essentially instigates this pathway that leads to pancreatic β-cell deaths. If Calcineurin can be inhibited, the pathway stops and the damage to transplanted islets can be drastically reduced.

Calcineurin Inhibitors

There have been several Calcineurin inhibitors discovered and studied over the past few decades, however the two versions that have become major elements of interest in the pharmaceutical industry are Cyclosporine and Tacrolimus, the latter of which will be referred to for the remainder of this paper by its alternative name, FK506. These compounds inhibit immune responses by blocking various points in the T-cell signaling pathway that leads to the production of interleukin-2 and other cytokines.[49]

FK506

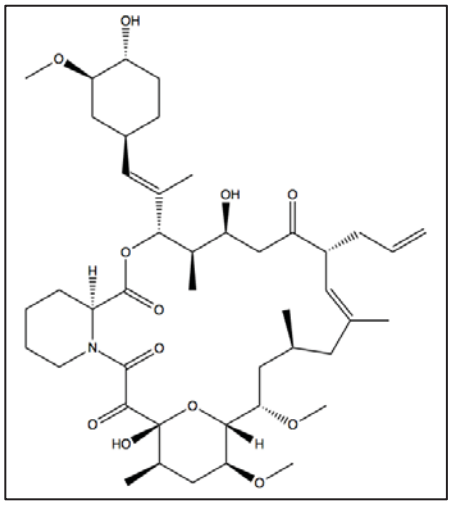

Figure 11: FK506 Molecular Structure

FK506 functions by forming five hydrogen bonds to the intracellular immunophilin, FK Binding Protein 12 (FKBP12, or simply FKBP).[50] This interaction eliminates Calcineurin's ability to act as a phosphatase, thereby suppressing immune function by preventing NFAT from entering the nucleus and complexing with interleukin-2 DNA, as well as the transcription factors Fos and Jun (Figures 12 and 13).[47] FK506 inhibits the T-cell's cycle early, between the G_0 (resting) to G_1 (post cytokinesis and pre DNA-synthesis) phases.[49,51]

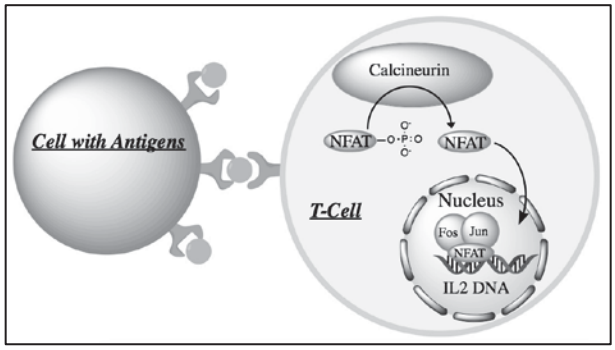

Figure 12: Progression of uninhibited calcineurin signaling

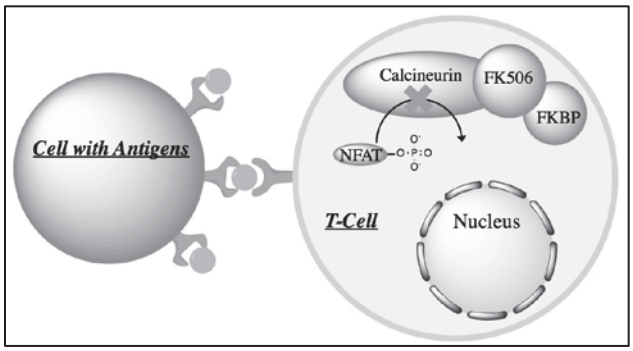

Figure 13: Calcineurin signaling inhibited by FK506 complex

Mechanistic Target of Rapamycin

Another important structure in the immune responses that threaten the success of transplanted islets is Mechanistic Target of Rapamycin (mTOR). mTOR possesses the exact opposite function of Calcineurin— while Calcineurin is a serine and threonine phosphatase, mTOR is a serine and threonine kinase, a phosphate-group adding enzyme.[52] mTOR is also distinct in that it proceeds through two unique pathways via the formation of two separate sub-complexes, mTORc1 and mTORc2. mTORc1 contains the regulatory-associated protein of mTOR (RAPTOR), while mTORc2 contains the Rapamycin-insensitive companion of mTOR binding protein (RICTOR).[53]

mTORc1 is activated by the cytokine interleukin-2 and is thereby a key component in the activation of T-cell lymphocytes.[52] mTORc2 is also activated by cytokines, but it is involved in the differentiation of helper T-cells and has not been suggested to have a strong impact on the activation of T-cell lymphocytes.[54] Inhibition of mTORc2 is therefore less relevant to pancreatic islet transplant viability than is the inhibition of mTORc1.

