2006

ISOPRENOID ANALOGS AS CHEMICAL GENETIC TOOLS TO PROVIDE INSIGHTS INTO FARNESYL TRANSFERASE TARGET SELECTION AND CELLULAR ACTIVITY

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ABSTRACT OF DISSERTATION

Jerry Troutman

The Graduate School

University of Kentucky

2006
ISOPRENOID ANALOGS AS CHEMICAL GENETIC TOOLS TO PROVIDE INSIGHTS INTO FARNESYL TRANSFERASE TARGET SELECTION AND CELLULAR ACTIVITY

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy in The College of Medicine at the University of Kentucky

By
Jerry Troutman
Lexington, Kentucky

Director: Dr. H. Peter Spielmann, Associate Professor, Molecular and Cellular Biochemistry
Lexington, KY

2006

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ABSTRACT OF DISSERTATION

ISOPRENOID ANALOGS AS CHEMICAL GENETIC TOOLS TO PROVIDE INSIGHTS INTO FARNESYL TRANSFERASE TARGET SELECTION AND CELLULAR ACTIVITY

Protein farnesylation is an essential post-translational modification required for the function of numerous cellular proteins including the oncoprotein Ras. The farnesyl transferase (FTase) catalyzed reaction is unique because farnesyl diphosphate (FPP), the farnesyl group donor for the reaction, forms a significant portion of a target protein binding site. The major goal of this research was to exploit this unique property of the FTase reaction and determine if changing the structure of the farnesyl donor group would affect FTase protein targeting. A small library of structural analogues of FPP was synthesized. Michelis-Menten steady-state kinetic analyses and competition reactions were used to determine the effect of these structural modifications on FTase targeting. We found that the analogues did affect FTase protein selectivity and that this could be exploited to induce unnatural target selectivity into the enzyme.

The second goal of this research was to determine the effect of FPP analogues on the function of FTase target proteins. To test the effect of these analogues we determined whether the unnatural lipid could ablate oncogenic H-Ras biological function in a *Xenopus laevis* model system. Several analogues were able to disrupt oncogenic H-Ras function while others mimicked the activity of FPP. These results indicated that some of the FPP analogues may act as prenyl group function inhibitors that could lead to an important new class of anti-cancer therapeutics.

Another major goal of this research was to use the FPP analogues as unnatural probes for the endogenous cellular activity of FTase target proteins. We developed antibodies to two of the unnatural FPP analogues to study their activity in cell culture. Utilizing these antibodies we found that alcohol prodrugs of the FPP analogues could be incorporated into cellular proteins in an FTase dependent manner. The ability of cell permeant analogues to be incorporated into live cells enhances the chances that such a molecule could be used to modify oncogenic cellular proteins with a prenyl group function inhibitor.

KEYWORDS: Protein Farnesyl Transferase, Enzyme Kinetics, Prenyl Transferases, Solid-Phase Organic Synthesis, Hapten Synthesis

Jerry M. Troutman

August 27, 2006
ISOPRENOID ANALOGS AS CHEMICAL GENETIC TOOLS TO PROVIDE INSIGHTS INTO FARNESYL TRANSFERASE TARGET SELECTION AND CELLULAR ACTIVITY

By

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August 27, 2006
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Lexington, Kentucky

2006
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In Memory of Michael Sheehan
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<td>AGPP</td>
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</tr>
<tr>
<td>AdoHCy</td>
<td>Adenosyl homocysteine</td>
</tr>
<tr>
<td>Ado-Met</td>
<td>Adenosyl methionine</td>
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<tr>
<td>AFOH</td>
<td>Anilinofarnesol</td>
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<td>AFPP</td>
<td>Anilinofarnesyl diphosphate</td>
</tr>
<tr>
<td>AG</td>
<td>Anilinogeranyl</td>
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<tr>
<td>AGRas</td>
<td>Anilinogeranylated Ras</td>
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<tr>
<td>anti-AG</td>
<td>Anti-anilinogeranyl polyclonal antibody</td>
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<tr>
<td>anti-NAG</td>
<td>Anti-nitroanilinogeranyl polyclonal antibody</td>
</tr>
<tr>
<td>bs</td>
<td>Broad singlet</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>bt</td>
<td>Broad triplet</td>
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<tr>
<td>CaaX</td>
<td>Cysteine-aliphatic-aliphatic-any residue amino acid sequence motif</td>
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<tr>
<td>CDI</td>
<td>Carbonyl diimidazole</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
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<td>DABCO</td>
<td>1,4-Diazabicyclo[2.2.2]-octane</td>
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<td>DBU</td>
<td>1,8-Diazabicyclo(5.4.0)-undec-7-ene</td>
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<td>DCE</td>
<td>Dichlorethane</td>
</tr>
<tr>
<td>DCM</td>
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<td>DM</td>
<td>n-dodecyl- β-D-maltoside</td>
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<tr>
<td>DMAP</td>
<td>Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>Dimethyformamide</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>Dithiothreitol</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>ESI-MS</td>
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<td>EtOAc</td>
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<tr>
<td>FLU</td>
<td>Fluorescence Units</td>
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<td>Guanosine triphosphate</td>
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<tr>
<td>GTPase</td>
<td>GTP hydrolyzing enzyme</td>
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<td>GVBD</td>
<td>Germinal vesicle breakdown</td>
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<td>Hepatitis delta virus</td>
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<tr>
<td>HEK</td>
<td>Human endocrine kidney</td>
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<td>Hydroxymethyl glutaryl CoA</td>
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<td>Acetic acid</td>
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<td>ppi</td>
<td>diphosphate</td>
</tr>
<tr>
<td>PPTS</td>
<td>Pyridinium $p$-toluenesulfonate</td>
</tr>
<tr>
<td>PT</td>
<td>PBS Tween</td>
</tr>
<tr>
<td>ras</td>
<td>Rat sarcoma viral oncogene</td>
</tr>
<tr>
<td>RhoGDI</td>
<td>Rho GDP dissociation inhibitor</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse phase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SPOS</td>
<td>Solid phase organic synthesis</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethyl amine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoro acetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydofuran</td>
</tr>
<tr>
<td>THP</td>
<td>Tetrahydropyran</td>
</tr>
<tr>
<td>TKCVIC</td>
<td>N-dansylated TKCVIC hexapeptide</td>
</tr>
<tr>
<td>TKCVIL</td>
<td>N-dansylated TKCVIL hexapeptide</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
</tbody>
</table>
CHAPTER 1 BACKGROUND AND OBJECTIVES

Background

Membranes and Lipid Anchoring in Cell Biology

Biological membranes are uniquely organized arrays of lipids and proteins that constitute the boundaries of cells and intracellular organelles [1, 2]. They provide a surface for many important biological processes to take place, and contain proteins that mediate the transport of metabolites, macromolecules and ions. Important biological processes ranging from electron transport to muscle contraction all depend on membranes and membrane proteins. Proteins are the “workhorses” of a cell and often require membrane localization to exert their cellular function. Proteins can interact with membranes in a variety of ways. Peripherally bound membrane proteins interact with polar groups on the membrane surface, through electrostatic interactions; integrally membrane bound proteins are molecules with large hydrophobic regions embedded into lipid bilayers; and a third class of proteins associate with membranes via small lipid groups attached to the protein. Four different types of lipid anchoring motifs have been identified to date (Figure 1-1): amide linked myristoyl anchors, thioester linked fatty acyl anchors, thioether linked prenyl anchors, and amide linked glycosyl phosphatidylinositol (GPI) anchors[3-6].
Figure 1-1 Lipid Anchoring Motifs

Note: GPI anchored proteins have variable length hydrophobic chains
Prenyl anchors are made up of isoprenoids, which are molecules derived from the mevalonate biosynthetic pathway (Figure 1-2) [6-9]. Isoprenoids are formed through a head to tail condensation of isopentyl diphosphate and dimethylallyl diphosphate to give the precursor molecule geranyl diphosphate 1 (GPP) [10-12]. Sequential additions of the isopentyl diphosphate to geranyl diphosphate leads to elongation of the isoprenoid chain to 3 and 4 isoprene units to give farnesyl diphosphate 2 (FPP) and geranylgeranyl diphosphate 3 (GGPP), respectively. FPP can be divided into three isoprene units the first (α) isoprene which is the isoprene unit closest to the diphosphate, then the β-isoprene which is the middle isoprene unit, and the third isoprene which is often referred to as the terminal or ω-isoprene unit. The farnesyl and geranylgeranyl groups of FPP and GGPP are post-translationally added to proteins in a reaction catalyzed by protein prenyltransferase enzymes. The mevalonate biosynthetic pathway is essential for the production of FPP and GGPP as well as other biomolecules including cholesterol, numerous steroid hormones, ubiquinone and dolichol [6].
Figure 1-2 Mevalonate Pathway

Acetyl-CoA → 3-hydroxy methylglutaryl-CoA → Mevalonate

Dimethylallyl diphosphate ↔ Isopentyl diphosphate

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{SCoA} & \quad -\text{O} \quad \text{O} \\
& \quad \text{C} \quad \text{CH}_3 \quad \text{SCoA} \\
& \quad -\text{O} \quad \text{C} \quad \text{CH}_3 \quad \text{OH}
\end{align*}
\]

Geranyl diphosphate 1

Farnesyl diphosphate 2 → Geranylgeranyl diphosphate 3

\[
\begin{align*}
\text{OPP} & \quad \text{OPP} \\
\text{farnesylated proteins} & \quad \text{Squalene} \quad \text{Dolichol} \quad \text{Ubiquinone} \quad \text{geranylgeranylated proteins}
\end{align*}
\]

Cholesterol

Steroid Hormones

Bile Acids

Note: FPP isoprene unit designation (α, β and ω).
Prenylated proteins have received a great deal of attention in recent years due to the fact that the protein product of the ras oncogene requires post-translational attachment of a prenyl group in order to function [13-18]. The ras oncogene is present in 10-30% of all human cancers and has become a major target of anti-cancer therapeutics [19]. Ras proteins are small molecular weight GTPases (Enzymes that bind and hydrolyze Guanosine Triphosphate or GTP) that act as molecular “on/off” switches that control cellular signaling cascades leading to cell proliferation and cell growth [19]. The Ras switching mechanism occurs through the cycling of the protein between a GTP bound “on” state, to a Guanosine diphosphate (GDP) bound “off” state. Ras proteins must be localized to membranes to carry out this crucial molecular switching function, and membrane association requires the attachment of an isoprenoid group. The best characterized Ras signaling pathway is control of the Mitogen Activated Protein Kinase (MAPK) signaling cascade (Figure 1-3) [20-23]. The MAPK signaling cascade can be activated by the binding of an external cellular signal to a Receptor Tyrosine Kinase (RTK) leading to receptor autophosphorylation. Autophosphorylation of the RTK recruits Guanine Nucleotide Exchange Factors (GEFs) to the membrane of a cell [19, 24-27]. GEF proteins assist the exchange of GDP for GTP and activate membrane localized Ras proteins [28-31]. GTP bound Ras then signals the initiation of cell proliferation through effector proteins such as Raf [32-36]. The signal then progresses through a series of phosphorylation events that eventually results in the activation of transcription factors essential for cell division. The Ras proteins are essential to cell survival making them an excellent model protein for studying prenylated protein function. In addition, inhibiting the activity of Ras proteins may be a key mechanism for the inhibition of cancer cell proliferation.
**Figure 1-3 Ras Signaling**

This is a simple Ras signaling network leading to cell proliferation via binding of epidermal growth factor (EGF) to a receptor tyrosine kinase (RTK). The Figure highlights the molecular switching function of Ras from a GDP bound on state to a GTP bound off state. Switching between the two states requires guanine nucleotide exchange factors (GEF), and GTPase activating proteins (GAP).
There are at least four hypothesized roles for the prenyl modification of a protein (Figure 1-4) [37-40]. The best understood is the direct interaction of the isoprenoid group with the lipid bilayer of a cell. However, small isoprenoid anchors such as the 15 carbon farnesyl isoprenoid poorly partition into membranes as evidenced by a 5-40 µM half-maximal binding to liposomes relative to 0.2-0.8 µM binding of a 20 carbon geranylgeranyl isoprenoid moiety [41]. Another potential role of an isoprenoid protein modification may be for the lipid moiety to directly interact with hydrophobic regions of other proteins such as chaperones. One example of this type of activity is the interaction between the small molecular weight GTPase Rac and the Rho GDP dissociation inhibitor (RhoGDI) [42]. Rac proteins are important in cytoskeleton formation, and are prenylated with a 20 carbon geranylgeranyl moiety. The geranylgeranyl moiety inserts directly into a hydrophobic groove in the RhoGDI protein and mediates the interaction between the two proteins. RhoGDI is thought to act as a carrier or transport protein that masks the hydrophobic prenyl group from the cytosolic aqueous environment, and delivers Rac to specific target membrane locations. The lipid groups attached to proteins may also interact with membranes through a mixed lipid:lipid and lipid:protein interaction. Another potential role for prenyl protein modifications may be to induce conformational changes in the modified protein. Such changes in the structure of a protein may lead to new protein:protein interactions with binding partners that would not otherwise interact with the unmodified protein [39]. Other types of protein modifications including phosphorylation and glycosylation have been shown to affect protein structure and folding, and it is possible that prenyl group modification may also support changes in the folding of a modified protein [43, 44]. The different roles of prenyl protein modification may be important for the function and cellular localization of a variety of proteins, and the role of the prenyl modification may differ depending on the protein modified.
Figure 1-4 Models for Isoprenoid Function
Four models for prenyl group function on modified protein.
Protein Prenylation and Processing

The protein prenyltransferases farnesyltransferase (FTase), geranylgeranyl transferase type I (GGTasel) and geranylgeranyl transferase type II (GGTaselII) are heterodimeric zinc metalloenzymes that catalyze the prenylation of proteins. FTase and GGTasel catalyze the transfer of FPP 2 or GGPP 3 to a conserved cysteine residue four amino acids from the C-terminus of target proteins (Figure 1-5) [45-48]. Protein substrates of both FTase and GGTasel contain a Ca1a2X group at their C-terminus, where C is a conserved cysteine residue, a1 and a2 are often aliphatic amino acids and X is frequently methionine, glutamine or serine for FTase and leucine or phenylalanine for GGTasel [49, 50]. However, there are overlapping Ca1a2X substrate specificities for the prenyltransferases, where some canonical GGTasel substrates can be farnesylated by FTase, and visa versa [49, 51-53]. The Ca1a2X motif alone is all that is required for FTase and GGTasel protein recognition and small Ca1a2X peptides often mimic the reactivity of full length proteins [54, 55]. The sequence of the Ca1a2X motif is also often unique to a single protein or subsets of proteins, and the reactivity of different Ca1a2X sequences varies from one sequence to another [56].
Figure 1-5 Prenyltransferase Reaction and Downstream Processing

PROTEIN

\[
\begin{align*}
&\text{NH} \\
&\text{HS} \\
&\text{Xaa}
\end{align*}
\]

FTase (cytoplasm)

\[
\begin{align*}
&\text{FPP} \\
&\text{PPI}
\end{align*}
\]

PROTEIN

\[
\begin{align*}
&\text{NH} \\
&\text{S} \\
&\text{Xaa}
\end{align*}
\]

RCE1 (endoplasmic reticulum)

\[
\begin{align*}
&\text{aaX}
\end{align*}
\]

PROTEIN

\[
\begin{align*}
&\text{NH} \\
&\text{S} \\
&\text{O}
\end{align*}
\]

ICMT (endoplasmic reticulum)

\[
\begin{align*}
&\text{Ado-Met} \\
&\text{AdoHcy}
\end{align*}
\]

PROTEIN

\[
\begin{align*}
&\text{NH} \\
&\text{S} \\
&\text{O} \\
&\text{H}_3\text{CO}
\end{align*}
\]

MEMBRANE LOCALIZATION
Protein prenylation directs an ordered series of processing events (Figure 1-5) to further increase hydrophobicity leading to the eventual localization of a modified protein to cell membranes [57-60]. Through a currently unidentified mode of transport, a target protein is prenylated then directed to the endoplasmic reticulum (ER). At the ER the protein undergoes two additional processing events. The first of these events is the cleavage of the a1a2X residues of the Ca1a2X sequence motif. The endoproteolysis of the protein is catalyzed by the integrally membrane bound Ras converting enzyme, RCE1 [58, 61-64]. Following the Ca1a2X cleavage reaction the now prenylated C-terminal cysteine residue remains associated with the ER. The carboxyl terminus is then methylated by a second ER bound enzyme Isoprenylcysteine carboxymethyl transferase (ICMT) [59, 60, 65, 66]. The ICMT enzyme donates a methyl group from S-adenosyl methionine (Ado-Met) to the carboxyl terminal residue. The reaction results in the formation of the hydrophobic methylated carboxyl terminus and adenosyl homocysteine (AdoHCy). A prenylated Ca1a2X motif is the minimum requirement for recognition by RCE1, and a prenylated cysteine residue is the minimum requirement for protein recognition by ICMT. Once the protein has been methylated it is then transported to cell membranes. Carboxyl group methylation and endoproteolysis of the protein is thought to increase the hydrophobicity of the C-terminus to enhance direct lipid:lipid interactions with membranes or hydrophobic interactions with protein chaperones. Once an isoprenylated protein has reached the target membrane it may undergo further post-translational lipid modification reactions [67-70]. Acyl transferases can attach palmitoyl hydrocarbon chains to further increase the protein hydrophobicity, but this modification is not essential for the membrane localization of all isoprenylated proteins.

**Protein Prenyltransferase Reaction Mechanism**

FTase and GGTaseI appear to have similar unexpectedly complex reaction mechanisms (Figure 1-6) [51, 55, 71-82]. Using steady-state kinetic analyses FTase was initially identified to follow a random ordered binding of the isoprenoid and Ca1a2X substrates where both FPP and the Ca1a2X substrate could bind to the free FTase enzyme [55]. However, the FTase reaction showed significant substrate inhibition by the Ca1a2X substrate making the standard Michaelis-Menten interpretations of the enzyme
activity difficult. Later using isotope trapping experiments, the reaction mechanism was found to progress preferentially with FPP binding first to the free enzyme (E$\rightarrow$E•FPP) followed by the Ca$_1$a$_2$X substrate (E•FPP$\rightarrow$E•CaaX) [72]. In these experiments an E•FPP complex was formed by mixing FTase and tritiated FPP at stoichiometric concentrations. Excess Ca$_1$a$_2$X peptide and a large excess of unlabeled FPP were then added to the “hot” E•FPP complex. The incorporation of radiolabel into product was then measured, and nearly the entire reaction product (>90%) was tritiated. To test the ability of the E•CaaX complex to form product a biotinylated Ca$_1$a$_2$X peptide was mixed stoichiometricly with FTase. The E•CaaX complex was then treated with tritiated FPP and a large excess of unbiotinylated Ca$_1$a$_2$X peptide. In these reactions very little of the tritiated product was biotinylated (<5%). These results suggested that the E•CaaX complex was unreactive, or at the very least dissociated rapidly allowing FPP to bind to the free enzyme followed by the excess unbiotinylated peptide. The preferred reaction pathway was therefore, through FPP binding first to the enzyme followed by the Ca$_1$a$_2$X substrate.
Figure 1-6 FTase Mechanism

a) FTase kinetic pathway where E is the FTase enzyme, E•FPP is the FTase•FPP complex, E•FPP•CaaX is the FTase•FPP•CaaX peptide complex, E•Product is the FTase bound product complex, E•Product•FPP is the FTase bound to both FPP and the reaction product, and E•CaaX is the peptide bound FTase inhibitory complex. b) Crystal structures published by Long et al. [80] of the individual steps along the FTase reaction pathway. Only substrates are shown other than the free E complex with individual kinetic constants provided for each step of the pathway. The kinetic constants are for a CVIM peptide and FPP as the isoprenoid donor.

a)

\[
\begin{align*}
E & \underset{[FPP]}{\rightleftharpoons} E-FPP & \underset{[CaaX]}{\rightleftharpoons} E-FPP-CaaX & \rightarrow E-Product \\
& & & \downarrow \text{[FProduct]} \\
& & & \text{E-Product-FPP} \\
E-CaaX
\end{align*}
\]

b)

Note: crystal structure diagram from Long et al. [80]
FPP was later found to have a much higher affinity for the free FTase enzyme (Kd=5nM) than a Ca₁a₂X peptide (Kd=4µM GCVLS) [54]. FTase and GGTasel are α,β heterodimers with identical α subunits, but vary in the amino acid composition of the β subunit. FPP binds along one side of a hydrophobic cavity on the FTase β-subunit made up of the highly conserved aromatic residues Trp303β, Tyr251β, Trp102β, Tyr205β and Tyr200α [83-85]. When FPP was replaced with a non-hydrolyzable analog (Figure 1-7, 4) and mixed with FTase to form a non-reactive E•FPP complex, the analog bound to the same region of the FTase enzyme [83-85]. Surprisingly, the affinity for the Ca₁a₂X peptide to the E•FPP complex was increased up to 70 fold (Kd=58nM) relative to the free enzyme [76]. These results indicated that the isoprenoid directly interacted with the peptide target of the FTase reaction, and the isoprenoid increased the peptide binding affinity. Later, Beese et al. solved the crystal structure of a ternary complex containing FTase, a non-hydrolyzable FPP analog and a Ca₁a₂X peptide (E•FPP•CaaX) [49, 86]. The non-hydrolyzable analog bound to the enzyme with the same configuration as found in the crystal structure of the E•FPP complex. In the E•FPP•CaaX crystal structure the isoprenoid formed a significant portion of the binding site for the target protein Ca₁a₂X sequence (Figure 1-6). Interestingly, a similar interaction was found for the GGTasel enzyme, with a non-hydrolyzable GGPP analog and a canonical GGTasel target Ca₁a₂X sequence [49, 87].
Figure 1-7 Inhibitors of FTase, RCE1 and ICMT

**FARNESYL TRANSFERASE INHIBITORS**

- Non-hydrolyzable FPP analog, 4
- Tipifarnib, 5
- Lonafarnib, 6

**RAS CONVERTING ENZYME INHIBITORS**

- RPI 7
- BFCCMK 8
- TLCK 9

**ISOPRENYLCysteine Methyl Transferase INHIBITORS**

- AFC 10
- 3-isobutenylAFC 11
- Cysmethyl 12
FTase and GGTaseI have identical α subunits (48 kDa), but differ in the composition of the β-subunit (25% identity, 46 and 43 kDa respectively) [88]. To determine the structural parameters that affect Ca₁a₂X sequence binding, the crystal structures of different Ca₁a₂X sequences bound to an E•4•CaaX complex were solved (4 was the non-hydrolyzable FPP analog in Figure 1-7 and was extended by one isoprene unit for GGTaseI) [49]. FTase to GGTaseI Ca₁a₂X discrimination was highly dependent on the identity of the Ca₁a₂X sequence X residue. The X residue binds to what is referred to as the specificity pocket of the protein prenyltransferase enzymes. The specificity pocket is made up primarily of the β-subunit residues Ala98β, Ser99β, Trp102β, His149β, Ala151β, and Pro152β of FTase and Thr49β, His121β, Ala123β, Phe174β of GGTaseI. Interestingly, the X binding site of the GGTaseI enzyme also included the 4ᵗʰ isoprene of GGPP. A second X residue binding site was found for FTase with Ca₁a₂X sequences that are normally considered substrates for GGTaseI. The second binding site in FTase was made up of Leu92β, Ser99β, Trp102β, Ala151β, the third isoprene of FPP, and the Ca₁a₂X sequence a₂ residue. This second binding site in FTase allowed for some of the overlap in the binding specificity of the two enzymes. The a₂ binding site of both FTase and GGTaseI accept the same Ca₁a₂X sequence a₂ residues. The a₂ residue binding site in FTase was made up of the ω-isoprene of FPP and the aromatic residues Trp102β, Trp106β and Tyr361β. The a₂ residue binding site of GGTaseI was made up of the 3ʳᵈ and 4ᵗʰ isoprenes of GGPP, Thr49β, Phe53β and Leu320β. The peptide binding sites of both prenyltransferase enzymes were made up of the terminal isoprene units of FPP and GGPP interacting directly with the terminal Ca₁a₂X residues of the prenylation target.

The chemical step of the FTase reaction is thought to primarily proceed through an S₉₂ addition of the zinc activated thiol to the C1 atom of the isoprenoid donor [89, 90]. However, there is some evidence suggesting dissociative character in the isoprenoid diphosphate suggesting at least a partial S₉₁ contribution [91]. Crystal structures have been solved representing the E•4•CaaX and E•product complexes of both FTase and GGTaseI (Figure 1-6, FTase) [80, 87]. These structures suggest that the α-isoprene and β-isoprene unit of FPP and GGPP undergo a significant repositioning in the active site to place the C1 atom in close proximity to the zinc
activated thiol of the target Ca₁a₂X sequence. Interestingly, the ω-isoprene of FPP and GGPP is in the same position throughout the prenyltransferase reaction.

The overall rate of the prenyltransferase reactions are dependent on the rate of product release from the enzyme [72]. After the chemical step of the reaction the resulting product remains bound to the enzyme active site. An unusual feature of the protein prenyltransferase reaction mechanism is that, in order for product to be released from the enzyme, a new substrate molecule must bind to displace it [74]. Studies have shown that both FPP and a Ca₁a₂X peptide can stimulate product release from FTase. However, FPP stimulated release is faster than peptide stimulated, and the general consensus is that FPP stimulated product release is the pathway primarily responsible for product dissociation from the enzyme. A crystal structure of FTase bound to both a farnesylated peptide and an FPP molecule (Figure 1-6) has revealed that the prenyl chain of the product is displaced from the active site onto a hydrophobic groove on the outer surface of the protein (“exit groove”) [80]. The new FPP binds to the active site in the same position previously occupied by the farnesyl moiety of the product in the E•product complex. The hypothesized consequence of FPP stimulated product release is that the free enzyme does not form after the first turnover of the reaction. Instead, the E•FPP complex is formed as a direct consequence of product release [74].

**Farnesyl Transferase Inhibitors**

The importance of oncogenic Ras proteins and other prenylated proteins in cancer biology has led to the development of numerous farnesyl transferase inhibitors (FTI) to block prenylated protein membrane localization and activity [13, 14, 92-98]. Several of these inhibitors are currently undergoing phase I, II, and III clinical trials as anti-neoplastic agents [15, 99]. The FTI Tipifarnib 5 (Figure 1-7) has been evaluated in phase III clinical trials and shows promise in the treatment of blood and breast malignancies [15, 94, 100-102]. Crystal structures of Tipifarnib and other FTIs (Lonafarnib 6) bound to FTase show a similar binding pattern. The molecules bind as a ternary complex with FPP already bound to the enzyme [102-105]. Extensive Van der Waals contacts are made between the FTI and the FPP lipid indicating an ordered binding inhibition mechanism where the inhibitor binds to the E•FPP complex not the free enzyme. The inhibition mechanism was therefore exactly analogous to the ordered
binding mechanism of the FTase enzyme substrates. Six potent FTIs bound to FTase have been crystallized and are positioned in such a way that the aromatic rings of the inhibitor interact directly with the $a_2$ binding site of the enzyme. The inhibitors form face-on-face or edge-on-face stacking interactions with one or more of the aromatic $a_2$ binding site residues. These aromatic stacking interactions are thought to provide the binding energy required for the non-peptidomimetic inhibitors to efficiently bind and inhibit the FTase enzyme.

FTIs have shown considerable activity in a number of clinical trials. However, the overall response in patients has been less than hoped for [106-108]. One possible explanation for the lack of FTI clinical efficacy is the process of alternative prenylation where some FTase substrates can become alternatively prenylated by GGTaseI [107]. Proteins such as K-Ras can be substrates for either FTase or GGTaseI. K-Ras is the most prevalent mutated Ras isoform found in human cancers, yet FTIs are ineffective at blocking the proliferation of cells transformed by oncogenic K-Ras. Alternative K-Ras geranylgeranylation is thought to be responsible for the proteins ability to evade the effects of an FTI. FTIs also do not appear to function solely by blocking farnesylation of Ras proteins. A number of pre-clinical studies have shown that FTI sensitivity does not correlate with the presence of constitutively active Ras; thus, FTIs must also target other, unknown FTase substrate(s) [109-111].

Recently the use of FTIs have shown promise for the treatment of numerous other important disease states including hepatitis δ,[112-114] African sleeping sickness[115] and malaria [116-118]. Each of these disease states are dependent upon the farnesylation of a protein essential for the development of the disease. For example, the hepatitis δ virus (HDV) causes chronic and acute liver disease in patients infected with both HDV and the hepatitis B virus. HDV viral assembly is dependent on the farnesylation of the HDV large antigen protein. Preclinical studies have shown that treatment of HDV infected mice with an FTI is highly effective in clearing HDV viremia [119-121].

**Downstream Processing Inhibition**

Recently the farnesylated protein processing enzymes have become increasingly popular targets for the inhibition of prenylated protein function in transformed cells [60,
Targeting the downstream enzymes rather than FTase may avoid issues related to alternative prenylation of proteins such as K-Ras by targeting post-prenylation processing. In cells deletion of RCE1 induces mislocalization of Ras, but the affect on oncogenic transformation of cells has been less pronounced relative to FTI treatment [58]. Elimination of RCE1 does however markedly increase the sensitivity of cells to an FTI [58]. In contrast to the effects induced by elimination of RCE1, elimination of ICMT has profound affects on cell proliferation. Conditional deletion of ICMT completely blocks the transformation of cells by oncogenic K-Ras [65].

Few inhibitors of RCE1 and ICMT have been developed, due to the fact that these enzymes are relatively new targets for anti-proliferation drugs. Inhibitors targeting the RCE1 enzyme have been limited primarily to substrate analogs. RPI (Figure 1-7, 7) is one such analog that potently inhibits RCE1 activity in vitro. Oncogenic Ras supports the growth of cells in soft agar and disruption of Ras cellular signaling cascades inhibits anchorage independent cell growth. A series of chloromethylketone Cala2X substrate analogs (Figure 1-9, 8-9) [125-128] also inactivate RCE1 and appear to reduce anchorage-independent growth of K-Ras transformed cells.

Inhibitors of the ICMT enzyme fall into two classes which are mimics of either substrates or products of the enzyme [60, 129]. The first class of inhibitor is the reaction product adenosyl homocysteine (AdoHCy) and methods to increase cellular concentrations of this molecule [130-134]. AdoHCy is the product of many methyltransferase reactions including ICMT; therefore, it is not specific for the post-prenylation processing enzyme. The second class of ICMT inhibitors is based on the minimal substrate, farnesylcysteine [135, 136]. N-acetyl farnesylcysteine (Figure 1-7, AFC 10) is a substrate for ICMT and can be viewed as a competitive inhibitor of the enzyme[59, 137, 138]. The AFC molecule is effective at inhibiting Ras post-translational processing, but has also been reported to affect numerous other unrelated cellular processes. Recently Gibbs group has focused on altering the structure of the AFC molecule (Figure 1-7, 11) to create new more potent inhibitors of the ICMT enzyme with limited success [139, 140]. Recently, through small molecule library screening, Winter-Vann et al. discovered a potent and specific ICMT inhibitor, cysmethynil (Figure 1-7, 12) [123]. Cysmethynil blocks anchorage independent cell growth and treatment of cancer
cells with this molecule results in Ras mislocalization and impaired epidermal growth factor signaling. Cysmethynil is one of the first inhibitors discovered for one of the downstream processing enzymes that is not a substrate or product analog.

**Prenyl Function Inhibition**

Another important alternative to the inhibition of prenylated protein function by farnesyl transferase inhibition is alternative prenylation of the protein with non-functional analogs of the farnesyl lipid moiety. The farnesyl lipid group is required for recognition by both of the downstream processing enzymes. In addition the prenyl group may participate in direct protein:protein interactions with chaperone and effector proteins. Numerous analogs of FPP have been designed and synthesized that are inhibitors as well as substrates for the FTase enzyme (Figures 1-8 13-30). Changing the structure of the farnesyl moiety has been shown to disrupt the function of cellular Ras proteins. The analog 3vFPP 13a (Figure 1-8) is a substrate for FTase *in vitro* and in cell culture [141]. Treatment of cells with 3vFPP 13a blocks anchorage-independent growth of ras-transformed cells. Presumably, this effect is due to FTase catalyzed modification of proteins with the 3-Vinyl-farnesyl group. These observations suggest that alternative prenylation with farnesyl analogs may be an effective way to inhibit prenylated protein function.
Figure 1-8 FPP Analogs

13 a-f: R_1=CHCH_2 a; tBu b; isobutenyl c; CH_2CHCH_2 d; CH_3 e; Ph f
R_2=H; X=O
14 a-e: R_2=CH_2CH(CH_3)_2 a; iPr b; Et c; CHCH_2 d; CH_2CHCH_2 e
R_1=H; X=O
15 a-b: X=CH_2, X=CF_2
R_1=H; R_2=H

DHFPP 16

THF 17

MD 18

n=1-6 BNPP 19-24

25 a-b: R_3=mCOPh a; pCOPh b
26 a-d
R_4=H a, AGPP; pNO_2 b, NAGPP; pentafluoro c, PFAGPP; oCO_2CH_3 d

NBD-GPP 27

ETAZ-AGPP 28

DATFP 29

farnesyl azide 30
The affect of the farnesyl group structure on the activity of an oncogenic H-Ras proteins was directly studied in a *Xenopus laevis* oocyte model system [142-147]. The *Xenopus laevis* oocyte is a convenient *in vitro* system for studying farnesylation dependent cellular signal transduction. Oocytes are naturally arrested at the G2-M boundary of the first meiotic cell division. Microinjection of bacterially expressed, oncogenic H-Ras (H-Ras Q61L) promotes meiotic maturation in a process accompanied by activation of MAPK. Since, bacteria lack the enzymes required for protein isoprenylation and subsequent processing, the ability of bacterially expressed recombinant H-Ras to induce meiotic maturation is completely dependent on intracellular farnesylation of the protein or *in vitro* farnesylation of the protein prior to injection [142, 143]. FPP analogs stripped of most isoprenoid features such as methyl groups and unsaturation to more resemble simple fatty acids (16-18) can be transferred to Ras by FTase and support Ras function in the *Xenopus* signal transduction system. However, analogs that were shorter and lower in hydrophobicity than the natural FTase substrate were transferred to Ras, but unable to support Ras function. These results suggested an isoprenoid structure dependent activity of the H-Ras protein in this system.

**Analogs of Farnesyl Diphosphate**

Numerous analogs of FPP have been developed to probe the structural relationship of FPP with the activity of FTase and target proteins (Figure 1-8) [141, 148-163]. These analogs include molecules with altered diphosphate groups (Figure 1-8, 15a-b) and a plethora of changes in the structure of the α, β and ω isoprene units of FPP (Figure 1-8, 13a-f, 14a-e, 19-30). Small changes in the structure of FPP apparently have significant affects on FTase reactivity [154, 161]. Substitutions in the diphosphate leaving group have been used to study the interaction of the highly polar diphosphate leaving group with the enzyme active site. One of the most important of this class of analogs was the non-hydrolyzable analog 4 which allowed crystallographers and kineticists to study the interaction of the isoprenoid and Ca₁a₂X substrate [76]. Other diphosphate analogs have been utilized to show that the diphosphate linkage is simply a leaving group and energy release from phosphoanhydride hydrolysis is not required for the FTase reaction to occur (Figure 1-8, 15a-b) [164].
Alterations in the structure of the α, β and ω isoprene units of FPP have shed light on the importance of the lipid isoprene structure on FTase reactivity. FTase is incapable of efficiently binding GGPP as a substrate, but GGTaseI can bind both FPP and GGPP as a substrate. This gave rise to the “molecular ruler” hypothesis which ascribes FTase isoprenoid substrate binding discrimination to the length of the isoprenoid diphosphate [149]. Micali et al. synthesized a series of benzyloxy FPP analogs in which the ω-terminal isoprene of FPP was replaced with a benzyloxy group and the β-isoprene was replaced with variable length methylene chains (Figure 1-8, 19-24) [160]. Modeling and kinetic studies indicated that even the longer chain analogs could bind to the FTase enzyme although the reactivity and binding of the analogs was best where there were only 4 or 5 methylene units between the α and ω isoprenes. Modeling of the longer chain isoprenoid analogs into the FTase active site indicated that these analogs bound to the enzyme with a bent conformation in which part of the lipid chain protruded into the binding site of the target Ca\textsubscript{1}a\textsubscript{2}X sequence. This protrusion of the molecule may lead to Ca\textsubscript{1}a\textsubscript{2}X peptide exclusion from the enzyme active site suggesting that larger isoprenoids reacted poorly due to a second substrate exclusion rather than inability to bind. Additional support for the second substrate exclusion hypothesis was later generated in crystallographic studies of GGPP or a benzophenone analog (Figure 1-8, 25a-b) bound to the FTase enzyme [149]. In the GGPP•FTase crystal structures the longer prenyl chain did fit into the FTase active site, but the structure of the molecule was “kinked” where part of it protruded into the region typically occupied by the target Ca\textsubscript{1}a\textsubscript{2}X sequence.