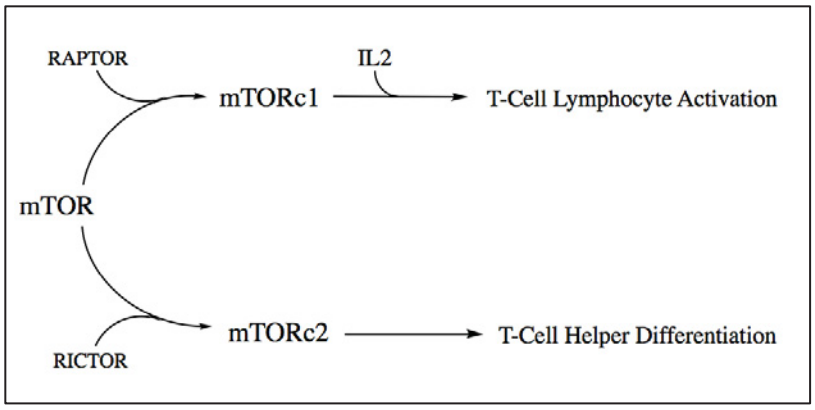

Figure 14: Simplified mTOR signaling pathway

Mechanistic Target of Rapamycin Inhibitors

Unsurprisingly, due to its name, the inhibitors of mTOR are Rapamycin and its derivatives, collectively called Rapalogs. Just as Calcineurin inhibitors block immune functions by complexing and preventing the T-Cell proliferation cycle, Rapalogs inhibit immune responses by complexing with mTOR and preventing the substrates necessary to continue the signal chain from binding to mTOR.[55] Of the two mTOR complexes only mTORc1 is sensitive to Rapalog inhibition, but as mentioned previously only mTORc1 has been indicated to participate in relevant immune responses.[53,54] For the remainder of this paper, mTOR will be referring exclusively to mTORc1.

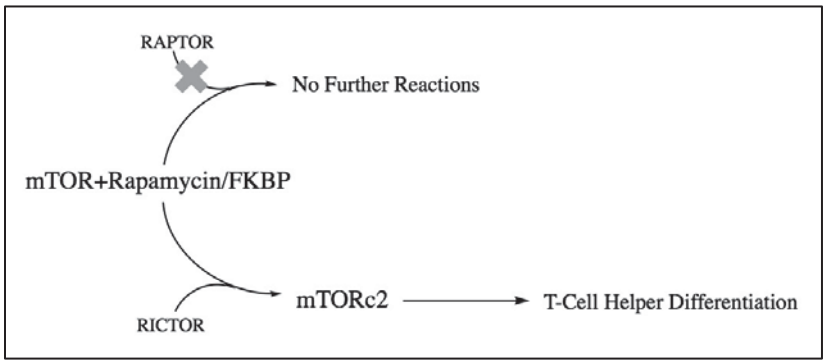

Figure 15: Simplified mTOR pathway when inhibited by Rapamycin/FKBP complex

Rapamycin

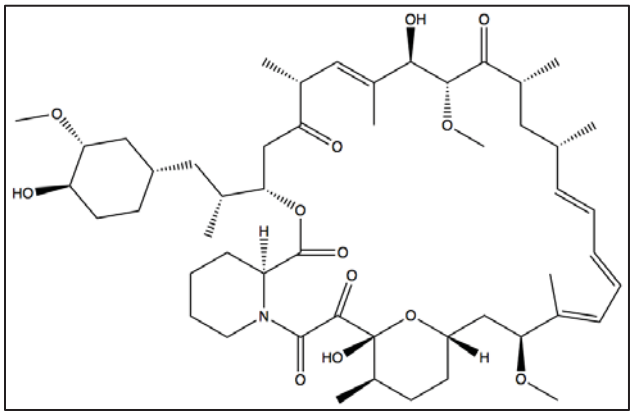

Figure 16: Rapamycin Molecular Structure

Rapamycin functions similarly to FK506 in that it interferes with IL2's activation of T-cell lymphocytes and binds to FKBP to form an inhibitory complex, but instead of precluding the production of the cytokine by intercepting the T-cell pathway between the G_0 to G_1 phases it intercepts the pathway further downstream at the end of the G_1 phase.[49,51] While mTOR's function as a kinase is well documented how the compound participates in T-cell lymphocyte activation and therefore, how Rapamycin suppresses it is only speculative.[53,56]

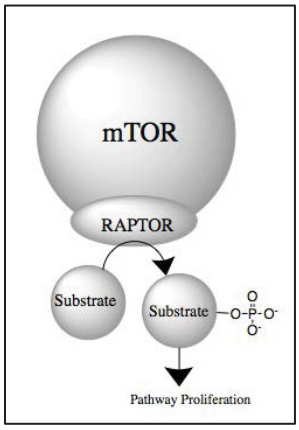

Figure 17: Uninhibited functioning of mTORc1

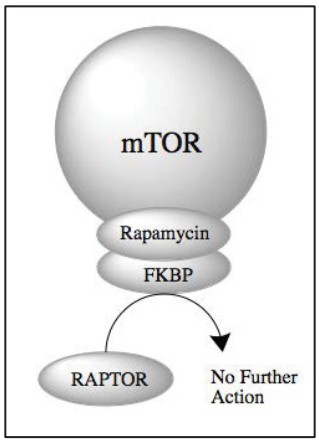

Figure 18: Inhibition of mTORc1 formation by Rapamycin-FKBP

Proposal: Islet Transplantation and Slow-Release Immune Suppression

As discussed previously, methods are being developed for immune suppression utilizing modification of the transplanted pancreatic islets. The following paper outlines a proposal for an alternative method of immunosuppression— the immobilization of immunosuppressant drugs through bioconjugation with a slow release, ß-eliminative cleavable linker that releases the free, active immunosuppressant molecule into a localized region of the body. The released molecules can then exert their normal effects on their target molecules, Calcineurin or mTOR for FK506 or Rapamycin, respectively. By modifying the immunosuppressant drugs to afford slow release, a single large dosage could theoretically be administered and the resulting activity prolonged. This would give an immunosuppressive effect equivalent to multiple small dosages of the drugs. Furthermore, reduction of the number of active immunosuppressant drugs in the system at any single point in time reduces the potential for toxic effects from these immunosuppressants.