Gibb’s group attempted to exploit the interaction of the isoprenoid and target peptide with the α-isoprene substituted FPP analog 3-BFPP (Figure 1-8, 13c) [154]. The hypothesis was that changing the structure of FPP may lead to alterations in the activity of particular Ca\textsubscript{1}a\textsubscript{2}X targets due to the direct interaction of the substrates in the enzyme active site. 3BFPP 13c was a substrate for FTase when the target Ca\textsubscript{1}a\textsubscript{2}X sequence mimicked the proteins H-Ras and K-Ras4B, but was poorly utilized by FTase when the target Ca\textsubscript{1}a\textsubscript{2}X structure mimicked the RhoB Ca\textsubscript{1}a\textsubscript{2}X motif. However, FPP was an efficient substrate with all three Ca\textsubscript{1}a\textsubscript{2}X sequences. The analog apparently changed the ability of FTase to react with a particular Ca\textsubscript{1}a\textsubscript{2}X peptide without significantly affecting
the reactivity of other Ca$_{1}$a$_2$X peptide sequences. Interestingly, the reactivity of the analog did not appear to be due to the interaction of the isoprenoid with the Ca$_{1}$a$_2$X target prior to the chemistry step of the reaction. These results suggested that the pathway after the chemistry step of the reaction may also be important for FTase Ca$_{1}$a$_2$X sequence selectivity.

Chehade et al previously developed a series of analogs where the ω-terminal isoprene of FPP was replaced with an aniline moiety or substituted aniline moieties [159, 161]. 8-anilinogeranyl diphosphate (AGPP, Figure 1-8 26a) was a substrate for FTase and the analog dependent steady-state transfer kinetics to H-Ras and an H-Ras based Ca$_{1}$a$_2$X peptide were nearly identical to the natural substrate of the enzyme. Modeling studies indicated that the aniline moiety behaved as an isostere for the ω-isoprene of FPP, and AGPP likely binds to FTase in a conformation similar to FPP. However, addition of a ρ-NO2 group (26b) to the aniline moiety or substitution of the aniline H atoms with fluorine (26c) decreased FTase catalytic efficiency. Understanding the structural relationships between FTase reactivity and lipid structure will be crucial for the development of new FTase transferable, Ca$_{1}$a$_2$X selective analogs of FPP.

In addition to studying the prenyl transferase reaction and inhibiting the function of FTase protein targets, analogs of FPP may also be useful as unique cellular tags to study protein prenylation in cells. The anthranillate analog of AGPP 26d (Figure 1-8)[165] and the nitro-benzo-oxadiazol analog 27 (Figure 1.8, NBD-GPP)[166] are fluorescent AGPP based analogs. The ortho- substituted anthranillate analog 26d is an FTase inhibitor while the NBD-GPP 27 isoprenoid analog is a substrate for FTase. The NBD-GPP 27 analog has proved very useful in studying cellular prenylation of FTase substrates allowing researchers to follow cellular protein prenylation in real time.

The analogs ETAZ-AGPP (Figure 1-8, 28)[161], the benzophenone analogs (Figure 1-8, 25a-b)[150], and DATFP (Figure 1-8, 29)[167] are photoactivatable crosslinking compounds. In these compounds the ω-terminus of FPP is replaced with photoactivatable units that can be covalently attached to regions of the FTase enzyme or protein binding partners that interact directly with the lipid. The benzophenone analogs 25a-b are FTase inhibitors, yet the ETAZ-AGPP 28 and DATFP 29 analogs are substrates for FTase. Another azido function containing molecule, farnesyl azide 30,
has been used by Kho et al for a tagging-via-substrate methodology to specifically label cellular proteins and isolate those proteins for mass spectral identification [168]. A better understanding of the structural parameters that distinguish substrate analogs from inhibitors will be essential for the design of new probes of the prenylation pathway and the prenyltransferase enzymes.

**Objectives**

**Solid Phase Parallel Chemistry, Structural Diversity, and Prenyl Function Inhibition**

The lipid structure of FPP appears to affect both transferability of target peptides and protein target function. The FPP binding site is made up primarily of aromatic amino acid residues, and the molecule interacts directly with a target protein throughout FTase catalysis [80]. AGPP is a transferable analog of FPP with kinetics nearly identical to the natural substrate, yet it has drastically different chemical and physical properties [159, 161]. We hypothesized that with the right combination of interactions between FTase, the protein target, and alternative lipid structures, some analogs could be efficiently transferred to proteins by FTase, yet unable to support the protein function normally supported by a farnesyl lipid.

In order to study the affect of changing the FPP structure on protein target activity we needed an array of compounds with varying chemical structures and properties. The AGPP skeleton allows simple structure diversification. AGPP was therefore an excellent lead compound for the development of structurally diverse transferable FTase analogs that could be used to probe the relationship of the isoprenoid structure and isoprenoid function. The typical solution phase route to producing AGPP and AGPP analogs was slow and not amenable to a library synthesis [159, 161]. In order to develop large numbers of ω-terminally substituted FPP analogs we utilized a solid-phase parallel chemistry method. Solid-phase chemistry allows the production of small molecules rapidly without tedious purification steps between every chemical manipulation by doing all reaction on solid-phase. Parallel chemistry is the synthesis of multiple compounds simultaneously and is especially amenable to solid-phase methodologies.
We designed and synthesized a library of AGPP analogs with various functional groups in the ortho-\textit{-}, meta-\textit{-}, and para- positions of the AGPP aniline ring. We found that a number of analogs appeared to be FTase transferable. As expected, based on the interaction of the isoprenoid and peptide target in the FTase active site the analog ability to transfer was dependent on the identity of the Ca$_1$a$_2$X sequence. However, the ability of the analog to transfer did not appear to be dependent on the hydrophobicity of the analogs. Instead, the analog reactivity was much more dependent on the size and position of the aniline ring substitution. Since many of the transferable analogs were much more hydrophilic than the natural substrate of the reaction, we tested the ability of these analogs to support oncogenic H-Ras function in the Xenopus signal transduction model system. We found that several analogs that were efficiently transferred to FTase target substrates were unable to support normal farnesyl group function in this system. These results indicated that FPP analogs could be designed with low hydrophobicity that could be transferred to FTase protein targets, yet were unable to support normal prenyl group function in target proteins. These analogs will be key lead compounds for the development of a new class of prenyl group function inhibitors.

**FTase Mechanism of Product Release**

The FTase reaction mechanism involves multiple interactions between FTase the isoprenoid donor and Ca$_1$a$_2$X sequence [80]. It has been supposed that the mechanism of the FTase reaction occurs through FPP binding to the E•product complex to stimulate the release of product from the enzyme [74]. The consequence of this is direct regeneration of the E•FPP complex which is highly committed toward catalysis, and association of FPP to the free enzyme is tight. FPP dissociation from the free enzyme is in fact slower than the overall rate of catalysis. However, it appears that the Ca$_1$a$_2$X peptide can inhibit the FTase reaction [55, 71]. In the generally accepted kinetic mechanism Ca$_1$a$_2$X peptide inhibition occurs through binding of the Ca$_1$a$_2$X sequence to the free enzyme apparently blocking access of the isoprenoid diphosphate to the enzyme active site. However, if FPP is so tightly bound to the enzyme and the E•FPP complex is so highly committed to catalysis it is unreasonable for the Ca$_1$a$_2$X peptide to inhibit the FTase reaction especially at concentrations well below isoprenoid concentrations.
We hypothesized that the generally accepted FTase mechanism was incomplete and required inclusion of peptide stimulated product release to account for Ca_{1}a_{2}X substrate inhibition. We tested this, using peptide competition reactions and found that the Ca_{1}a_{2}X selectivity of the FTase enzyme was highly dependent upon peptide stimulated product release rather than isoprenoid stimulated product release. These results suggested that peptide selectivity for the FTase enzyme was highly dependent on the kinetic flux between the FPP stimulated and peptide stimulated product release pathways. These results also suggested that although FPP stimulated product release may be faster than peptide stimulated, the peptide stimulated release pathway may be more efficient.

Chemical Manipulation of FTase Ca_{1}a_{2}X peptide Selectivity

Chemical genetics is a research method that uses small molecules to change the way proteins work. The FTase reaction is an excellent target for chemical genetic research because the isoprenoid lipid forms a significant portion of the binding pocket for a target Ca_{1}a_{2}X sequence motif. We hypothesized that by changing the ω-terminal isoprene unit of FPP the interactions with a target Ca_{1}a_{2}X motif would be altered leading to chemical genetic changes in the activity of the FTase enzyme and different protein targets. Such alterations would be analogous to amino acid mutations in the enzyme active site, potentially leading to enhanced reactivity of particular Ca_{1}a_{2}X sequences as well as decreases in the reactivity of other Ca_{1}a_{2}X peptides. The reactivity of the FPP analogs with different Ca_{1}a_{2}X peptide structures varied a great deal, and did not correlate with the differences in reactivity with FPP as the isoprenoid donor. Most importantly we have found that by changing the structure of FPP the target selectivity of FTase can be altered.

FPP analogs as unique cellular protein tags

FPP analogs are not only useful as inhibitors and FTase reactivity probes they have also been used to tag endogenous proteins with unique properties that could be used to track cellular FTase targets. Tritiated farnesyl diphosphate analogs and farnesyl diphosphate precursors have been used for years to track the endogenous modification of proteins by FTase [169, 170]. Treatment of cells with tritiated farnesol led to the
discovery that a cell contains endogenous kinases that phosphorylate the allylic alcohol producing intracellular farnesyl diphosphate that can be utilized by FTase. We hypothesized that the isoprenoid alcohol precursors of AGPP would be taken up into a cell, endogenously diphosphorylated and incorporated into cellular proteins by FTase. To test this we developed antibodies to the anilinogeranyl modification and discovered that the anilinogeranyl moiety of AGPP is incorporated into cellular proteins, and the analogue is competitive with endogenous pools of FPP. In the event that a particular allylic alcohol analog structure would not be converted to diphosphate endogenously, we also developed a diphosphate anion masking scheme to build cell permeant analogs of FPP.

GGPP analogs and GGTasel

We found important effects with the FPP analogs on FTase activity. We hypothesized that the technology developed for studying FTase would also be applicable to the related GGTasel enzyme. We developed two analogs of AGPP with an extended isoprenoid chain and measured the affect of changing the terminal isoprene of GGPP on the kinetics of GGTasel. Like FTase with AGPP, the longer chain analog was a substrate for GGTasel with kinetics similar to GGPP. The ability of GGTasel to utilize target peptides was also altered by the GGTasel substrate analogs, which indicated similar interactions between the isoprenoid diphosphate and target peptides in the GGTasel reaction as in the FTase reaction. In addition, we found that the antibody reagents developed for the AGPP analogs were also useful for the longer chain anilinofarnesyl analogs.
CHAPTER 2 PRENYL FUNCTION INHIBITION

Introduction

A wide variety of proteins, including Ras, require post-translational prenylation for their proper membrane localization and activity [171-175]. Protein farnesyltransferase (FTase) catalyzes the transfer of a farnesyl group from farnesyl diphosphate (FPP, 1 Figure 2-1) to proteins with a cysteine residue located in a C-terminal Ca1a2X sequence motif where C is the modified cysteine, a1 and a2 are often an aliphatic amino acid, and X is typically Ser, Met, or Gln [50, 84, 176, 177]. Farnesylation is the first and obligatory step in an ordered series of post-translational modifications that direct membrane localization and potentially protein:protein interactions for a variety of proteins involved in cellular regulatory events [19, 42, 178, 179]. Subsequent to farnesylation, the a1a2X peptide is cleaved by the endoprotease RCE1 [65, 180] followed by methylation of the now terminal farnesylated cysteine residue by the carboxymethyl transferase ICMT.
Figure 2-1 Analog structures

FPP, 1

GGPP, 2

GPP, 3

AGPP, 4a

NAGPP, 4b

x=NH 4a-2al; CH₂, 13; O, 14; S, 15; CH₂O, 16
R=See Table 2-1 for 2a-al; R=H 13-16

R= H, F, PhO 17a-c

18

19

20

21

22a x=H
22b x=F
The *Xenopus laevis* oocyte is a convenient in vitro system for studying farnesylation dependent cellular signal transduction [143, 181]. Microinjection of bacterially expressed, oncogenic H-Ras (H-Ras Q61L) promotes meiotic maturation in a process accompanied by activation of mitogen-activated protein kinase (MAPK) [143, 181, 182]. Maturation of the oocytes can be easily scored by the appearance of a white spot on the dark hemisphere of the oocytes which signifies germinal vesicle breakdown (GVBD) in the oocytes. Since, bacteria lack the enzymes required for protein isoprenylation and subsequent processing, the ability of recombinant H-Ras to induce meiotic maturation is completely dependent on intracellular farnesylation of the protein [183]. Depletion of the endogenous isoprenoid pools by treatment of the oocytes with inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) such as lovastatin, blocks the ability of bacterially expressed H-Ras to induce maturation. Microinjection of H-Ras modified *in vitro* by FTase with FPP analogs into *Xenopus laevis* oocytes allows the biological signal transduction functions of the lipidated protein to be studied [142, 181].

Proteins modified with the 20 carbon geranylgeranyl group from geranylgeranyl diphosphate (GGPP 2, Figure 2-1) are more hydrophobic than the 15 carbon farnesyl group and both classes of prenylated proteins are processed by the same RCE1 endoprotease and ICMT methyltransferase. Replacement of the farnesyl group with FTase transferable lipid analogs of similar or greater hydrophobicity than FPP are effective in allowing all of the subsequent downstream processing events to take place [181]. Substitution of the smaller, less hydrophobic 10 carbon geranyl group from geranyl diphosphate (GPP, 3 Figure 2-1) for the farnesyl group significantly delays the membrane association of H-Ras [181]. Importantly, it was concluded that the reduced hydrophobicity of the geranyl group impaired processing downstream of prenylation by slowing either the endoproteolysis or carboxyl methylation steps [181]. Once C-terminal processing of the geranylated H-Ras was complete, normal palmitoylation followed by membrane association was observed. These observations suggest that the precise structure of the farnesyl group is not critical for the function of the protein as long as it is sufficiently hydrophobic to allow downstream processing and membrane localization. However, it was not possible to distinguish whether the significantly smaller size of the
geranyl group impeded the processing of H-Ras by the RCE1 endoprotease, the ICMT methyltransferase and/or other unknown farnesyl lipid interactions.

The above observations suggest that FTase catalyzed transfer of FPP analogs with hydrophobicity lower than GPP 3 or with significantly different structures may completely block the membrane localization of Ras by preventing the downstream processing or membrane interactions of the alternatively prenylated C-terminus. Altering the downstream biological function of a prenylated protein with unnatural analogs of FPP is an attractive antineoplastic strategy. Synthetic modifications to the farnesyl moiety of FPP have generated both FTIs and alternative substrates transferable for FTase [153, 157, 159-161, 184, 185]. Definition of the structural features responsible for efficient transfer of unnatural analogs to Ras requires the synthesis of additional, structurally diverse FPP analogs that are transferable by FTase, and a rapid method for determining the ability of the analogs to act as FTase substrates.

Parallel synthesis of directed libraries is an effective strategy for rapidly introducing focused diversity into a target template [186-189]. Directed libraries are particularly useful for generating groups of closely related molecules to explore structure-activity relationships [190]. We have previously prepared a limited set of alternative substrates for FTase by replacing the terminal isoprene of FPP with substituted anilines in an effort to define some of the structural features responsible for efficient transfer of unnatural analogs to Ras [159, 161]. These 8-anilinogeranyl diphosphates AGPP 4a (Figure 2-1) were prepared by solution phase synthesis where the key step was reductive amination of an α, β unsaturated aldehyde to form the anilinogeranyl skeleton. This approach was appealing because it was carried out under mild conditions. However, all intermediates and products required purification by chromatography.

In this chapter, I will describe the synthesis of a series of FPP analogs with a wide range of molecular surface areas, shapes and lipophilicities by both solid and solution phase methods. An assay for the rapid determination of FTase transferability was developed to test the ability of these analogs to react with a variety of Ca$_{1}$a$_{2}$X sequences that represented the C-terminal sequence motifs of H-Ras, K-Ras4B, RDJ-2 and a canonical GGTaseI Ca$_{1}$a$_{2}$X sequence. A number of the analogs were alternative
substrates for mammalian FTase, and the reactivity was dependent on the Ca$_1$a$_2$X sequence target. Utilizing the *Xenopus laevis* oocyte system, we found that H-Ras modified with hydrophobic anilinogeranyl diphosphate derivatives support H-Ras dependent oocyte maturation. However, hydrophilic anilinogeranyl diphosphate derivatives prevent H-Ras dependent oocyte maturation. These hydrophilic FPP analogs are Ras function inhibitors (RFIs) and serve as lead compounds for a unique class of potential anti-cancer therapeutics.

**Results**

**Analog Synthesis**

We have adapted our solution methods to provide a solid phase organic synthesis (SPOS) route to anilinogeranyl FPP analogs (Figure 2-2). The advantage of an SPOS approach is that it allows for the rapid preparation of additional analogs and reduces the number of tedious purification steps needed. The THP resin 5 was prepared from the Merrifield-Cl resin as previously described [191]. Attachment of synthetic intermediates to the resin through the THP group provides a traceless linker for the alcohols 10a-am and the final diphosphate compounds 4a-am (Table 2-1).

Preparation of resin 5 was achieved by combining a 5-fold excess of 8-oxo-geraniaol 6 with support 5 in the presence of 0.2 equiv of PPTS at 60 °C. Under these conditions, excess alcohol 6 can be recovered for reuse. The optimized reaction conditions gave a resin loading of 72% for the solid support-linked α,β-unsaturated aldehyde 7. Aldehyde loading was determined by cleavage and recovery of alcohol 6 by treatment of resin 7 with PPTS/MeOH/DCE at 60 °C. With resin 7 in hand, we employed a solid-phase parallel synthetic strategy to prepare a directed library of the anilinogeranyl analogs 10a-am. Diversity was introduced into the library by selecting the commercially available substituted anilines 8a-am listed in Table 2-1. Optimum yields of the desired resin-bound anilinogeraniols 9a-am were obtained by performing the reductive amination with 10 equiv of the anilines and 12 equiv of acetic acid in 1:1 THF/DCE and subsequent reduction with 10 equiv of NaBH(OAc)$_3$. Cleavage of the anilinogeraniols 10a-am from the resin-bound amines 9a-am was achieved by treatment of the solid support with DCE/MeOH/PPTS at 60 °C.
In solution, FPP analogs are typically prepared by sequential conversion of the allylic alcohols into corresponding chlorides followed by diphosphorylation to provide diphosphates [159, 192, 193]. Utilizing this approach reduces some of the advantages of SPOS, as it requires two solution-phase manipulations after release of the anilinogeraniols 10a-am from the resin. In previous solution-phase work, we employed an excess of Ph$_3$PCl$_2$ to convert alcohol 10a to its corresponding chloride, which was immediately transformed to the diphosphate 4a. Direct cleavage of the resin-linked THP ethers to the corresponding allylic chlorides or bromides would reduce the number of solution steps to one, diphosphorylation. We observed that addition of Ph$_3$PCl$_2$ to the resin-bound ethers and subsequent diphosphorylation resulted in poor yield of diphosphates. Alternatively, stirring the THP resin 9a-am with 3 equiv of Ph$_3$PBr$_2$ in DCM for 4 h followed by addition of 10 equiv of tris(tetra-n-butylammonium)hydrogendiphosphate in MeCN gave the desired diphosphates 4a-am in moderate yield. Employing Ph$_3$PBr$_2$ not only reduced the reaction time but also increased the yield of the desired diphosphates. The release of bromides 11a-am from the THP resin by Ph$_3$PBr$_2$ provides a traceless linker path to the FPP analogs 4a-am. In practice, bromides 11a-am were not isolated but were converted directly to the anilinogeranyl diphosphates 4a-am by addition of tris(tetra-n-butylammonium)hydrogendiphosphate in dry MeCN in the same reaction vessel.
Figure 2-2 Synthesis of AGPP library of analogs
Reagents and yields: (a) PPTS, DCE, 60°C; (78%); (b) aniline 8a-am, HOAc, NaBH(OAc)$_3$, THF/DCE(1:1), rt; (c) PPTS, MeOH/DCE(1:1), 60°C; (70-41%); (d) PPh$_3$Br$_2$, MeCN, rt; (e) PPh$_3$Br$_2$, MeCN, rt; (f) (nBu$_4$N)$_3$HP$_2$O$_7$, MeCN

![Chemical diagram of the synthesis process.](image-url)
Table 2-1 Analog FTase Reaction Rates and Structural Properties

Reaction rates measured using 3 µM peptide and 12 µM analog in Tris-HCl buffer (pH=7.4) containing DTT reducing agent and DM as the detergent. Rates are reported as µM per sec per µM enzyme. Rates below detection limits are reported as < values. Each rate was measured in the 96 well plate assay as described in the experimental section. Sizes of the terminal isoprene or aniline are reported in cubic angstroms and were estimated using the PreADME online molecular descriptor software. The partition coefficient LogP was estimated for the alcohol precursors based on RP-HPLC capacity factors. Note the large difference in rates depending on the peptide target.

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<td>&lt;0.6</td>
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<td>4o</td>
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<td>155</td>
<td>&lt;0.9</td>
<td>&lt;0.6</td>
<td>&lt;1</td>
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<td>&lt;0.9</td>
<td>6.6 ± 0.9</td>
<td>&lt;1</td>
<td>&lt;0.4</td>
</tr>
<tr>
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<td>5.1</td>
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<td>149</td>
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<td>&lt;0.4</td>
</tr>
<tr>
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<td>&lt;1</td>
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<td>16 ± 3</td>
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<td>0.4 ± 0.1</td>
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<td>6.0 ± 0.7</td>
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<td>&lt;0.4</td>
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<td>142</td>
<td>7.7 ± 0.4</td>
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<td>8 ± 2</td>
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<td>&lt;1</td>
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<tr>
<td>oMeO</td>
<td>4ae</td>
<td>4.1</td>
<td>140</td>
<td>9 ± 1</td>
<td>4 ± 1</td>
<td>1.4 ± 0.1</td>
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<tr>
<td>mCF₃</td>
<td>4af</td>
<td>4.6</td>
<td>139</td>
<td>5.8 ± 0.4</td>
<td>1.8 ± 0.3</td>
<td>5 ± 1</td>
<td>5.1 ± 0.5</td>
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<tr>
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<td>13.6 ± 0.7</td>
<td>1.6 ± 0.3</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>mCN</td>
<td>4ah</td>
<td>3.1</td>
<td>135</td>
<td>3.2 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>pMe</td>
<td>4ai</td>
<td>3.6</td>
<td>129</td>
<td>12 ± 1</td>
<td>3.1 ± 0.2</td>
<td>3.4 ± 0.4</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>mMe</td>
<td>4aj</td>
<td>3.3</td>
<td>129</td>
<td>4.9 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>4.3 ± 0.6</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>pF</td>
<td>4ak</td>
<td>3.5</td>
<td>118</td>
<td>7.7 ± 0.4</td>
<td>0.7 ± 0.1</td>
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<td>mF</td>
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<td>4am</td>
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<td>3.7 ± 0.5</td>
<td>&lt;0.4</td>
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</table>
The resulting diphosphates 4a-am were first converted to the NH₄⁺ form by ion exchange chromatography and then purified by reverse-phase HPLC (RP-HPLC). Complete removal of the tetra-n-butylammonium counterions from the sample prior to reverse-phase HPLC was required for effective purification. However, efficient recovery of the NH₄⁺ form of the diphosphates 4a-am from the ion exchange chromatography was highly dependent on the buffer conditions employed. This observation is unsurprising, as the various diphosphates 4a-am are expected to have a wide range of solubility in aqueous buffer related to the structure of the parent anilines 8a-am. Optimization of the ion exchange conditions by altering the concentration of NH₄HCO₃ and including compound-dependent proportions of MeCN cosolvent had a profound influence on the yield of individual diphosphates 4a-am recovered.

FTase Analog Transfer

Fluorescence assay for FTase transfer

In order to examine the reactivity of large numbers of FPP analogs, we desired a method that could rapidly sort the molecules into groups of FTase substrates and non-substrates. Poulter et al. developed a continuous fluorescence assay with N-dansyl-GCaaX pentapeptides (Figure 2-3) that is often used to analyze FTase activity [194]. In this assay a fluorescence enhancement results upon farnesylation of an N-dansyl-GCaaX peptide due to the increase in local hydrophobicity near the dansyl reporter group. However, this assay was not immediately amenable for our purposes; because, the assay utilized only a single cuvette and was therefore limited in throughput. We adapted the single cuvette method to a microtiter plate assay which would allow the analysis of many analogs at once. In individual wells of a 96-well plate, FPP and a Ca₁a₂X peptide (N-dansyl-GCVLS) were mixed, and the initial fluorescence was read in a microplate fluorescence reader. The reaction was then initiated by the addition of FTase and allowed to continue until fluorescence enhancement had ceased. The final fluorescence value was expected to represent complete consumption of the input peptide substrate. (Figure 2-4) To confirm total consumption of the peptide reactant the reaction mixture was separated by RP-HPLC with fluorescence and absorbance detection of the dansyl moiety. We found with excess FPP all peptides were completely consumed in the reaction. Fluorescence enhancement from the FTase reaction was

37
similar to that previously found in the single cuvette assay, and the increase in RP-HPLC retention time of the dansyl peak corresponded to the increase in hydrophobicity due to peptide farnesylation. The micro-titer plate assay allowed simple and rapid analysis of the transferability of FPP to the dansyl-GCVLS pentapeptide (Table 2-1).

**FTase reaction rate is enhanced by AGPP analogs**

In order to measure the reactivity of the analogs with the CVLS peptide we measured the fluorescence increase of FTase reactions containing excess analog and the CVLS peptide. The fluorescence enhancement differed depending on the hydrophobicity of the isoprenoid. Reactions that did not go to completion within several hours were stopped using a solution of acetic acid and isopropanol and the extent of modification was determined by RP-HPLC analysis of the dansyl absorbance peaks. A time-dependent increase in fluorescence was observed for over half of the analogs in the AGPP analog library suggesting that a wide variety of functional groups in various positions in the aniline ring were acceptable to the FTase enzyme (Table 2-1, Figure 2-5a).

The analog reactivity ranged from non-transferable to analogs that transferred as fast as, or faster than FPP. Interestingly, the *ml*-AGPP 4y and *pBr*-AGPP 4aa analogs reacted faster than FPP with the CVLS peptide. However, the *mBr*-AGPP 4ab and *oI*-AGPP 4z analogs were poorly transferred to the CVLS peptide. In addition, none of the analogs with functional groups larger than an EtO group were efficiently transferred by FTase. These results indicated that the transfer rate of the analogs was highly dependent upon the size of the aniline substituent as well as the position of the substituent in the aniline ring of AGPP.
Figure 2-3 Dansyl peptide structures

Structures of dansylated peptides representing the Ca$_1$a$_2$X motifs of H-Ras, RDJ2, K-Ras4B, and a canonical GGTaseI substrate.
Figure 2-4 Fluorescence enhancement of N-dansyl-GCVLS modified with FPP

FPP and the N-dansyl-GCVLS peptide were mixed in individual wells of a 96 well plate in Tris-HCl buffer pH=7.4, DTT and DM. The (+) FTase reaction was treated with FTase enzyme and the (-) FTase reaction was treated with buffer only and the fluorescence increase over time was measured in a microplate reader at 2 second intervals. Fmax-Fmin is the difference between the (+) FTase and (-) FTase reactions, and the slope is the rate of fluorescence change over time. Note that the initial velocity of the fluorescence increase over time and the Fmax-Fmin can be used to calculate the rate of the FTase reaction.
Figure 2-5 Reaction rates of analogs with N-dansyl-GCaaX motif peptides

FTase catalyzed analog reaction rates with each peptide relative to the reaction rate with FPP. Rates are given in table 2-1. Cross-hatched bar is to show the location of FPP. a) analog reactions with N-dansyl-GCVLS b) analog reactions with N-dansyl-GCAHQ c) analog reactions with N-dansyl-GCVIM d) analog reactions with N-dansyl-GCVIL. Note that FPP is not always the fastest analog transferred and peptide identity as well as analog identity affects transfer rates relative to FPP.

a. CVLS

b. CAHQ
Reaction rate changes depending on the structure of the $Ca_1a_2X$ sequence and the analog structure

The crystal structure of a non-hydrolyzable FPP analog bound to FTase and a $Ca_1a_2X$ target solved by Reid et al. indicated that the $\omega$-terminal isoprene of FPP and the $a_2$ position of the target protein $Ca_1a_2X$ motif are in van der Waals contact with one another [49]. In addition, the position of the $a_2$ residue affects the placement of the C-terminal $X$ residue in the FTase specificity pocket. We hypothesized that by changing the $\omega$-isoprene of FPP the ability of FTase to catalyze the modification of different $Ca_1a_2X$ sequences will be affected by the structure of the isoprenoid lipid. To determine the effects of different $Ca_1a_2X$ structures on the reactivity of different $Ca_1a_2X$ sequences we measured the rate of modification of three additional $Ca_1a_2X$ sequences. The $Ca_1a_2X$ sequences corresponded to the C-terminal sequence of two canonical FTase protein substrates RDJ-2 (CAHQ) and K-Ras4B (CVIM) (Figure 2-3 structures, Table 2-1 and Figure 2-5 reactivity). We also tested the ability of the analogs to react with a canonical GGTasel $Ca_1a_2X$ substrate CVIL. GGTasel peptide substrates can often readily bind to the FTase enzyme. However, the canonical GGTasel $Ca_1a_2X$ sequences are thought to bind to the E•FPP complex in a different conformation than the canonical FTase substrates. In this alternate conformation the GGTasel $Ca_1a_2X$ substrates have an even more pronounced interaction with the isoprenoid substrate, where the $\omega$-isoprene of the isoprenoid forms part of the binding site for both the $a_2$ and $X$ residues of the canonical GGTasel peptide substrates. The increased interaction between the peptide and isoprenoid may therefore be even more sensitive to the structure of the $\omega$-isoprene.

We hypothesized that the hydrophobicity of the peptide would affect the FTase reaction rate since the rate limiting step is product release and the overall product volume is dominated by the peptide portion of the product. We found that the rate of the peptide reactions with FPP correlated with the retention time of the product in the FPP reaction (Figure 2-6). The most hydrophilic peptide, CAHQ, reacted the fastest, followed by the CVLS, CVIM and CVIL peptides. The FTase active site is relatively hydrophobic and these results are consistent with the idea that product release is rate-limiting due to
interactions with the enzyme active site. The interactions of the more polar farnesylated peptide products appear to be more easily disrupted than the less polar.

Since the peptide reactivity appeared to correlate with the hydrophobicity of the peptide substrate with FPP, we tested whether the reactivity of the AGPP analogs also correlated with peptide target hydrophobicity (Table 2-1, Figure 2-5 a-d). We found that with the analogs the peptide reaction rate did not correlate with the HPLC retention time of the peptide substrates, and differed substantially depending on both the structure of the analog and the peptide. The CVIM peptide reacted readily with a much larger number of analogs than any of the other peptides, where several analogs that were not reactive with the CVIL, CAHQ and CVLS peptides reacted rapidly with the CVIM peptide. In addition, the analogs that transferred with a rate faster than FPP with the other peptides were not necessarily the same analogs that were good substrates with the CVLS peptide. The $m$-AGPP 4y and $p$Br-AGPP 4aa analogs were still rapid substrates reacting three times faster than FPP with the CVIM peptide. However, the $p$Me-AGPP 4ai and $m$CF$_3$-AGPP 4af analogs which were among the fastest transferred to CVLS were considerably slower than FPP when the Ca$_1$a$_2$X substrate was the CVIM peptide rather than CVLS. These results indicated that there were major differences between the reactivity of the analogs with different peptides and that the change in reactivity did not correlate with the change in reactivity with FPP as the isoprenoid donor.
Figure 2-6 Correlation of peptide HPLC retention and reaction rate with FPP
C18 retention capacity of each FTase target peptide correlated with the rate of the FTase reaction with FPP from table 2-1. The HPLC retention time of each of the peptides was measured and the capacity factors calculated as described for the LogP measurements in the experimental section. Reaction rates are reported as reciprocal seconds • 10^{-2}. Note that there is an excellent linear correlation between peptide hydrophobicity and reaction rate with FPP as the isoprenoid donor for the reaction, yet this was not the case with the various analogs.
Analog reactivity does not correlate with analog hydrophobicity

Since the reactivity of the analogs did not appear to correlate with the hydrophobicity of the peptide substrate we tested whether the reactivity of the AGPP analogs correlated with the analog hydrophobicity. In order to determine the hydrophobicity of the AGPP series of analogs we used an RP-HPLC assay to estimate the apparent LogP ($\text{LogP}^{\text{app}}$). LogP is a measure used for the hydrophobicity of a molecule and represents the partition coefficient of a compound in water saturated octanol verses octanol saturated water [195]. The RP-HPLC method uses the HPLC capacity factor for a range of compounds with known LogP values as standards to determine the apparent LogP of unknown compounds. We found that the LogP$^{\text{app}}$ of the analogs ranged from 2.9 for $\rho$CN-AGPP 4ag and 5.3 for ol-AGPP 4z. Surprisingly, the hydrophobicity of the analogs did not correlate with the rate of the FTase reaction. In order to have a molecule with similar size to FPP but greatly reduced hydrophobicity we synthesized an additional analog containing an isoxazole moiety in the position of the $\omega$-isoprene. The isoxazole-GPP analog 12 was transferable by FTase and had an extremely low LogP$^{\text{app}}$ of 0.7. Taken together these results suggest that very hydrophilic analogs of FPP can be made that are substrates for the FTase enzyme depending on both the analog structure and the target peptide structure(Table 2-1).

Additional Isoprene Substitutions

To further explore the ability of the $\omega$-terminal isoprene substituted analogs to be utilized as substrates for FTase, Thangaiah Subramanian synthesized four analogs in which the aniline nitrogen atom was replaced with an O (13), CH$_2$ (14) S (15), or CH$_2$O (16). All four substitutions were acceptable for the FTase catalyzed modification of CVLS (Figure 2-7). However, there were major differences in the transfer rate of the analogs. When the nitrogen atom was replaced with a methylene or CH$_2$O group, the rate of the reaction with CVLS was comparable to that with FPP and AGPP. With both sulfur and oxygen substituting the aniline nitrogen, the reaction rate was considerably slower. These results indicated that the aniline nitrogen could be replaced, but the reactivity of the analogs will change relative to the rate with AGPP.
**Figure 2-7 Transfer of non-aniline analogs to a CVLS peptide**

FTase reactions were prepared as described in table 2-1 and the experimental section with each of the exploratory analogs and the CVLS peptide. In these analogs the AGPP nitrogen atom is replaced with an O, CH$_2$, S or OCH$_2$. Note that the sulfur and oxygen substituted compounds are transferred much slower than the other analogs.
Analogs with aromatic rings replacing the ω-isoprene of FPP were acceptable substrates for the FTase enzyme. We were interested in whether this was also true when the β-isoprene of FPP was replaced with an aromatic ring. In order to determine whether the β-isoprene could also be replaced Subramanian Thangiah synthesized six additional phenoxy substituted isoprene analogs 17-20. We tested the ability of these analogs to transfer to the CVLS peptide, and found that several of the β-substituted phenoxy analogs were FTase transferable substrates. The analogs 18, 19 and 20 were all acceptable substrates for FTase. Surprisingly, the fluorine substituted diaromatic compound 17b was a substrate, while 17a and 17c were not substrates. Interestingly, although 17a was not a substrate for the enzyme, switching the position of the ω-PhO residue from the meta- to the para- position (18) resulted in a transferable analog. These results indicated that the β, ω substituted analogs could be acceptable substrates for the FTase enzyme. In addition, the results from compounds 17b, 17a and 18 indicated that the transferability of these molecules will be highly dependent on the structural features. Additional AGPP analogs were also synthesized with increasing functionalization in the aniline ring of AGPP. The methylene dioxy-AGPP analog 21 was an acceptable substrate for the FTase enzyme. Two other analogs were synthesized as potential fluorescent transferable FTase substrates where coumarin units (22a-b) were placed in the position of the ω-isoprene unit. However, it is currently not clear whether these analogs are substrates.

**H-Ras Function Inhibition**

**H-Ras biological function depends on prenyl analog hydrophobicity and hydrophilic analogs are Ras Function Inhibitors.**

The delay in post-prenylation maturation for geranylated H-Ras relative to farnesylated H-Ras in Xenopus has been attributed to the difference in hydrophobicity of the two lipids [181]. The significantly less hydrophobic geranyl group appears to interfere with maturation of the lipidated H-Ras by delaying one or more downstream processing events. However, it is also possible that the downstream processing enzymes or membrane localization may require a lipid with a minimum size for high activity. The reduction in lipid hydrophobicity by shortening the 15 carbon farnesyl to the 10 carbon geranyl group cannot address this point. With the suite of FPP analogs in
table 2-2, we were able to separate the effect of lipid size from hydrophobicity in the H-Ras stimulated maturation of oocytes.