CHAPTER TWO

Current Modifications Known for FK506 and Rapamycin

FK506 Modifications

FK506 is known to have potent immunosuppressive properties but is also known to be toxic, potentially leading to the development of Calcineurin Inhibitor Nephrotoxicity (CNI) which can cause permanent damage to renal functions.[57] Synthesis of chemical derivatives of FK506, which retain immunosuppressive properties but lack nephrotoxic properties are therefore of great interest to transplant medicine. Chemical derivatives of FK506 have also been generated for dimerization functions rather than immunosuppression.[58]

The two most commonly encountered methods of generating FK506 chemical derivatives are olefin cross-metathesis of the C_{21} allyl group (sometimes referred to as the C_{39} terminal alkene depending on the specific paper's convention) and esterification of the C_{32} hydroxyl group.

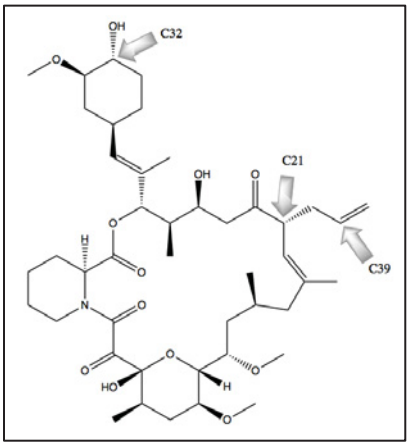

Figure 19: Structure of FK506 with common modification locations labelled

Allyl Modifications

Most olefin cross-metathesis reactions produce highly stable products, but in instances of large-molecule cyclization with second generation ruthenium carbene Grubb's Catalyst complexes (those with a 1,3-Dimesityl-4,5-dihydroimidazol-2-ylidene substituent in the place of one of the PCy_3 groups) reactions with products that can be fairly easily reverted to their initial states have been observed and utilized for retrosynthesis.[59,60] While some modifications of FK506 do utilize second generation Grubb's catalysts, these reactions result in an alkylation (R-group addition) to the end of the terminal alkene rather than a cyclization.[61] Allyl modifications of FK506 can thereby be considered irreversible in the absence of a catalyst.

The addition of a substituent to the C_{40} position on the terminal alkene is typically done in a dichlorinated solvent and uses a substituent that also contains a terminal alkene, a Grubbs catalyst (either first or second generation), and either heat or microwaves as a driving force for the reaction (Figure 20).[61,62]

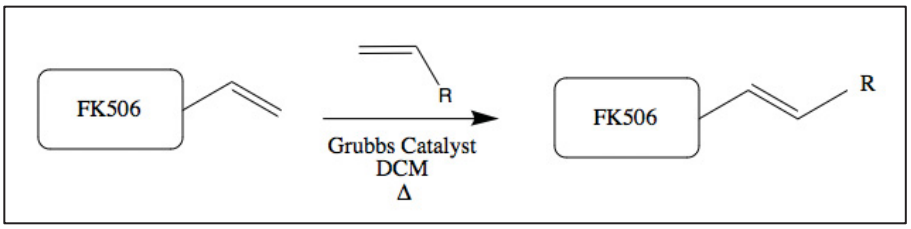

Figure 20: Basic olefin cross-metathesis modification of the C21 allyl of FK506 for the selective substitution at C40

These modifications have been shown to have no effect on the complexation of FK506 to FKBP, cell membrane permeability, or solubility.[62] This makes it a good candidate for immobilization techniques. However, FK506's allyl group has been shown to be part of the effector region (the region responsible for binding) of the FK506-FKBP

complex to Calcineurin.[63] Modifications of the allyl group have therefore been shown to decrease the efficiency in inhibiting Calcineurin, likely due to the effects of steric hindrance.[62] This drastically reduces or eliminates any immunosuppressive functions of FK506, its primary purpose.

C_{32} Hydroxyl Modifications

Another common modification point on FK506 is the C_{32} hydroxyl group. This hydroxyl is located on the cyclohexane ring at the periphery of the molecule, making it easily accessible for reaction purposes. There are three hydroxyl groups in the FK506 structure, however, the C_{32} hydroxyl is almost exclusively modified, likely due to having less steric hindrance. Complete removal of the C_{32} hydroxyl has been shown to completely eliminate immunosuppressive activity, though the exact reason for this is not yet known.[64] The cyclohexane ring has been indicated to be part of the Calcineurin effector region of FK506, which may be the source of this decrease in immunosuppression.[63] A better understanding of the specific interactions lost or gained with the removal of the hydroxyl would provide better insight into steric or bonding effects needed for the generation of possible chemical derivatives with intact immunosuppressive abilities.