Incorporation of aromatic rings and heteroatoms into the analogs decreases their hydrophobicity relative to hydrocarbons of the same size. As a result, the size and hydrophobicity of the analogs are no longer directly related. FPP 1 and AGPP 4a are almost identical in size but differ substantially in hydrophobicity. On the other hand, both the 10 carbon geranyl group 3 and the anilinogeranyl group 4a have a logP of 3.6 near the middle of the range, but differ in surface area by 135 Å². In order to evaluate the effect of the lipid physical properties on Ras function, we prepared H-Ras modified in vitro with FPP 1, GPP 3 and the analogs in table 2-2 (AGPP 4a, pNO₂-AGPP 4b, pCF₃O-AGPP 4r, mCF₃O-AGPP 4s, mEt-AGPP 4v, mI-AGPP 4y, pBr-AGPP 4aa, pCN-AGPP 4ag, and isoxazole-GPP 12) for microinjection into oocytes. The extent of H-Ras modification for each analog is shown in table 2-2. Lipidated H-Ras is somewhat unstable in solution, forming aggregates and precipitates upon standing for very short periods. The rate and extent of aggregation was dependent on the lipid analog structure. In particular, in vitro modification of H-Ras with pBr-AGPP 4aa results in complete denaturation of the H-Ras within five minutes of initiating the FTase reaction. The aggregated H-Ras was clearly visible in the microinjection needle under the microscope. Except for pBr-AGPP 4aa, aggregation could be controlled by allowing no more than 20 minutes to elapse from initiation of H-Ras lipidation and completion of oocyte microinjection. Previous studies employed detergent to keep lipidated H-Ras in solution. In our hands, the use of sufficient detergent to prevent aggregation of the anilinogeranyl-analog modified H-Ras resulted in inconsistent oocyte maturation results.
Table 2-2 *In vitro* modification of H-Ras with isoprenoids

Ras was modified in an *in vitro* reaction with each of the given prenyl analogs and the extent of modification is given as a percentage of total Ras protein in the reaction mixture. Note that even though all of the analogs transfer they do not appear to transfer to Ras with equal efficiency.

<table>
<thead>
<tr>
<th>Isoprenoid</th>
<th>% H-Ras modified in <em>vitro</em> for microinjection*</th>
</tr>
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<tbody>
<tr>
<td>1 FPP</td>
<td>$&gt;90$</td>
</tr>
<tr>
<td>3 GPP</td>
<td>70</td>
</tr>
<tr>
<td>4a AGPP</td>
<td>$&gt;90$</td>
</tr>
<tr>
<td>4b $p$NO$_2$-AGPP</td>
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</tr>
<tr>
<td>4r $p$CF$_3$O-</td>
<td>$&gt;90$</td>
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<tr>
<td>4s $m$CF$_3$O-</td>
<td>$&gt;90$</td>
</tr>
<tr>
<td>4t $o$CF$_3$O-</td>
<td>0</td>
</tr>
<tr>
<td>4v $m$Et-AGPP</td>
<td>70</td>
</tr>
<tr>
<td>4y $ml$-AGPP</td>
<td>$&gt;90$</td>
</tr>
<tr>
<td>4aa $p$Br-AGPP</td>
<td>$&gt;90$</td>
</tr>
<tr>
<td>4ag $p$CN-</td>
<td>$&gt;90$</td>
</tr>
<tr>
<td>12 isoxazole-</td>
<td>50</td>
</tr>
</tbody>
</table>

*The extent of H-Ras lipidation was estimated from images of SDS-PAGE separations of in vitro FTase reactions.*
Lovastatin treated oocytes were microinjected with 5 pmols of \textit{in vitro} analog modified H-Ras and scored for germinal vesicle breakdown (GVBD) over the subsequent 24 to 48 hour period. Microinjection of H-Ras pre-modified with the lipid analogs was employed to ensure that the intracellular behavior was not dependent on the ability of endogenous FTase to effectively transfer the analogs. The effective dose of analog modified H-Ras microinjected into each oocyte was greater than 4.5 pmols each for FPP 1, AGPP 4a, $p$NO$_2$-AGPP 4b, $p$CF$_3$O-AGPP 4r, $m$CF$_3$O-AGPP 4s, $ml$-AGPP 4y, $p$Br-AGPP 4aa, and $p$CN-AGPP 4ag, but only 2.5 pmols for isoxazole-GPP 12 and 3.5 pmols for $m$-Et-AGPP 4v and GPP 3 due to incomplete lipidation (Table 2-2). The time required for 50% maturation of the oocytes was calculated from sigmoidal curves fitted to the GVBD time dependence for each of the analogs (Table 2-3). FPP 1, GPP 3, AGPP 4a, $m$CF$_3$O-AGPP 4s, $p$CF$_3$O-AGPP 4r, $ml$-AGPP 4y, and $m$Et-AGPP 4v, stimulated GVBD, while $p$CN-AGPP 4ag, $p$NO$_2$-AGPP 4b and isoxazole-GPP 12 did not. Relative to FPP 1, GVBD was delayed for all the analogs other than AGPP 4a. Farnesylated H-Ras is the natural substrate for the downstream maturation steps and is expected to undergo efficient processing and appropriate membrane localization in the oocyte. However, the time required for maturation of 50% of oocytes injected with farnesylated H-Ras was highly dependent on the frog from which they were harvested. Maturation times varied from 4-10 hours for different batches of oocytes stimulated by farnesylated H-Ras. Accordingly, individual experiments were conducted with oocytes simultaneously harvested from a single frog. We found that the order in which the analog modified H-Ras stimulated 50% maturation relative to farnesylated H-Ras was essentially identical from experiment to experiment. This observation gave us confidence to combine and analyze data obtained from experiments using oocytes from multiple frogs after appropriate normalization. We expressed the maturation half-time for analog modified H-Ras as the ratio of the time required for 50% maturation of analog modified H-Ras to that of farnesylated H-Ras (Table 2-3).
Table 2-3 Maturation kinetics of microinjected oocytes

Stage V or VI oocytes were incubated with lovastatin for 16 hr and then microinjected as described below. Microinjected oocytes were monitored for GVBD at the indicated times and the results expressed as a percentage of the number of viable injected oocytes (14-20/group). The small number of oocytes that did not survive 3 hr post injection were removed from the experiment. A. Induction of GVBD following microinjection of pre-modified activated H-Ras into lovastatin treated oocytes. The $t_{1/2}$ was 8.8 hr for farnesylated H-Ras and 13 hr for anilinogeranylated H-Ras. The ratio of $t_{1/2}$(AGPP) to $t_{1/2}$(FPP) is 1.5. B. Induction of GVBD following co-microinjection of activated H-Ras and isoprenoid diphosphate into lovastatin treated oocytes. The $t_{1/2}$ was 5 hr for FPP and 6.9 hr for AGPP coinjected with H-Ras. The ratio of $t_{1/2}$(AGPP) to $t_{1/2}$

<table>
<thead>
<tr>
<th>Isoprenoid</th>
<th>Fold increase in maturation time relative to 50% GVBD for FPP 1*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vitro modified H-Ras</td>
</tr>
<tr>
<td>1 FPP</td>
<td>1</td>
</tr>
<tr>
<td>3 GPP</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>4a AGPP</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>4r pCF$_3$O-AGPP</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>4s mCF$_3$O-</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>4t oCF$_3$O-AGPP</td>
<td>nd**</td>
</tr>
<tr>
<td>4v mEt-AGPP</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>4y ml-AGPP</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>4aa pBr-AGPP</td>
<td>No assay</td>
</tr>
<tr>
<td>4b pNO$_2$-AGPP</td>
<td>nd$^b$</td>
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<tr>
<td>4ag pCN-AGPP</td>
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<tr>
<td>12 isoazole-</td>
<td>nd$^b$</td>
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</tbody>
</table>

*The time required for 50% GVBD for FPP ranged from 4-10 hours (see text for details). Average and standard deviation for fold increase are calculated from at least three independent experiments.
**Not detected.
The 5 pmols/oocyte dose of modified H-Ras is in excess of that required to induce maximal rates of oocyte maturation. Previous studies of prenyl function showed that 2 pmols/cell of farnesylated H-Ras induced maximal rates of maturation [181]. Under suboptimal conditions of 0.5 pmols/cell of modified H-Ras, the oocyte system is very sensitive to structural alterations of the lipid, which affects its biological activity. For example, under suboptimal conditions, microinjection of 0.5 pmols of geranyl modified H-Ras results in maturation half-times that are 2.5 to 3.3 times longer than farnesyl modified H-Ras [181]. The increase in maturation time is closely associated with delays in the downstream endoproteolysis, carboxyl methylation and subsequent palmitoylation [181]. In contrast, the maturation of oocytes microinjected with 5 pmols/cell of 70% lipidated geranyl H-Ras (3.5 pmols of modified protein) is only delayed by 40% relative to farnesylated H-Ras. Conditions of high H-Ras dose reveal any signal transduction activity, expressed as GVBD, which is supported by the analogs. These observations suggest that lipid analogs that do not support GVBD in oocytes microinjected with these high doses of modified H-Ras are extremely compromised in their ability to support oncogenic Ras function.

In each experiment, unmodified H-Ras was injected into both lovastatin treated and untreated oocytes to test for the completeness of the isoprenoid depletion and as an additional check on the quality of the oocytes. As previously reported, untreated oocytes injected with activated H-Ras underwent GVBD, whereas those pre-incubated with lovastatin failed to mature. Progesterone stimulates GVBD via a Ras-independent pathway, and is therefore unaffected by lovastatin treatment [196]. In each experiment, a small number of the oocytes injected with the individual analog modified H-Ras were withheld and treated with progesterone to test the overall oocyte quality and to ensure that the manipulations did not interfere with maturation. In all cases, the progesterone treated oocytes matured.

Table 2-3 shows that the ability of the analogs to support oocyte maturation fell into three categories. The first category is represented by oCF$_3$O-AGPP 4t which does not support oocyte maturation because it is not transferred to H-Ras by FTase. Microinjection of the H-Ras/oCF$_3$O-AGPP 4t reaction mixture was no different from
injection of unlipidated H-Ras into the lovastatin treated oocytes. In the second category are GPP 3, AGPP 4a, mCF3O-AGPP 4s, pCF3O-AGPP 4r, ml-AGPP 4y, and mEt-AGPP 4v which are transferred by FTase to H-Ras in vitro and support GVBD. Interestingly, the half-time for maturation stimulated by these analogs was delayed from 20-60% relative to farnesylated H-Ras. The third category consists of analogs pCN-AGPP 4ag, pNO2-AGPP 4b, and isoxazole-GPP 12 which are transferred by FTase to H-Ras in vitro but do not support GVBD. These analogs are Ras function inhibitors (RFIs). The lipid chains of FPP 1 and the transferable analogs are similar in length, size and excluded volume, but have different hydrophobicities. The delay in oocyte maturation kinetics does not show any correlation with the length or surface area of the lipid moiety. Rather, it appears that H-Ras is capable of initiating GVBD if the lipid alcohol has a logP of at least 3.6, yet they were unable to support oncogenic H-Ras function when the LogP of the parent alcohol was less than 3.2.

In situ lipidation of H-Ras by hydrophobic FPP analogs rescues Ras biological function in X. laevis oocytes.

The loss of H-Ras function in the experiments described above is independent of differences in their rates of transfer by FTase. However, partial loss of the in vitro lipidated H-Ras to aggregation was inevitable and prevented evaluation of the pBr-AGPP 4aa analog. The possibility existed that different substitution patterns on the analog might affect the ability of the lipid to support oocyte maturation. Analogs mEt-AGPP 4v and pBr-AGPP 4aa share the same logP and have similar surface areas and excluded volumes. However, they differ in structure. Previous studies showed that the X. laevis FTase was competent to transfer alternative lipids with similar or greater hydrophobicity than FPP to H-Ras in situ [181]. The rate of transfer was sufficiently high that the maturation half-time for co-injected H-Ras and in vitro modified H-Ras was indistinguishable. These results suggest that the substrate specificity of X. laevis FTase is sufficiently similar to mammalian FTase to allow efficient modification of the H-Ras by other, structurally unrelated analogs.

We co-injected 5 pmols each of the lipid diphosphates and H-Ras into lovastatin treated oocytes and scored for GVBD. The half-times for oocyte maturation are shown in Table 2-3. Consistent with the in vitro modification results, coinjection of H-Ras and
FPP or the analogs GPP 3, mCF3O-AGPP 4s, pCF3O-AGPP 4r, ml-AGPP 4y, mEt-AGPP 4v, pBr-AGPP 4aa or AGPP 4a results in maturation of the oocytes, while coinjection of H-Ras and analogs oCF3O-AGPP 4t, pNO2-AGPP 4b, pCN-AGPP 4ag, and isoxazole-GPP 12 failed to promote maturation. Interestingly, the p-Br-AGPP analog 4aa, promotes oocyte maturation about as effectively as GPP 3. The mEt-AGPP 4v was the only FPP analog which showed a significant increase in the half-time to maturation upon co-injection with H-Ras. On the other hand, co-injection of the H-Ras with ml-AGPP 4y results in a slight enhancement in the rate of oocyte maturation compared to pre-modified protein. The half-time to maturation for co-injected H-Ras with both FPP and ml-AGPP 4y are identical. None of the analogs accelerated the maturation of the oocytes relative to farnesylated H-Ras. Thus, it is likely that the X. laevis FTase is able to transfer the FPP analogs to H-Ras in situ. However, these results suggest that mEt-AGPP 4v is a poor substrate for X. laevis FTase catalyzed transfer to H-Ras. With the exception of mEt-AGPP 4v it appears that the rate of in situ FTase catalyzed modification of H-Ras by the analogs was not rate limiting for oocyte maturation. From these data, it is not possible to determine if the X. laevis FTase was able to transfer the analogs unable to support maturation to co-injected recombinant H-Ras.

Discussion

Implications

These results have important implications for the development of RFIs as potential alternatives to FTIs. It is now well established that isoprenylation is required for Ras function, and that oncogenic Ras activity may be inhibited by pharmacologic inhibitors of FTase (FTIs). However, while the farnesylation of Ras, and a variety of additional proteins, is blocked by FTI treatment, the Ras protein that is most often mutated in human cancer (K-Ras) undergoes alternative prenylation. Since, geranylgeranyl-modified Ras is effectively targeted to cellular membranes and retains its transforming ability, K-Ras function is not disrupted by FTI treatment. Our work suggests that the hydrophilic RFI lipid structures may act by interfering with membrane localization rendering the modified protein non-functional. The results seen with H-Ras, lead us to predict that modification of oncogenic K-Ras4B with RFI prenyl analogs
would both prevent its transforming ability and block GGTase-mediated alternative prenylation.

Hydrophobicity and FTase Transfer

Another important implication from this chapter is that the structural parameters that control transferability do not appear to affect the ability of the analogs to support H-Ras biological function. This provides an orthogonal relationship between transferability and prenyl group function that could be exploited for the design of new potent transferable analogs that transfer very well to proteins, yet poorly support prenylated protein membrane localization. The assay used in this chapter to screen the majority of the FTase analogs was limited in its ability to characterize the efficiency of transfer of the analogs relative to FPP. The next important step for the characterization of these analogs would be to determine if they can compete with FPP and can they be incorporated into cellular proteins. The Xenopus model system avoids issues related to cell penetrance due to the fact that the compounds are directly injected into the cytoplasm of the cell.

Analog Reactivity Dependent on Both Substrate Structures

The lipid binding pocket of the FTase enzyme is considerably hydrophobic and evolutionarily designed to bind hydrophobic isoprenoid chains. However, the enzyme binds a wide variety of Ca₁a₂X sequences with very different chemical properties. In particular the a₂ binding site of the enzyme accepts a variety of amino acids that do not conform to the classical Ca₁a₂X aliphatic amino acid paradigm. Not only can this region of the enzyme bind a variety of different amino acids, the a₂ binding site also can form aromatic interactions with a number of potent FTase inhibitors. The aniline moiety of AGPP replaces the ω-isoprene of FPP which also forms part of the a₂ binding site. AGPP may be less polar and therefore less able to interact with the hydrophobic farnesyl binding site of FTase, but it is possible that the loss of binding energy to the hydrophobic groove may be made up for with aromatic stacking interactions.

FTase Accepts a Diverse Array of Substrate Structures

We also found that both the structure of the isoprenoid and the structures of the target peptide affect the transferability of the FTase analogs. A number of the analogs
could be rapidly transferred to particular Ca\textsubscript{1}a\textsubscript{2}X sequences, while they were unable to react with other Ca\textsubscript{1}a\textsubscript{2}X peptides. These studies imply that it may be possible to selectively modify particular Ca\textsubscript{1}a\textsubscript{2}X sequences of proteins with certain analogs even in the presence of multiple other Ca\textsubscript{1}a\textsubscript{2}X sequences. However, the assay used to determine peptide reactivity was limited and does not directly address the efficiency of the reactions with the different peptides. Enzyme catalytic efficiency is highly dependent on both the rate of an enzyme reaction and the enzyme affinity for particular substrates. Issues relating to target selectivity will be addressed in later chapters of this dissertation.

**Experimental**

**General**

Reaction temperatures refer to the external bath. All solvents were purchased from VWR (EM Science-Omnisolv high purity) and used as received. All other reagents were purchased from Aldrich, Alfa Aesar or GIBCO. Merrifield-Cl resin was purchased from Argonaut technologies. \textsuperscript{1}H and \textsuperscript{13}C NMR Spectra were obtained in CDCl\textsubscript{3} with a Varian Inova spectrometer operating at 400 and 100.6 MHz respectively. Chemical shifts are reported in ppm from CDCl\textsubscript{3} internal peak at 7.27 ppm for \textsuperscript{1}H and 77.4 ppm for \textsuperscript{13}C. ESI-MS were performed at the University of Kentucky Mass Spectra Facility. HPLC analysis was performed with an Agilent 1100 series purification system equipped with a diode array detector and autoinjection system. All the reactions except for resin preparation were performed in PTFE tubes using a Quest 210 apparatus manufactured by Argonaut Technologies.

Credits: Zhongwen Wang and I were responsible for the development and implementation of the solid phase synthetic route. Thangiah Subramanian was responsible for re-synthesis and optimization. All molecules for the Xenopus study were synthesized by me and all Xenopus assays were performed by Mike Roberts.

**Synthesis**

**Preparation of aldehyde resin 5**

THP resin 3 (0.50 g, 0.82 mmols), geranyl aldehyde 4 (1.35 g, 8.2 mmols), PPTS (41.2 mg, 0.164 mmols), and 2.5 ml of anhydrous DCE were combined and stirred at 60
ºC for 48 hours. The resin was recovered by filtration and washed sequentially with DCM (3x), DMF/H₂O (1:1) (4x), DMF (3x) and DCM (3x), followed by vacuum drying at rt. to afford 636 mg of light yellowish resin 5 (74% based on recovery of aldehyde 4 after treatment of resin 5 with PPTS/MeOH). Unreacted geranyl aldehyde 4 can be recovered from the filtrate and reused.