This modification has been achieved previously by reacting the desired substituent, which typically has a carboxylic acid functional group, with the coupling reagent phenylphosporochloridate-DMF (PhO(O)PCl2·DMF) to generate an intermediate with a reactive phosphorodichloridic anhydride structure, then coupling it directly to FK506 using the weak base trimethylamine as depicted in the figure below.[65]

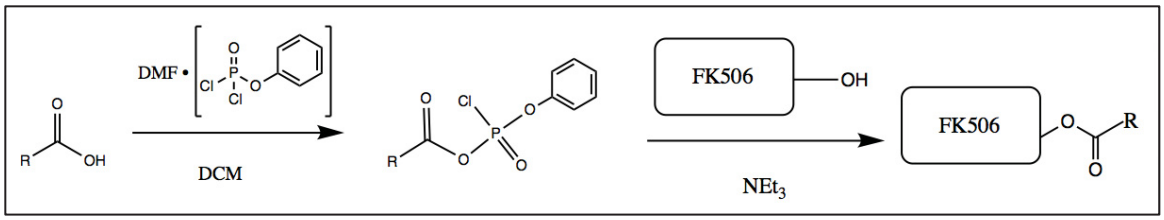

Figure 21: Basic depiction of the esterification of FK506's C32 hydroxyl group

Rapamycin Modifications

Rapamycin is widely known to have potent immunosuppressive properties, and several chemical derivatives of Rapamycin exhibit them as well. The most commonly encountered chemical derivatives of Rapamycin are the resulting products of esterification of the hydroxyl groups, specifically those on C_{40} (or C_{42}, depending on the specific paper's numbering convention) and C_{31} (or C_{28}, also dependent on numbering convention)

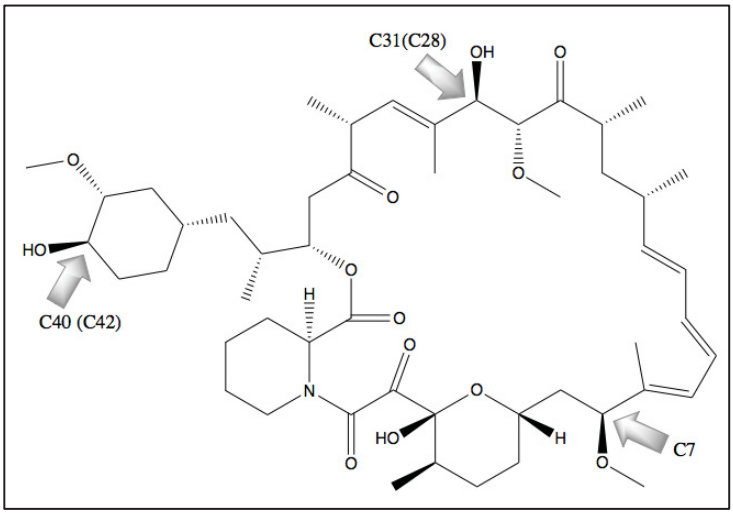

Figure 22: Structure of Rapamycin with common modification locations labelled

C_{40} Hydroxyl Modification

The C_{40} hydroxyl is the most reactive of the three Rapamycin hydroxyls, which is likely due to its lack of steric hindrance relative to the others.[66] Crystal structure analysis

of the Rapamycin/FKBP-mTOR complex has indicated that the C_{40} hydroxyl is involved in the binding of Rapamycin to the FKBP12-Rapamycin binding domain (FRB) of mTOR via hydrogen bonding.[67] In order to retain the immunosuppressive properties of Rapamycin, modifications at this position would therefore likely need to provide an alternative means of hydrogen bonding to FRB. Modifications at this position are typically achieved by esterification in a DCM solution using a carboxylic acid and a coupling agent such as dicyclohexyl carbodiimide (DCC) as shown in Figure 23 .[65,68]

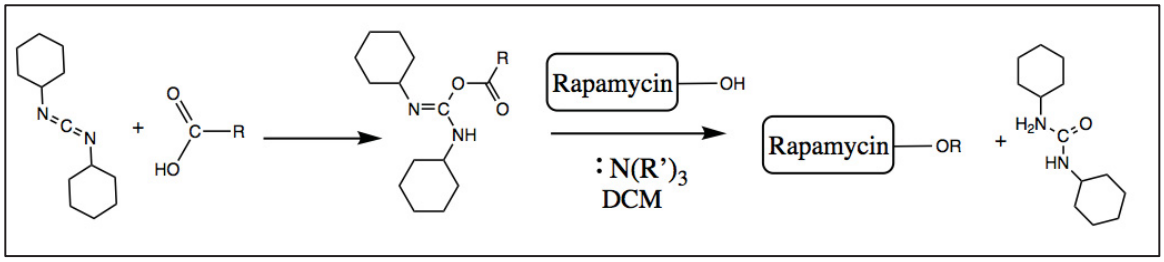

Figure 23: Basic depiction of a Rapamycin C40 hydroxyl esterification

There have been studies conducted on the attachment of photo-cleavable linkers at the C_{40} hydroxyl position which have provided good possible avenues for modification as well. It has been indicated that modification of the C_{40} hydroxyl with either a hydrophilic substituent or a large substituent, such as a macromolecule with a photo-cleavable linker, prevents the movement of Rapamycin into the cell and therefore any immunosuppressive functions.[68] In addition, some of the examined photo-cleavable linker modifications released an unaltered, active form of Rapamycin upon cleavage with ultraviolet irradiation, making it an area of interest for further study.[69]