**Reductive amination and cleavage (8a-8al, 2a-2al)**

**Reductive amination and cleavage (Compounds 8a-8al)**

Aldehyde resin 5 (100 mg, 0.10 mmols), the appropriate aniline 6a-ad (0.50 mmols), acetic acid (36 mg, 0.60 mmols), 2 ml anhydrous THF and 1 ml anhydrous DCE were combined in individual reactor tubes and agitated at rt. under N₂ for 12 hours. NaBH(OAc)₃ (42 mg, 0.20 mmols) was added and the slurry agitated at rt. under N₂ for an additional 60 hours. The resin was filtered and washed sequentially with THF (4x), aq. NaHCO₃ (3x), H₂O (3x), THF (4x) and anhydrous methanol (1x). PPTS (50 mg, 0.20 mmols) in 1 ml of anhydrous methanol and 1 ml of anhydrous DCE was added and the slurry and agitated at 60 ºC under N₂ for 20 hours. After cooling to rt., the resin was filtered and washed with 2 ml of methanol. The filtrate and washing were combined and evaporated to dryness under reduced pressure. Each residue was dissolved in 5 ml chloroform and washed with water (1x) and the organics evaporated to dryness under reduced pressure. The crude products were purified by RP-HPLC (Ranin Microsorb 89-203-C5, 5µm, 300 Å, C-18 (10mm x 250 mm) column with an isocratic mobile phase: 65%Acetonitrile-35%H₂O; Monitored at 254 nm and 298 nm).

**Reductive amination and cleavage (compounds 2a-ai)**

Aldehyde resin 5 (100 mg, 0.10 mmols), the appropriate aniline 6a-ad (0.50 mmols), acetic acid (36 mg, 0.60 mmols), 2 ml anhydrous THF and 1 ml anhydrous DCE were combined in individual reactor tubes and agitated at rt. under N₂ for 12 hours. NaBH(OAc)₃ (42 mg, 0.20 mmols) was added and the slurry agitated at rt. under N₂ for an additional 60 hours. The resin was filtered and washed sequentially with THF (4x), aq. NaHCO₃ (3x), H₂O (3x), THF (4x) and anhydrous CH₃CN (1x). Ph₃PCl₂ (66 mg, 0.20 mmols) in 2 ml of anhydrous CH₃CN was added and the slurry and agitated at room temperature under N₂ for 2 hours. The resin was filtered and washed with 1 ml of
anhydrous CH$_3$CN. The combined filtrate was directly introduced into glass tubes containing tris(tetra(tert-butyl)ammonium)diphosphate (451 mg, 0.50 mmols). The mixtures were stirred at room temperature for 30 mins under N$_2$. Volatiles were removed under vacuum after which the residue was suspended in 4 ml of 25 mM NH$_4$CO$_3$, and extracted with ether (3x). The aqueous phase was applied to an NH$_4^+$ form ion-exchange column and eluted with two-column volume 25 mM NH$_4$HCO$_3$ buffer. The eluent was applied to a fresh NH$_4^+$ form ion-exchange column and eluted with a further two-column volume 25 mM NH$_4$HCO$_3$ buffer. The combined aqueous phase was lyophilized, the residue dissolved in a minimum volume of 25 mM NH$_4$HCO$_3$ buffer and the diphosphates 2a-ad purified by RP-HPLC (Varian Dynamax, 10 µm, 300 Å, C-4 (10 mm x 250 mm) column with a gradient mobile phase: 100% 25 mM NH$_4$OAc buffer for 3 mins, 100% 25 mM NH$_4$HAcO buffer to 80% MeCN 20% 25 mM NH$_5$AcO buffer over 20 mins; Monitored at 254 nm and 298 nm).

**Protein Farnesyl Transferase Transfer Rates**

Reaction rates of the analogs with each N-dansyl-GCaaX peptide were determined in triplicate measurements using a continuous spectrofluorometric assay adapted for a 96-well plate. The following assay components were assembled in individual wells of a 96-well plate and incubated at 30°C for 20 minutes: 180 µL of assay buffer (52 mM Tris-HCl, 12 mM MgCl$_2$, 12 µM ZnCl$_2$ pH 7.4), 40 µL of detergent (0.125% n-dodecyl-β-D-maltoside in assay buffer), 40 µL of reducing agent (50 mM DTT in assay buffer) 20uL of FPP or AGPP (200 µM in 25 mM NH$_4$HCO$_3$) and 20 µL of N-dansyl-GCaaX peptide(final concentration 3 µM). Fluorescence was detected using a time based scan at 30°C for 120 minutes. The velocity of each reaction was determined by converting the rate of increase in fluorescence intensity units (FLU/s) to µM/s with equation 2-1.

**Equation 2-1:** $v_i = (R·P)/(F_{MAX}-F_{MIN})$

Where $v_i$ is the velocity of the reaction in µM/s. R is the rate of the reaction in FLU/s. P is equal to the concentration of modified product in µM (see below). $F_{MAX}$ is the fluorescence intensity of fully modified product. $F_{MIN}$ is the fluorescence of a reaction mixture that contained 20 µL of assay buffer in the place of FTase. Complete
modification of peptides was assumed when fluorescence stabilized for more than 10 minutes and was confirmed by RP-HPLC analysis of the reaction mixture.

**Analog Structure Properties**

**LogP determination**

The apparent LogP values for the corresponding alcohols of diphosphates were estimated from the capacity factors ($k'$) using high performance reverse phase liquid chromatography (HPLC) (added high performance and abbreviation) [197, 198]. A BioBasic-8 RP-C8 column was used as the stationary phase, and the mobile phase consisted of a mixture of 20mM phosphate buffer (pH=6.0) and methanol (3:7) as described by Niemi et al. [199]. Solutions with known LogP values were dissolved in 3:7 phosphate buffer in methanol [1mM progesterone (logP 3.87), 1mM norgestrel (logP 3.7), 100 mM anthracene (logP 4.54), 1mM hydrocortisone (logP 1.53), 0.01 mM nitrobenzene (logP 3.3), 0.01 mM anisole (logP 1.85), 1 mM perylene (logP 2.11) 0.05 mM naphthalene (logP 3.3)] 5 up of each standard was injected and monitored at 254 and 280 nm. A standard curve was generated from the log of the capacity factor $k$ calculated with equation 2 where $R_t$ is the retention time and $R_0$ is the time for a 5 µL injection of 1mM benzoic acid to pass through the column.

**Equation 2-2:** $k = (R_t - R_0)/R_0$

The log of the capacity factor was plotted against the known logP values to generate a standard curve $R^2 0.97$. A Pasteur pipette tip was placed into each of the neat alcohols and the drawn up solution was taken up in 1000 up of mobile phase and 1 µL was injected onto the column. The capacity factor for each of the alcohols was calculated from equation 2-2 and the standard curve was used to determine the apparent logP of the alcohol.

**Surface area calculation**

Surface area calculations were achieved using the online PreADME molecular descriptor calculation software.
H-Ras Preparation and Microinjection

H-Ras protein

Oncogenic H-RasQ61L was expressed in the Rosetta DE3 strain of *E. Coli* (Novagen, CA, USA) using the pTrc-His A plasmid (Invitrogen corporation, CA, USA). Expression was induced by 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) when the optical density of bacteria reached 0.6, and the bacteria harvested after 4 hrs [200]. Bacteria were pelleted by centrifugation, resuspended in 20 mM Tris, pH 8.0 containing 0.5 M NaCl, 1mM BME, 1mM MgCl₂, 10% glycerol and 20 mM imidazole, lysed using a French Press, and H-RasQ61L purified on a nickel column by Fast Protein Liquid Chromatography using an imidazole gradient. Aliquots from the resulting fractions were separated on a 12% SDS-PAGE gel, and proteins stained by incubation with coomassie blue (0.625 g in 250 ml 50% MeOH, 10% acetic acid) for 1 hr followed by destaining (3:1 MeOH:Acetic acid). Fractions shown to contain pure (>90%) H-RasQ61L by SDS-PAGE were dialyzed against Tris and protein content measured using the BCA Protein Assay Kit (Pierce, IL, USA), and this value was used as the concentration of protein in subsequent experiments.

Pre-Modification of Ras in vitro using FPP Analogs.

100 µM RasQ61L was incubated in assay buffer (52 mM Tris-HCl, 12 mM MgCl₂, 12 µM ZnCl₂, 5.8 mM DTT), with 100 µm FPP or FPP analogs, 1 µM recombinant FTase and 0.04% n-dodecyl-β-D-maltoside in a total volume of 15µl for 20 min at 37°C [159]. The reaction mixture containing the lipidated H-RasQ61L was then immediately injected into lovastatin treated oocytes as described below.

Oocyte extraction and microinjection

Oocytes were isolated from *Xenopus laevis* females (Xenopus Express, Inc., Florida, USA) that were primed with gonadotropin before shipment and allowed to acclimatize to their new environment for a minimum of 30 days after arrival before surgery. Frogs were housed in tanks with water and room temperature kept at 17°C and given a 12/12 hr light/dark cycle for optimal egg production. Frogs were anesthetized with 0.01% MS-222 (Argent, Washington, USA), the oocytes surgically removed, and the frog returned to the communal tank for recovery. Oocytes were defolliculated by
incubation with 3 mg/ml type 3 collagenase (Worthington Chemical Corp., NJ, USA), in 30 ml Barth’s medium (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 20 mM HEPES, pH 7.5), for 60-90 min. Following defolliculation, oocytes were washed 3X in Barth’s medium (30 ml each time), followed by 3 X in one half Leibowitz L15 medium (Invitrogen, NY, USA) (30 ml each time), and allowed to recover for at least 4 hr before manipulation. Following recovery, stage V and VI oocytes were selected and incubated overnight (16 hr) in one half L15 medium +/- 50 µm lovastatin (LKT laboratories, MN, USA). Following incubation in lovastatin, any oocytes that were not of uniform pigmentation and otherwise healthy were discarded, and the remaining oocytes microinjected with H-RasQ61L pre-modified as described above, or H-RasQ61L was co-injected with the FPP analogs synthesized in our laboratory [161, 163]. 50 nL total volume was injected per oocyte. All microinjections were done using a Nanoject II microinjector (Drummond Scientific Company, PA, USA) mounted on a micromanipulator (Narishige, NY, USA). Oocytes were scored hourly for germinal vesicle breakdown (GVBD), as evidenced by the appearance of a white spot in the otherwise dark colored animal hemisphere. Progesterone was used as a positive control to induce GVBD in lovastatin treated and untreated oocytes and was also used to determine whether any oocytes that did not undergo GVBD following microinjection of the FPP analogs were still responsive to maturation signals.

**Gel Shift Assay**

Following modification of Ras for exactly 20 min as described above, a 1 µl sample was taken and immediately mixed with 4 µl 2 X Laemmli SDS sample buffer. Samples were boiled for 5 min, then loaded onto 12% SDS-PAGE gels and modified Ras separated from unmodified Ras by running at 200V for 80 min. Proteins were stained by incubation with Coomassie blue (0.625 g in 250 ml 50% MeOH, 10% acetic acid) for 1 hr followed by destaining (3:1 MeOH:Acetic acid). Ras modified with a prenyl group shows higher electrophoretic mobility than unmodified Ras. The extent of protein modification by each analog was estimated from images of the gel analyzed using NIH Image 1.63.
The solid phase work has been reported in the following citation:


Credits:
1. Post-doctoral researcher, Michael J. Roberts was responsible for all Xenopus assays and Ras modifications
2. Post-doctoral researcher Thangiah Subramanian was responsible for yield optimization of the solid phase reaction scheme, resynthesis of many of the compounds, the use of Ph$_3$PBr$_2$ as a cleavage reagent replacing Ph$_3$PCl$_2$, and as cited in the text the synthesis of the exploratory FPP analogs.
3. Post-doctoral researcher Zhongwen Wang in collaboration with me was responsible for the development of the solid-phase synthetic scheme, most notably using the $\alpha$-$\beta$-unsaturated aldehyde as the first isoprenoid linked resin reagent.
CHAPTER 3 FARNESYL TRANSFERASE TARGET SELECTIVITY IS DEPENDENT ON PEPTIDE CATALYZED PRODUCT RELEASE

Introduction

The mechanism of the protein prenyltransferases is unexpectedly complex (Figure 3-1) [49, 82]. Substrate association is assumed to proceed through a functionally ordered mechanism where FPP (1) (Figure 3-2) binds FTase first, giving the enzyme-FPP complex (E•FPP) followed by Ca\(^{2+}\) substrate association forming the enzyme-FPP-Ca\(_{1}\)a\(_{2}\)X peptide complex (E•FPP•CaaX) [72, 201]. After thioether formation, the modified peptide remains associated with FTase in an enzyme-product complex (E•product) [72, 74]. Product release is the rate determining step \((k_{\text{cat}})\) for the FTase reaction mechanism [72, 80]. An unusual feature of the FTase mechanism is that product dissociation is greatly enhanced by binding of either a new FPP or Ca\(_{1}\)a\(_{2}\)X peptide substrate [74]. Apparently, two competing pathways can lead to the release of farnesylated peptide, depending on whether FPP or Ca\(_{1}\)a\(_{2}\)X peptide binds to the E•product complex. However, for the farnesylation of CVIM, FPP stimulates release slightly faster than does peptide [74]. It is generally accepted in the literature that FPP is the substrate primarily responsible for stimulating product release [74, 154]. The steady-state kinetic constant \(k_{\text{cat}}/K_{m}\) is often used to describe the selectivity of an enzyme for its substrates (Equation 3-1) [202]. The \(k_{\text{cat}}/K_{m}\) is also the catalytic efficiency of FTase, and measures the ability of the enzyme to catalyze a reaction at low substrate concentrations. Unlike the selectivity constant, catalytic efficiency does not depend on the competitive interaction of an alternative substrate target. In the case of FTase, the \(k_{\text{cat}}/K_{m}\) should describe FTase selectivity for different Ca\(_{1}\)a\(_{2}\)X peptide substrates [154, 203]. For example, \(k_{\text{cat}}/K_{m}\) for the H-Ras CVLS peptide is twice that measured for the K-Ras CVIM peptide. If \(k_{\text{cat}}\) is dominated by FPP stimulated product release, \(k_{\text{cat}}/K_{m}\) should describe FTase selectivity. Therefore, the expected ratio of products from the farnesylation of two competing Ca\(_{1}\)a\(_{2}\)X substrates is given by equation 3-1. Interestingly, the ratio of products from the FTase catalyzed farnesylation of competing Ca\(_{1}\)a\(_{2}\)X peptides has not been reported. However, there are a number of reports suggesting that FPP binding may not be the sole, or even dominant mechanism for product release [49, 74, 80, 203]. A crystal structure of FTase bound to both the
farnesylated peptide and FPP (E•FPP•product) revealed that the product prenyl chain is displaced from the active site into an external hydrophobic groove (“exit groove”) and the new FPP is bound in the active site [49]. Surprisingly, the farnesylated peptide was not dissociated from the crystal by the presence of excess FPP. However, the farnesylated product was dissociated by additional peptide. Complicating matters further, Hartman et al [203] found that some FTase reaction products do not dissociate from the enzyme even in the presence of excess FPP or peptide. However, the addition of a different un-farnesylated Ca₁a₂X peptide stimulated product release. These observations are inconsistent with FPP stimulated product release as the sole mechanism for dissociation, and indicate that Ca₁a₂X substrate stimulated product release may also be important.

Equation 3-1 kat/Km as a selectivity factor

\[
\frac{V_a}{V_b} = \frac{\frac{V_{max_a} \cdot A \cdot e_t}{K_{m_a} \cdot (1 + \frac{B}{K_{m_b}}) + A}}{\frac{V_{max_b} \cdot B \cdot e_t}{K_{m_b} \cdot (1 + \frac{A}{K_{m_a}}) + B}} = \frac{k_{cat_a} \cdot A}{K_{m_a}} \cdot \frac{k_{cat_b} \cdot B}{K_{m_b}}
\]
Figure 3-1 Minimum reported FTase reaction mechanism

FTase kinetic pathway where E is the FTase enzyme, E-FPP is the FTase•FPP complex, E•FPP•CaaX is the FTase•FPP•CaaX peptide complex, E-Product is the FTase bound product complex, E-Product-FPP is the FTase bound to both FPP and the reaction product, and E-CaaX is the peptide bound FTase inhibitory complex. Note that E•FPP is formed after product release and is not likely to dissociate to the free enzyme.
Figure 3-2 Analog Structures
Structure of farnesyl diphosphate (1, FPP) 8-anilinogeranyl diphosphate (2, AGPP)
A wide range of Ca$_{1}a_{2}X$ sequences have been identified as potential FTase substrates [56]. However, farnesylation has only been confirmed for some of them. Understanding FTase substrate specificity may be critical for understanding which Ca$_{1}a_{2}X$ motifs are farnesylated. Hartman et al. reported that the $k_{cat}/Km^{peptide}$ varies over 400 fold for 14 Ca$_{1}a_{2}X$ peptides with different X-groups [203]. However, it is unclear whether the product distribution for the competition of peptides for farnesylation by FTase will be adequately predicted by the $k_{cat}/Km^{peptide}$ since product release can be stimulated by either of the two peptides or FPP [74].

The FTase reaction is unique because the FPP lipid moiety forms a substantial part of the binding surface for the target Ca$_{1}a_{2}X$ peptide [80, 204]. Crystallographic studies of FTase with a bound Ca$_{1}a_{2}X$ substrate and a non-hydrolyzable FPP analog indicate that the ω-terminal isoprene unit of the lipid is in direct contact with the a$_{2}$ residue of the Ca$_{1}a_{2}X$ motif [80, 205]. Unnatural FPP analogs with structures that differ from FPP may be useful in determining the molecular features of both the lipid and Ca$_{1}a_{2}X$ peptide that contribute to the FTase mechanism [149, 154, 163, 192]. 8-anilinogeranyl diphosphate (AGPP (2)) contains an aniline moiety which replaces the ω-terminal isoprene of FPP (1). AGPP (2) is a good substrate for FTase with steady-state transfer constants $Km^{analog}$ and $k_{cat}/Km^{analog}$ nearly identical to those of FPP (1) [159, 161]. Recent studies with other FPP analogs have shown that the lipid structure can significantly affect the Ca$_{1}a_{2}X$ target selectivity of the FTase enzyme [154].

In this chapter, I describe studies to measure the ability of FTase to selectively modify peptides corresponding to six different Ca$_{1}a_{2}X$ motifs with the isoprenoid donors FPP and AGPP. The reactivity and the selectivity of FTase for the peptides were similar for both isoprenoid donors. Surprisingly, we found that the Ca$_{1}a_{2}X$ substrate selectivity of the enzyme is not predicted by the selectivity factor $k_{cat}/Km^{peptide}$. The ratio of products from competition reactions between two Ca$_{1}a_{2}X$ peptides correlated with the ratio of $Km^{peptide}$ instead of the $k_{cat}/Km^{peptide}$ ratios. We further analyzed the steady-state peptide dependent kinetics of the FTase reaction and found that there were at least three Ca$_{1}a_{2}X$ substrate concentration dependent binding states in the FTase reaction mechanism. We propose that the binding states are due to peptide binding to the E•FPP complex, the E•product complex and a substrate inhibition complex. These results
demonstrate the importance of peptide stimulated product release in the mechanism of FTase and the involvement of this pathway in the selectivity of the FTase enzyme for different Ca_{1a2}X targets.

**Results**

**Individual Ca_{1a2}X peptide reactivity is similar with both FPP and AGPP**

In order to determine if $k_{cat}/K_m^{peptide}$ is the selectivity factor for FTase, we measured the apparent $k_{cat}/K_m^{peptide}$ and $K_m^{peptide}$ for six dansylated-GCa_{1a2}X peptides with saturating concentrations of the isoprenoids FPP and AGPP utilizing a continuous fluorescence assay (Table 3-1). The steady state kinetics of the K-Ras4B (CVIM) and H-Ras (CVLS) full length proteins and the corresponding Ca_{1a2}X peptides with FPP have been extensively characterized [50, 76, 90, 154, 206]. We confirmed that $k_{cat}/K_m^{peptide}$ for the dansylated-GCVIM (Dns-GCVIM) and dansylated-GCVLS (Dns-GCVLS) substrates were identical to those previously reported in the literature [154]. The other four substrates correspond to Ca_{1a2}X motifs from physiologically important proteins, three of which are canonical FTase substrates: CenpF-F (CKVQ) [207], the DNAJ homologue, RDJ2 (CAHQ) [200], and the hepatitis delta virus (HDV) large antigen protein (CRPQ) [113], and the fourth, a canonical GGTaseI-I substrate Ca_{1a2}X motif (CVIL) [56, 208].

The catalytic efficiency ($k_{cat}/K_m^{peptide}$) of the Ca_{1a2}X substrates varied over a 11- and 20-fold range with FPP and AGPP, respectively (Table 3-1). The difference in reactivity of the peptides with each isoprenoid was similar, where CVLS was the most reactive peptide with both isoprenoids. FTase showed slightly enhanced catalytic efficiency with AGPP relative to FPP for the CRPQ peptide. Interestingly, the catalytic efficiency with the canonical GGTaseI substrate CVIL was decreased over three-fold with AGPP relative to FPP.

**Peptide selectivity is not predicted by $k_{cat}/K_m^{peptide}$ rather, it is correlated with $K_m^{peptide}$**

We performed competition reactions between pairs of Ca_{1a2}X peptides in order to determine whether $k_{cat}/K_m^{peptide}$ is the selectivity factor for FTase. The products from competition reactions for pairs of Ca_{1a2}X peptides with either FPP or AGPP were
separated by RP-HPLC and quantified by integration of the dansyl absorbance (Table 3-2). The competition reactions were stopped prior to 50% consumption of either Ca1a2X substrate in order to ensure that the peptide concentration did not fall below its $K_{m,peptide}$. We found that the ratio of $k_{cat}/K_{m,peptide}$ for any of the pairs of Ca1a2X peptides examined did not predict the actual ratio of products formed (Table 3-2). For example, a 1:1 product ratio of farnesyl-CVIM (fCVIM) to farnesyl-CVLS (fCVLS) was expected if $k_{cat}/K_{m,peptide}$ is the selectivity factor for FTase (Table 3-2). Instead, we found a product ratio of 3:1 fCVIM:fCVLS (Table 3-2). The CVIM peptide was three-fold more competitive over the CVLS peptide than predicted by the ratio of $k_{cat}/K_{m,peptide}$. We also found that the ratio of products was invariant for reactions where from 10% to 50% of the CVIM peptide was consumed. Results from competition reactions with AGPP as the isoprenoid donor mirrored those of FPP. However, the product ratios of competition reactions for pairs of Ca1a2X peptides with either 12 µM FPP or AGPP did correlate with the ratio of the apparent $K_{m,peptide}$ with a slope of 0.8 for FPP and 2.1 for AGPP (Figure 3-3). In order for equation 3-1 to accommodate these observations, the ratio of $k_{cat}$ for the competing substrates must be equal to an isoprenoid dependent constant. However, inspection of the experimentally determined values for $k_{cat}$ (Table 3-1) indicates that this is not the case.
Table 3-1 peptide steady-state kinetic parameters

Kinetic parameters $K_m^{peptide}$, $k_{cat}$ and $k_{cat}/K_m^{peptide}$ for N-dansyl-GCa$_1$a$_2$X peptides with FPP or AGPP as the isoprenoid donor at 6.7 µM and peptide at varying concentrations in Tris-HCl buffer (pH=7.4) plus reducing agent and detergent. Note that FPP and AGPP are nearly identical based on the steady state rate constants, except with the CVIL and CRPQ peptides.

<table>
<thead>
<tr>
<th>donor</th>
<th>peptide</th>
<th>$K_m^{peptide}$ (µM)</th>
<th>$k_{cat}$ ($s^{-1}$)•$10^{-2}$</th>
<th>$k_{cat}/K_m^{peptide}$ (µM$^{-1}$•$s^{-1}$)•$10^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPP</td>
<td>CVLS</td>
<td>0.8 ± 0.1</td>
<td>14 ± 2</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>FPP</td>
<td>CVIM</td>
<td>0.30 ± 0.06</td>
<td>4.3 ± 0.5</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>FPP</td>
<td>CAHQ</td>
<td>2.0 ± 0.3</td>
<td>20 ± 2</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>FPP</td>
<td>CKVQ</td>
<td>1.5 ± 0.3</td>
<td>8 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>FPP</td>
<td>CVIL</td>
<td>6.6 ± 0.6</td>
<td>23 ± 2</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>FPP</td>
<td>CRPQ</td>
<td>5 ± 1</td>
<td>8 ± 1</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>AGPP</td>
<td>CVLS</td>
<td>0.5 ± 0.1</td>
<td>12 ± 2</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>AGPP</td>
<td>CVIM</td>
<td>0.18 ± 0.06</td>
<td>1.6 ± 0.3</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>AGPP</td>
<td>CAHQ</td>
<td>2.4 ± 0.5</td>
<td>21 ± 3</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>AGPP</td>
<td>CKVQ</td>
<td>1.7 ± 0.2</td>
<td>11 ± 1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>AGPP</td>
<td>CVIL</td>
<td>16 ± 2</td>
<td>17 ± 1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>AGPP</td>
<td>CRPQ</td>
<td>10 ± 1</td>
<td>21 ± 2</td>
<td>2.1 ± 0.3</td>
</tr>
</tbody>
</table>
Table 3-2 Ratios of products

Ratios of products from reaction mixtures containing FPP or AGPP at 6.7 µM each with two N-dansyl-GCa_{1}a_{2}X peptides at 3 µM each. Product ratios were determined from HPLC analysis of competition reactions containing two peptides and FPP. Product was quantified by integration of 340 nm trace chromatogram. Note that the ratio of the $K_{m}^{peptide}$ change with the product ratio but not the ratio of the $k_{cat}$ or $k_{cat}/K_{m}^{peptide}$.

<table>
<thead>
<tr>
<th>Reactants</th>
<th>FPP</th>
<th>AGPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>peptide A</td>
<td>peptide B</td>
<td>FPP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$k_{cat}/K_{m}^{peptide}$ ratio</td>
</tr>
<tr>
<td>CVIM</td>
<td>CVLS</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>CVLS</td>
<td>CKVQ</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>CVLS</td>
<td>CAHQ</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>CKVQ</td>
<td>CAHQ</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>CAHQ</td>
<td>CVIL</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>CAIL</td>
<td>CRPQ</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>CVIM</td>
<td>CRPQ</td>
<td>13.4 ± 9 ± 3</td>
</tr>
<tr>
<td>CVLS</td>
<td>CRPQ</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>CVIL</td>
<td>CRPQ</td>
<td>1.5 ± 0.3</td>
</tr>
</tbody>
</table>

*ND: not determined  
**NA= not applicable
Figure 3-3 Comparison of the Ratio of products with the $Km^{\text{peptide}}$

Product ratio verses the ratio of the $Km^{\text{peptide}}$ for each competition reaction in Table 3-2 a) FPP or b) AGPP. Note that the slope of the two graphs differ where the $Km^{\text{peptide}}$ ratio correlates directly with the product ratio with FPP but is two times the product ratio with AGPP. R value for both lines is greater than 0.95

Note: different y-axis scales
Product ratio is dependent on the isoprenoid donor concentration

Since the observed product ratios were not predicted by the ratio of $k_{cat}/K_m^{peptide}$ but did correlate with an isoprenoid dependent ratio of the apparent $K_m^{peptide}$ we examined the dependence of the product ratios on the concentration of isoprenoid diphosphate. There are two isoprenoid diphosphate bound states in Figure 3-1 and the experimental $K_m$ for both FPP and AGPP is 46 nM with the CVLS peptide. We did not expect any change in the product ratios, as the isoprenoid concentrations used in the competition reactions were well above saturation for the isoprenoid donor. We measured the ratio of product from the CVIM and CVLS peptides over a range of FPP and AGPP concentrations. (Figure 3-4) The product ratio decreased with increasing concentrations of the isoprenoid donor, trending towards the ratio expected from equation 3-1. These unanticipated results suggest that the mechanism shown in Figure 3-1 is incomplete.
Figure 3-4 Product ratio dependence on isoprenoid concentration
CVIM/CVLS peptide pair product ratios were measured from reaction mixtures containing 3µM of the CVIM and CVLS peptide with a) FPP or b) AGPP at increasing concentrations. Note that the effect of increasing concentration of FPP on the product ratio is much greater than the effect with increasing AGPP concentrations.
Overall rate of competition reactions is governed by the rate of the peptide with the lowest $K_m^{peptide}$

In order to characterize the unusual dependence of the product ratios on $K_m^{peptide}$, we examined the overall velocity of the competition reactions relative to the velocity of reactions for the individual peptides. For competition reactions between CVIM and CVLS peptides the overall reaction rate was the same as for the CVIM peptide alone. CVIM has both a lower $K_m^{peptide}$ and rate of transfer than does CVLS. We examined the dependence of the overall competition velocity on the component Ca$_1$a$_2$X peptide $K_m^{peptide}$ and rates of transfer. Competition reactions between the CKVQ/CAHQ and CRPQ/CVIL peptide pairs with AGPP as the isoprenoid donor were run in parallel with the corresponding single peptide reactions at the same individual substrate concentrations (Figure 3-5). The total peptide concentration in the competition reactions was twice that of the single peptide reactions. The overall rate of the CKVQ/CAHQ competition reaction was the same as for the reaction of the CKVQ peptide alone. Of the pair, the CKVQ peptide has the lower $K_m^{peptide}$ and the slower reaction rate. Similarly, the overall rate of the CRPQ/CVIL reaction was the same as for the lower $K_m^{peptide}$ CRPQ substrate alone. However, under these conditions, the reaction rate of the CRPQ peptide is higher than that of the CVIL. We conclude that the overall rate of the competition reactions is governed by the rate of the peptide with the lowest $K_m^{peptide}$. 
**Figure 3-5 Rate of the competition and individual peptide reactions**

Reactions were prepared that contained the peptide pair indicated in parallel with each individual peptide reactions at the same enzyme and peptide concentration. Error bars represent values from triplicate reactions 4-5a: Competition of CAHQ and CKVQ peptides for AGPP modification. 4-5b: Competition of CAHQ and CKVQ peptides for AGPP modification. Note that the rates of the competition reactions are the same as the rates of the single peptide reaction where the peptide is the one with the lowest $K_{m}^{peptide}$.
There are a minimum of three peptide binding states in the FTase steady-state reaction

In the generally accepted FTase mechanism, FPP association with the E•product complex is solely responsible for stimulating product release (Figure 3-1). As noted above, Ca₁a₂X peptides can stimulate product release, suggesting that peptide also interacts with the E•product complex. In addition, substrate inhibition of the FTase reaction by both Ca₁a₂X peptides and full length proteins, including CVLS and H-Ras, have previously been reported [55, 71]. In order to characterize Ca₁a₂X peptide stimulated product release, we investigated the FTase reaction using peptide concentrations higher than required to reach maximum velocity. Much to our surprise, we found increasing concentrations of the CKVQ substrate in reactions with both FPP and AGPP had more complex effects than simple inhibition (Figure 3-6). As previously reported, the reaction rate increased to a maximum, before falling as the concentration of peptide was increased. Surprisingly, at even higher concentrations of peptide, the rate again increased slightly to a smaller second maximum before finally approaching zero. To our knowledge, the second peptide concentration dependent rate increase has not been previously described. To confirm these observations, we examined the FTase reaction for the Ca₁a₂X concentration dependence of the CVLS and CAHQ peptides with FPP. The appearance of two peptide substrate concentration dependent maxima in the CVLS and CAHQ reaction profiles was similar to what we observed for CKVQ. The second rate maximum for the CVLS peptide was less pronounced than for CKVQ. Interestingly, with increasing concentrations of CAHQ we found that the rate dipped slightly after the first maxima, then rose gradually to a second, higher maximum before decreasing.
Figure 3-6 Steady state reaction of FPP with peptides
Response of reaction rate with increasing concentrations of peptide well above concentration required to reach the first Vmax. Note the second increase in the reaction rate after the first Vmax point has been reached.

Note: The y-axis and x-axis scales are different with different peptides and that the lines are for illustrative purposes only.
The presence of two peptide substrate concentration dependent maxima and substrate inhibition in the steady-state reaction profile requires a minimum of three peptide binding states. The generally accepted mechanism (Figure 3-1) has only two peptide bound states. The second peptide bound state, E•CaaX is thought to result from dissociation of FPP from the E•FPP complex, followed by peptide binding to FTase. Previous reports have shown that E•CaaX is non-productive, and is unable to bind FPP to form the reactive E•FPP•CaaX complex [72]. Rather, the productive E•FPP complex is generated by peptide dissociation from E•CaaX followed by FPP binding. In this model, formation of the inhibitory E•CaaX requires peptide to out-compete FPP for association with the free enzyme. However, FPP has a much higher affinity for FTase than do the Ca_1a_2X peptides, and CVLS peptide affinity for E•FPP is 70 fold higher than for the free enzyme [90]. Also, the E•FPP complex is highly committed towards catalysis since FPP dissociates at a rate 2.5 times slower than \( k_{cat} \) [55].

Here we propose a more complex model for the FTase reaction mechanism that takes these observations into account (Figure 3-7). In common with the generally accepted mechanism, the first peptide bound state is the E•FPP•CaaX complex. The second state is the E•product•CaaX complex which is responsible for peptide stimulated product release. This state is formed in competition with E•product•FPP. Release of product from the E•product•CaaX complex would leave the third state, E•CaaX. In this scheme, product dissociation from E•product•CaaX (Figure 3-7) would form the inhibitory E•CaaX complex as a natural consequence of catalysis. Additionally, higher concentrations of Ca_1a_2X peptide will drive formation of the inhibitory E•CaaX complex at the expense of E•FPP by favoring formation of E•product•CaaX relative to E•product•FPP without requiring dissociation of FPP from E•FPP.
Figure 3-7  FTase reaction mechanism
Extended mechanism incorporating peptide stimulated product release. The mechanism is split into two pathways path A representing FPP stimulated product release and path B representing peptide stimulated product release. E is the FTase enzyme, E-FPP is the FTase•FPP complex, E•FPP•CaaX is the FTase•FPP•CaaX peptide complex, E-Product is the FTase bound product complex, E-Product-FPP is the FTase bound to both FPP and the reaction product, and E-CaaX is the peptide bound FTase inhibitory complex, E-Product-CaaX is the new peptide bound enzyme product complex.
Competition by two Ca\textsubscript{1}a\textsubscript{2}X peptides for the E•product complex suggests a mechanistic explanation for the observations that the peptide with the lowest $Km$ controls the overall FTase reaction rate. In this scheme, the overall rate will depend on the relative affinity of the peptides and isoprenoid diphosphate for the E•product complex, as well as the rate of product release from the E•product•CaaX and E•product•FPP complexes. If one of the competing peptides binds the E•product complexes more efficiently and/or stimulates faster product release from E•product•CaaX, then its rate will determine the overall rate. Consequently, the $k_{cat}$ for both peptides in equation 3-1 will be the same. Secondly, increasing isoprenoid will compete with the peptides for E•product and increase flux through path A of Figure 3-7. Consequently, the ratio of products formed under high concentrations of isoprenoid donor in a competition reaction between two peptides will be less dependent on peptide stimulated release. These conclusions are consistent with the experimental findings that the peptide with the lowest measured $Km_{peptide}$ determines the overall rate and that the ratio of $k_{cat}$ for each of the competing substrates will be equal to an isoprenoid dependent constant.

**Discussion**

**Peptide verses FPP stimulated release efficiency**

The efficiency of the FPP and peptide stimulated release pathway could be dependent on the binding affinity of the peptide or FPP to the E•product complex as well as the rate of release due to binding of either substrate. Under competition conditions this scheme becomes even more complex due to a third route to product release with the alternative peptide substrate. It is possible that peptide binding affinity to the E•product complex is attenuated depending on the identity of the isoprenylated Ca\textsubscript{1}a\textsubscript{2}X sequence in the active site of the enzyme. The Ca\textsubscript{1}a\textsubscript{2}X binding affinity to the E•product complex may be completely unrelated to the binding affinity to the free enzyme or the enhanced affinity with the E•FPP complex. For example, a TKCVIC peptide can stimulate the release of a farnesylated TKCVIL peptide product from the FTase active site, yet the affinity of TKCVIC for the E•FPP complex is 7-fold lower than the affinity of TKCVIL [203]. In addition, FPP binding affinity for the E•product complex will not necessarily correlate with the binding affinity to the free enzyme. The relative efficiency
of peptide stimulated release verses FPP stimulated release is not known. However, large quantities of isoprenoid were required for the competition reaction product distribution to approach the ratios predicted from the $k_{cat}/K_m^{peptide}$ suggesting that peptide stimulated release may be more efficient than isoprenoid stimulated. In addition, AGPP stimulated product release appears to be even less efficient than FPP stimulated. AGPP is less hydrophobic than FPP and may not associate with E•product complex as readily as the natural substrate.

**Altering FTase selectivity**

The isoprenoid and target peptide interact directly with one another throughout the FTase reaction, and changes in the structure of the isoprenoid may alter the interactions between them. Changing the interactions of the isoprenoid with the target Ca$_1$a$_2$X motif could lead to the selective modification of particular Ca$_1$a$_2$X substrates with unnatural probes specific for certain Ca$_1$a$_2$X sequences. The reactivity of the canonical FTase Ca$_1$a$_2$X substrates was nearly identical with both FPP and AGPP as the isoprenoid donors. However, with AGPP as the isoprenoid donor the selectivity was markedly decreased for the canonical GGTaseI substrate over the CRPQ Ca$_1$a$_2$X sequence. AGPP is more readily utilized to modify the HDV large antigen based Ca$_1$a$_2$X peptide over the GGTaseI target peptide in contrast to FPP which is more readily utilized to modify the GGTaseI Ca$_1$a$_2$X substrate. To our knowledge this is the first example of an analog switching the Ca$_1$a$_2$X selectivity of the FTase enzyme. Further alterations of the isoprenoid diphosphate may therefore lead to analogs that further alter FTase target selectivity for different Ca$_1$a$_2$X sequences.