C_{31} Hydroxyl Modification

Another, less common, hydroxyl modification of Rapamycin is that of the C_{31} hydroxyl. As previously mentioned, this hydroxyl group is less reactive than the C_{40} hydroxyl so in order to effectively modify the C_{31} hydroxyl without altering the more reactive C_{40} hydroxyl as well, the C_{40} must be protected prior to the modification. A selective-substitution study achieved this modification by strategically shielding the C_{40} and C_{31} hydroxyls with trimethylsilyl (TMS) and tert-butyldimethylsilyl (TBDMS) followed by hydrolysis with acid to direct the substitution towards the less reactive group (Figure 24).[66]

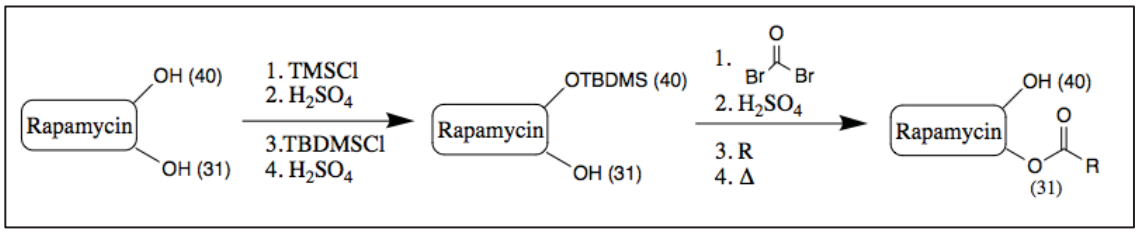

Figure 24: [66] Selective modification of the C_{31} hydroxyl using shielding groups and acid workups

There is very little information regarding the effects of substitution at the C_{31} hydroxyl. The selective substitution study mentioned above reported that even with the substitution of bulky quaternary ammonium salts, the chemical environment of the rest of the molecule was relatively unaffected and that C_{31} modifications increased aqueous solubility more than modifications at the C_{40} hydroxyl.[66]

C_7 Methoxy Modification

In addition to the hydroxyl groups, the methoxy groups of Rapamycin can be modified as well. There are two methoxy groups in the Rapamycin structure, however the

group at C_7 is far more reactive than that at C_{29} because the modifications proceed through an S_N1 mechanism and the triene π-system adjacent to the C_7 methoxy is able to participate and delocalize the positive-charge of the mechanism's intermediate.[70,71] This charge delocalization, however, opens up the possibility for a substitution at any of the carbons that are a part of the π-system. The C_7 methoxy group of Rapamycin is part of the effector domain for binding to FKBP, yet it does not make direct contact with the FKBP binding domain and is therefore not vital for binding.[70] This allows for some variation within the substituents without significantly compromising the affinity of the molecule for FKBP so long as the substituents are not hydrophobic and sterically hindering.[70]

Similar observations have been made regarding C_7-modified Rapamycin's binding ability towards mTOR. Crystal structures of the FKBP-Rapamycin molecular complex have indicated that the C_7 methoxy group is in a region close to the mTOR binding domain, meaning again that sterically hindering substituents could inhibit binding of the complex to mTOR.[71] This in turn would therefore eliminate any immunosuppressive effects. The C_7 methoxy position is also interesting in that its modification can undergo either esterification or alkylation. Aromatic ring systems or other electron-rich carbon systems must be used as the alkyl group in order to achieve alkylation at this position due to the need for a nucleophilic substituent in the second step of the cationic S_N1 reaction. Modifications at this position have been achieved previously by directly reacting a Rapamycin molecule with a nucleophilic-group containing substituent and using trifluoroacetic acid (TFAA) as a catalyst (Figure 25).[70]

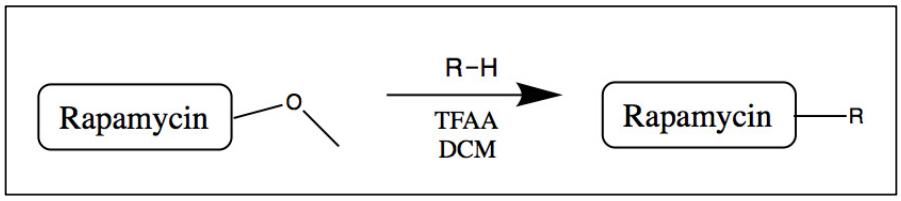

Figure 25: Modification of the C_7 methoxy position where R is a nucleophilic carbon

CHAPTER THREE

Proposal for FK506 and Rapamycin Modifications for Long-Term Immunosuppression

My proposal is the deactivation of FK506 and Rapamycin by covalently attaching a sterically-hindering β-eliminative cleavable linker, shown below, to the drugs via a hydroxyl-esterification reaction. The linker possesses a terminal azide which allows for the possibility of subsequent bioconjugation of the linker-drug complex to the surface of pancreatic islets for a localized slow release as the linker cleaves.

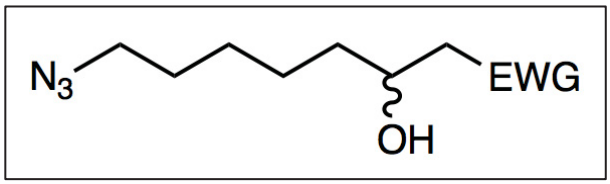

Figure 26: The proposed β-eliminative cleavable linker with a generic electron withdrawing group modulator

Final Synthesis Strategies for FK506 and Rapamycin Drug-Linker Complexes

While there are many possible strategies for the modification of FK506 and Rapamycin with a β-eliminative cleavable linker, not all possibilities are equal in their reaction complexity, selectivity, and overall likelihood of success. All factors considered, the best strategies for the modification of FK506 and Rapamycin appear to be those involving the C_{32} hydroxyl and C_{40} hydroxyl, respectively, on each of the compounds—more simply, the cyclohexane hydroxyls of the respective compounds.

Rapamycin

The best option for the successful installation of a β-eliminative linker on Rapamycin is using the C_{40} hydroxyl as the site of modification. This position offers easy accessibility and reactivity due to its position on the outermost parts of the structure. This modification can be done in one of two ways, forming either a carbonate linkage between the two structures, or a carbamate. Each of these options has its own set of advantages and disadvantages.

Carbonate Linkage Method. To bond the linker molecule to the Rapamycin hydroxyl, the linker itself must first be modified to make it labile for reaction. This can be done by adding a triphosgene to the linker at its own hydroxyl, generating a chlorocarbonate. The chlorocarbonate should then be able to be directly reacted with the Rapamycin hydroxyl to form a carbonate linkage.

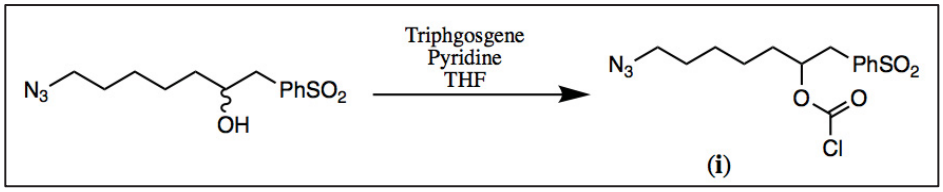

Figure 27: Modification of the cleavable linker to generate a reactive chlorocarbonate substituent

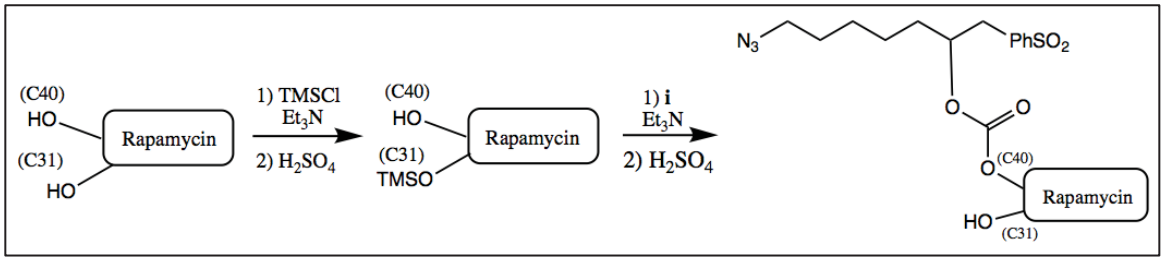

Figure 28: Modification of Rapamycin with the β-eliminative cleavable linker

Rapamycin has three hydroxyls, and though the cyclohexane hydroxyl is more reactive than the other two in the structure, there is still a moderate risk that the linker will complex with one of the unintended hydroxyls, most likely the one at the C_{31} position. This potential problem can be easily avoided by shielding the hydroxyls using TMSCl followed by reaction with a small amount of low-concentration H_2SO_4 to regenerate the hydroxyl at the most reactive position, the C_{40}. This solution, unfortunately, presents another potential problem in itself—the hydrolysis of the carbonate.

After the modifications to the C_{40} hydroxyl have been made, the TMS protecting groups that were added to the other two hydroxyls must be removed. This is easily done by treating the molecule with H_2SO_4, however, in this case, there is also a potential for the acid to react with the carbonate and cleave the linker from the Rapamycin. The carbonate should theoretically be considerably more stable than the TMS ether and therefore the H_2SO_4 should not pose a problem, however, if it is found that acid hydrolysis of the carbonate is in fact occurring, careful regulation of the amount of H_2SO_4 added may prevent undesired cleavage of the Rapamycin from the linker.

Carbamate Linkage Method. The carbamate-forming reaction is far more complex, both in the workups required for the linker molecule and for the Rapamycin itself. Carbamate linkages on β-eliminative linkers have been previously formed by reacting a carbonate ester containing a terminal NHS group with an amine functional group on the target drug.[72] The desired Rapamycin modification position, however, has a hydroxyl rather than an amine making this methodology invalid for our purposes without some alteration. In order to generate a carbamate linkage, a nitrogen-containing group must be added to either the Rapamycin hydroxyl or the carbonate of the linker. As the intention of

the Rapamycin modification is to eventually release the drug in its original form, the nitrogen for the carbamate linkage must be added to the linker rather than the drug. This has been done before by adding a para-disubstituted benzyl amine to the carbonyl generated on the linker, then adding a chloromethyl residue to the nitrogen to generate a reactive position (Figure 29).[28]

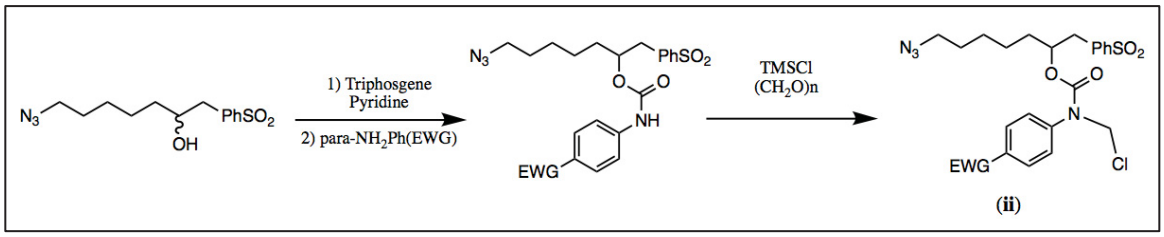

Figure 29: Modification of the β-eliminative cleavable linker with a para-disubstituted benzyl amine and methyl chloride reactive site

This linker modification, unfortunately, is not sufficient for the carbamate linkage formation. It may be necessary to deprotonate the Rapamycin hydroxyl to generate a nucleophile strong enough to replace the chloromethyl group. Lithium di-isopropyl amide (LDA), sodium hydride, K_2CO_3, or another strong base should be sufficient to achieve this.

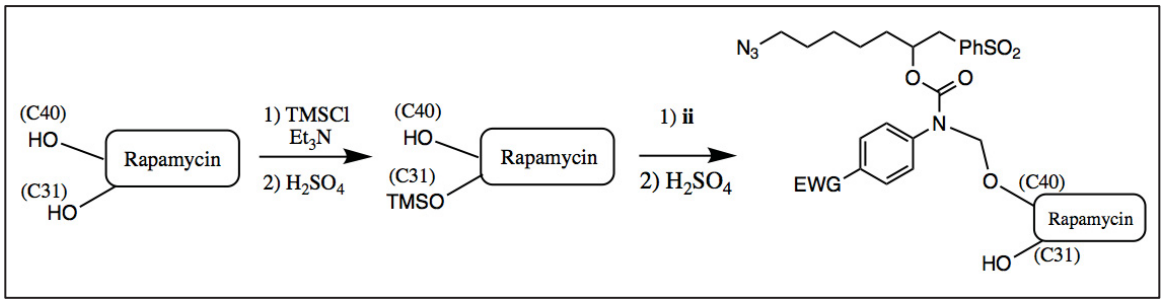

Figure 30: Shielding, deprotonation, and binding of Rapamycin with a modified linker to form a carbamate linkage

While the carbamate method is considerably lengthier and more involved than the carbonate method, there are benefits to utilizing the technique, most importantly, likelihood of success. The successful formation of carbamate linkages with similar β-eliminative linkers has been previously published by Santi et. All along with kinetic data for the cleavage of the linkages, but formation of carbonate linkages with β-eliminative linkers has not been thoroughly explored, and therefore the success of the method is only speculative.[29]

The carbamate method has the same potential pitfalls as the carbonate method. There is a small risk that the Rapamycin hydroxyl modification can occur at the unintended C_{31} hydroxyl, therefore, like with the carbonate method, the undesired hydroxyl positions can be shielded with TMSCl prior to the reaction with the linker and, in this case, prior to the hydroxyl deprotonation step. As for removal of the TMS protecting groups, same as with the carbonate method, the possibility of the linkage being hydrolyzed by the H_2SO_4 is minimal but can be effectively avoided by carefully managing the concentration of the acid added.

FK506

The most promising modification site on the FK506 structure is the cyclohexane C_{32} hydroxyl. Though distinct in their functional targets, Rapamycin and FK506 have several similar elements, one of which is the cyclohexane moiety with this hydroxyl group. Like the cyclohexane hydroxyl on Rapamycin, the cyclohexane hydroxyl of FK506 offers a sterically-unencumbered reaction site that is more reactive than the others in the molecule. This structural similarity allows the C_{32} hydroxyl of FK506 theoretically to be modified following the exact same modification procedures used for the C_{40} hydroxyl of Rapamycin,

including the linker workups for the respective methods. For these reason, only the drug-modification schemes are shown below. This parallel in synthesis strategies between Rapamycin and FK506 also results in the same pitfalls and solutions for the FK506 reactions as were addressed in the Rapamycin section above.

Carbonate Linkage Method.

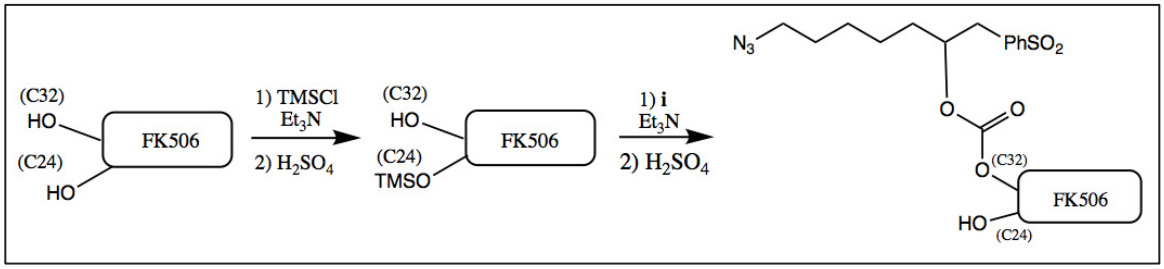

Figure 31: Synthesis of a FK506-linker complex via the generation of a carbonate linkage

Carbamate Linkage Method.

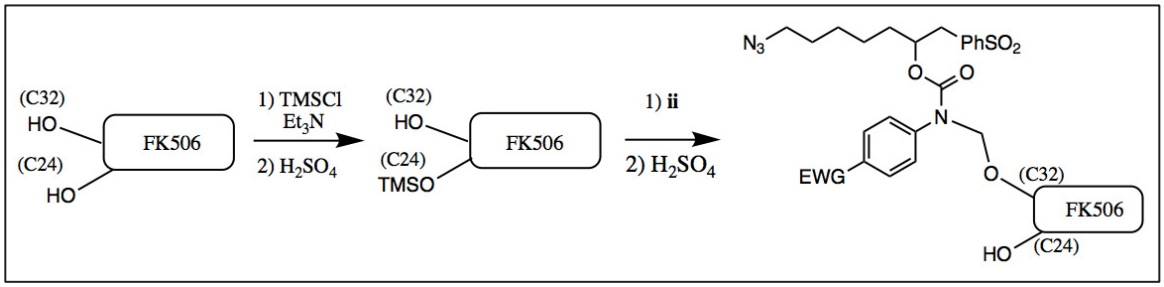

Figure 32: Synthesis of the drug-linker complex via the generation of a carbamate linkage

Characterization of the Modified Rapamycin and FK506 Complexes

Once the procedures for the modifications to Rapamycin and FK506 have been performed, characterizations must be done to confirm the success of the modification. The first element of the modification that must be confirmed to be successful is the linkage of each of the drugs with the ß-eliminative linker.

Complexation Confirmation Characterization

Confirmation of the modification can be achieved used a fluorescence assay. This assay could be easily prepared by covalently attaching cyclooctyne-containing substituents to the surface of agarose beads via an amine or PEG-phospholipid. The cyclooctyne substituents then have the capability able to undergo strain-catalyzed copper-free cycloaddition click chemistry with the azide of the ß-eliminative linker and covalently bond the complex to the agarose bead. Prior to the addition of the complex, however, further modifications must be done to the drug.

For the purposes of the fluorescence test on the assay, a fluorophore must be added to the drug. As the addition must be stable enough to resist reaction during the proposed methods to add the ß-eliminative linker, alkylation is the best option. The C_{24} allyl of FK506 and the C_7 methoxy group of Rapamycin each are able to alkylate upon modification, making these positions the best candidates for the covalent attachment of a fluorophore. Specifics on how to achieve alkylation at these positons are discussed in the previous chapter. After the fluorophore has been added, the drug can then be treated as if it were the original compound, and then modified with the ß-eliminative linker using the modification proposals.

Once the linker-drug-fluorophore complex is synthesized, the complex can then be bonded to the beads using the click chemistry cycloaddition. The beads can then be washed to remove any compounds not bound to the beads and tested for fluorescence. As the fluorophore is bound to the drug rather than the linker, presence of fluorescence is indicative of a successful complexation between the drug and the ß-eliminative linker.

Inhibition of Immunosuppressive Activity of the Complex Confirmation

Confirmation of the inhibition of immunosuppressive properties of the modified drugs can be achieved through the utilization of activity assay kits which can be purchased from almost all biotechnology or laboratory equipment providers.

CHAPTER FOUR

Further Considerations: Immobilization and Timeframe Extensions

While the proposed method inactivates FK506 and Rapamycin, it does not rigidly restrict its movement within the body, making localized treatment difficult. A possibility to remedy this issue is through the partnering of islet-surface modifications with the proposed drug modifications.

Installation of a PEG-phospholipid anchor with a cyclooctyne-containing substituent onto the islets prior to implantation would help to remediate this issue. The cyclooctyne could then be reacted with the azide group of the linker in a strain-promoted cycloaddition, immobilizing the drug in an inactive form until the β-elimination ultimately cleaved the drug off in an unmodified form. Once free in its unmodified from, the drug can then go on to exert its normal immunosuppressive effects of T-cell pathway inhibition.

As the strength of the electron withdrawing group modifier on the β carbon of the linker determines the half-life of the carbonate or carbamate linkage prior to cleavage, it may be beneficial to generate multiple Rapamycin-linker complexes with different strength electron withdrawing group modifiers to provide more varied release rates. At physiological conditions some modifiers have been indicated to have half-lives that are over a year-long (10,500 hours) as is the case with the modifier $(Et)_2NSO_2$, others, as short as 14 hours as is the case with CF_3PhSO_2.[29] Theoretically, this method could to provide same-day, effective, immunosuppression which lasts well over a year.

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