**FTase clearance mechanism**

Peptide stimulated product release may be essential to allow slowly turned over FTase substrates to be cleared from the enzyme active site. A wide variety of peptides including GGTaseI substrate peptides have a high affinity for the FTase•FPP complex and thioether formation occurs. However, some of these Ca$_1$a$_2$X sequences such as the GGTaseI substrate TKCVIL are not readily released from the active site with excess FPP or substrate peptide [203]. In order for FTase to be functional after formation of a slowly dissociating or single turnover product a cell may require product release to be
stimulated by an alternative Ca$\text{a}_2\text{a}_2\text{X}$ substrate. The GGTasel reaction mechanism is similar to that of FTase and it is likely that the peptide stimulated release mechanism also exists for the GGTasel enzyme.

**Experimental**

**General**

AGPP and FPP were synthesized as described previously. All RP-HPLC was performed on an Agilent 1100 HPLC system equipped with a microplate autosampler, diode array and fluorescence detector. The HPLC analysis was performed with a microsorb C18 column with 0.01% TFA in water (A) and 0.01% TFA MeCN (B) as the mobile phase. Peptides were purchased from peptidogenics and each contained a dansyl group N linked to a glycine linker followed by a C residue then a variable aaX sequence (VLS, VIM, AHQ, RPQ, VIL or KVQ). Spectrofluorometric analyses were performed in a 96-well flat bottom, non-binding surface, black polystyrene plate (Excitation wavelength, 340 nm; emission wavelength 505 nm with a 10 nm cutoff). The plate reader was a SpectraMax GEMINI XPS fluorescence well-plate reader. Absorbance readings were determined using a Cary UV/Vis spectrophotometer.

**Steady-state peptide kinetics**

The kinetic constants $K_{m}^{\text{peptide}}$ and $k_{cat}$ with FPP, and AGPP for each dansyl-GCaaX peptide were determined in triplicate measurements using a continuous spectrofluorometric assay adapted for a 96-well plate. The following assay components were assembled in individual wells of a 96-well plate and incubated at 30° C for 20 minutes: 180 µL of assay buffer (52 mM Tris-HCl, 12 mM MgCl$_2$, 12 µM ZnCl$_2$ pH 7.4), 40 µL of detergent (0.125% n-dodecyl- β-D-maltoside in assay buffer), 40 µL of reducing agent (50 mM DTT in assay buffer) 20uL of FPP or AGPP (200 µM in 25 mM NH$_4$HCO$_3$) and 20 µL of N-dansyl-GCaaX peptide (variable concentration). Fluorescence was detected using a time based scan at 30° C for 120 minutes. The velocity of each reaction was determined by converting the rate of increase in fluorescence intensity units (FLU/s) to µM/s with equation 3-2.

**Equation 3-2:** $v_i = \frac{(R-P)}{(F_{\text{MAX}}-F_{\text{MIN}})}$
Where $v_i$ is the velocity of the reaction in µM/s. R is the rate of the reaction in FLU/s. P is equal to the concentration of modified product in µM (see below). $F_{\text{MAX}}$ is the fluorescence intensity of fully modified product. $F_{\text{MIN}}$ is the fluorescence of a reaction mixture that contained 20 µL of assay buffer in the place of FTase. Complete modification of peptides was assumed when fluorescence stabilized for more than 10 minutes and was confirmed by RP-HPLC analysis of the reaction mixture.

Final analysis peptide concentrations were chosen based on preliminary determination of the $K_m^{\text{peptide}}$ with 0.5, 1, 5 and 10 µM dansyl-GCaaX concentrations (see below). The final analysis used 8 peptide concentrations which were: 1/6, 1/5, 1/4, 1/2, 3/4, 1, 2, and 3 times the estimated $K_m^{\text{peptide}}$ value except with the CVIM peptide where we used concentrations at 0.3, 0.45, 0.675, 0.9, 1.8, 2.7, 3.6, and 4.5 times the estimated $K_m^{\text{peptide}}$. The reaction was then initiated by the addition of 20 µL of FTase (final concentration 5 nM with the CLVS, CAHQ, CVIL and CRPQ peptide, 10nM with the CVIM peptide). Fluorescence enhancement determined as described above.

Complete modification of peptides was assumed when fluorescence stabilized for more than 10 minutes and was confirmed by RP-HPLC analysis of the reaction mixture. If the reactions that contained higher concentrations of peptides did not go to completion the $F_{\text{MAX}}$-$F_{\text{MIN}}$ value was extrapolated using a linear plot of the $F_{\text{MAX}}$-$F_{\text{MIN}}$ for each of the lower concentration reactions that did go to completion. Alternatively the extent of input peptide modified was determined by RP-HPLC analysis of the dansyl moiety peak absorbance corresponding to the unmodified and modified peptides. The percent of modified peptide relative to the total input peptide was used to calculate the concentration of product in the mixture.

The velocities of the reactions were plotted against the concentration of peptide and were fit to the Michaelis-Menten equation (Equation 3-3) to give the apparent $k_{\text{cat}}$ (app$k_{\text{cat}}$) and $K_m^{\text{peptide}}$ (app$K_m^{\text{peptide}}$) values. Where $e_t$ was the total enzyme concentration and [Pep] was the total input peptide. The $k_{\text{cat}}/K_m^{\text{peptide}}$ value was derived from a Lineweaver-Burke plot of the same data points.

**Equation 3-2:** $v_i/e_t=(\text{app}k_{\text{cat}}'[\text{Pep}])/(\text{app}K_m^{\text{peptide}}+[\text{Pep}])$
Substrate inhibition analysis

The steady-state reaction profiles with high concentrations of peptide were analyzed as described above with peptide concentrations 4, 5, 6, 7, 8, 9, and 10 fold higher than the app\(K_m^{\text{peptide}}\).

Peptide competition

Competition reactions were prepared with the same components given for the \(k_{\text{cat}}/K_m^{\text{peptide}}\) analysis except two peptides were added to the mixture at a single concentration. Peptides A and B were diluted to 45 µM in Tris-HCl buffer (pH=7.4) and the concentration checked by reading the Abs of the solution at 340 nm (\(\varepsilon_{\text{dansyl}}=4250\) Abs·M\(^{-1}\)·cm\(^{-1}\)). 20 µL of peptide A and 20 µL of peptide B were then added to reaction mixtures as described above except only 160 µL of assay buffer was added. Separate reactions were prepared as a standard with FPP or AGPP and only one peptide as described above. The reactions were then initiated with the addition of FTase and analyzed spectrofluorometrically (Reactions containing CVIM peptide used 20 nM final FTase concentration all other used 10 nM). The reactions were stopped prior to consumption of more than 50% of either peptide by adding 20 µL of a stop solution (isopropyl alcohol and acetic acid 8:2). HPLC analysis was then performed with 100 µL of the reaction mixture loaded onto C18 column and eluted with a linear gradient of 0-30 min 10% B to 100% B at a flow rate of 1 mL/min. Peaks on the 340 nm trace chromatogram that corresponded to both the standard reactions and fluorescent peaks were then integrated. The ratios of products were calculated according to equation 3-4.

Equation 4-4: \((I_{A\text{mod}}/I_{A\text{tot}})/(I_{B\text{mod}}/I_{B\text{tot}})\)

Where \(I_{A\text{mod}}\) is the integral of the modified peptide A, \(I_{A\text{tot}}\) is the integral of the modified peptide A plus the unmodified peptide A, \(I_{B\text{mod}}\) is the integral of the modified peptide B and \(I_{B\text{tot}}\) was the integral of the modified peptide B plus the unmodified peptide B. Alternatively, the ratios were calculated using the integrals of the standard peptides fluorescence relative to competition product fluorescence giving less than a 5% difference in the calculated ratio.

Overall rate of competition reactions

The overall rate of the competition reaction was determined using equation 2 and the amount of total peptide modified based on RP-HPLC analysis. Alternatively peptide
competition reactions were scaled up to a 1000 µL solution and sampled for RP-HPLC analysis every 45 minutes for 6 hours.

**Increasing FPP and AGPP concentrations effect on product ratio**

To determine the effect of increasing isoprenoid concentration the FPP and AGPP concentrations in the competition mixtures were increased from 0.5-66 µM with FPP and 0.5-400 µM AGPP.

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CHAPTER 4 FARNESYL DIPHOSPHATE ANALOG COMPETITION FOR FARENSYL TRANSFERASE

Introduction

An interesting feature of the FTase reaction mechanism (Figure 4-1) is the association between the isoprenoid lipid and the target CaG2X sequence in both the E•product•FPP and E•FPP•CaaX complexes [80]. In each of these complexes, FPP binds at the same hydrophobic surface and interacts directly with the terminal residues of the target CaG2X sequence. Pre-steady state kinetic studies have shown that FPP association with the free enzyme enhances CaG2X peptide affinity up to 70 fold [90]. The interaction between the target CaG2X motif and the isoprenoid suggests that changes in the structure of FPP may be analogous to amino acid mutations in the active site of the enzyme, where the reactivity of the CaG2X motif can be modulated by changes in the structure of FPP [154]. The ω-isoprene of FPP associates directly with the a2 residue of the target CaG2X peptide, but it is not clear how altering the ω-isoprene structure affects CaG2X peptide reactivity.

Isoprenoid analogs of FPP have been used to study the physical interactions between the isoprenoid, FTase and the CaG2X peptide. The analog, 8-anilinogeranyl diphosphate (AGPP, 2a, Figure 4-2) contains an aniline moiety in the place of the ω-terminal isoprene of FPP. AGPP is transferable to CaG2X substrates with steady-state kinetics nearly identical to FPP, and the aniline moiety appears to act as an isostere for the terminal isoprene of FPP [159, 161]. AGPP has been used to probe the endogenous modification of proteins by FTase and is competitive with FPP both in vitro and in cell culture [159, 209]. However, when the aniline moiety of AGPP is replaced with a p-NO2 aniline 2b or a pentafluoro-aniline the analog dependent steady-state transfer efficiency is severely reduced [161].
Figure 4-1 FTase reaction mechanism
Extended mechanism incorporating peptide stimulated product release. The mechanism is split into two pathways path A representing FPP stimulated product release and path B representing peptide stimulated product release. E is the FTase enzyme, E-FPP is the FTase•FPP complex, E•FPP•CaaX is the FTase•FPP•CaaX peptide complex, E-Product is the FTase bound product complex, E-Product-FPP is the FTase bound to both FPP and the reaction product, and E-CaaX is the peptide bound FTase inhibitory complex, E-Product-CaaX is the peptide bound enzyme product complex.
Figure 4-2 Analog Structures
Structure of farnesyl diphosphate (1, FPP) 8-anilinogeranyl diphosphate (2a, AGPP)

1: FPP

2a: R=H AGPP
2b-aq see table 4-1
Understanding the ability of substrate analogs to compete with the natural substrate will improve the ability to develop new analogs to probe the FTase reaction mechanism as well as the development of potential inhibitors of prenylated protein function. In chapter 2, I described the synthesis of a library of AGPP analogs with a variety of functional groups in the ortho-, meta- and para- positions of the AGPP aniline ring. In this section, I utilize this library of molecules to probe the relationships of the AGPP structure on Ca\textsubscript{1}\textsubscript{a}\textsubscript{2}X peptide reactivity and competitiveness with FPP. We have found that the position and size of the aniline ring substitution has a major affect on the reactivity of the AGPP analogs. With simple conservative substitutions in the AGPP aniline ring, the reactivity of the analogs range from molecules that transfer more efficiently than FPP to analogs that are inhibitors of the FTase catalyzed farnesylation of Ca\textsubscript{1}\textsubscript{a}\textsubscript{2}X substrates. We also found that the structure of the analogs affected the competitiveness with FPP and that the molecular features that improved peptide reactivity improved FPP competitiveness for the CVLS peptide. These results demonstrate that with only subtle alterations in the structure of an FPP analog, FTase activity can be effectively modulated depending on the structure of the analog. These results also suggest that the natural substrate of FTase can be improved upon, to develop molecules that can efficiently and competitively be transferred to FTase target Ca\textsubscript{1}\textsubscript{a}\textsubscript{2}X sequences.

**Results**

**Peptide target reactivity is dependent on size and position of substitutions in the aniline moiety of AGPP**

In order to determine how changing the structure of AGPP affects the reactivity of an FTase target peptide we measured the steady-state transfer kinetics of a dansylated-GCVLS (CVLS) pentapeptide with a library of structurally diverse AGPP analogs. (Table 4-1) The CVLS peptide corresponds to the Ca\textsubscript{1}\textsubscript{a}\textsubscript{2}X sequence of H-Ras, and the steady-state transfer kinetics of both H-Ras and the CVLS peptide have been extensively characterized. Utilizing a continuous fluorescence assay we found $k_{cat}$, $Km_{peptide}$ and $k_{cat}/Km_{peptide}$ values with CVLS and FPP that were identical to those previously reported for both CVLS and a full length H-Ras protein.
Over half of the 50 molecule AGPP library 2a-aq was transferred to the CVLS peptide by FTase. The ability of the peptide to react with the various analogs was highly dependent on the size and position of the functional group substitution in the AGPP aniline ring. Analogs with functional groups larger than an ethoxy group 2ac-2aq were poorly transferred to the CVLS peptide independent of the aniline ring position that was substituted. However, when the aniline ring was substituted with a fluorine atom at either the meta- 2d or ortho- 2c positions the CVLS peptide reacted with a catalytic efficiency ($k_{cat}/K_m^{peptide}$) nearly identical to that with FPP and AGPP. Surprisingly, with the pF-AGPP 2e analog the catalytic efficiency of the enzyme with the CVLS peptide was increased.
Table 4-1 Michaelis-Menten Peptide Kinetics

Steady state kinetic parameters were measured with varying concentrations of N-dansyl-GCVLS peptide FPP and each of the AGPP analogs with FTase in Tris-HCl buffer (pH=7.4) plus detergent and reducing agent. Note that some analogs increase efficiency and/or decrease the $K_m^{\text{peptide}}$

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<th>#</th>
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<th>$K_m^{\text{CVLS}}$ (µM)</th>
<th>$k_{\text{cat}}/K_m^{\text{CVLS}}$ (µM$^{-1}$·s$^{-1}$) $\times 10^2$</th>
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<td>2aq</td>
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</tr>
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</table>

* NR=No Reaction
** slow indicates that some reaction did occur, but not enough to characterize
*** ND=not determined due to the inability to measure $k_{\text{cat}}$
When the functional group in the aniline ring was smaller than an ethoxy group, the reactivity of the molecules was highly dependent on the position of the aniline ring that was functionalized. Aside from the fluorine substituted analogs, the reactions with the meta-substituted analogs were in general the most efficient. In particular, the position of an iodine and methyl functional group in the aniline ring drastically altered the catalytic efficiency of FTase with the CVLS peptide. The efficiency of the CVLS reaction with both the mI-AGPP 2o and mMe-AGPP 2g analogs was much higher than the efficiency with the natural substrate. However, when the functional group of the iodo and methyl AGPP analogs was shifted to the para- and ortho-positions, the efficiency of the reactions was drastically reduced. In particular, reactions containing the oI-AGPP 2n analog and the CVLS peptide resulted in little to no product formation even in the presence of enzyme concentrations well above what was required for complete modification of the CVLS peptide with the other iodine substituted analogs. The individual $k_{cat}$ and $K_m^{peptide}$ were significantly affected by the functionalized position of the aniline ring, even though the effects may not have been observed in the $k_{cat}/K_m^{peptide}$. We found that AGPP analogs with para-functional group substitutions, in general, increased both the $k_{cat}$ and $K_m^{peptide}$ of the CVLS substrate relative to the ortho- and meta-isomers. On the other hand, we found that the ortho-substituted analogs tended to reduce the $k_{cat}$ of the CVLS peptide reactions. These results indicated that the reactivity of the Ca1a2X peptide was highly dependent on the position and size of the aniline ring substitution in AGPP.

**Non-substrate ortho-substituted analogs are potent FTase inhibitors**

A number of the AGPP analogs were not substrates for the FTase catalyzed modification of the CVLS peptide, but analogs that were not substrates may still bind to the enzyme and act as an inhibitor. To determine if the non-substrate analogs were FTase inhibitors, we mixed FPP and each analog 1:1 in excess of the CVLS peptide, and determined the reaction rate relative to the rate with FPP alone (Figure 4-3). We found that the rate of farnesylation was decreased over 50% with the ortho- but not the meta- and para-substituted AGPP non-substrate analogs. In order to more closely examine the ability of these analogs to inhibit the FTase reaction, we measured the analog concentration dependence on the FTase inhibition. The $K_i$ of the ortho-
substituted non-substrate analogs were well below the previously reported $K_m$ of FPP ($K_m^{FPP} = 46$ nM). (Table 4-2) These results indicated that these analogs could readily bind to the enzyme, but the reaction with the CVLS peptide was compromised.

**Analogs are competitive with FPP for target modification**

In order for an analog to be utilized in cells they must be competitive with FPP. Previous studies to determine the kinetic factors that predict FTase selectivity for peptide targets suggested that simple steady-state catalytic efficiency measurements do not predict the selectivity of the enzyme for $Ca_{1}a_{2}X$ peptides in competition with one another. AGPP is competitive with FPP both *in vitro* and in cell culture [159], but it is not clear how well steady-state kinetic measurements predict the ability of AGPP to compete. Instead of relying on steady-state kinetic measurements we directly measured the ability of AGPP and the analogs to compete with FPP. We mixed AGPP and FPP 1:1 in excess of the CVLS peptide and analyzed the resulting CVLS modified product by HPLC and integration of the dansyl peak absorbance. We found that the resulting product ratio was 2:1 farnesyl to anilinogeranyl modified CVLS peptide. This was significantly less anilinogeranylated peptide than would have been predicted based solely on previous steady-state $k_{cat}/K_m$ measurements ($k_{cat}/K_m^{AGPP} = 1.8 \ \mu\text{M}^{-1}\cdot\text{s}^{-1}$ $K_m^{AGPP} = 46$ nM $k_{cat}/K_m^{FPP} = 2.2 \ \mu\text{M}^{-1}\cdot\text{s}^{-1}$ $K_m^{FPP} = 46$ nM)[159, 161].

We found that a number of the analogs were utilized by FTase even in the presence of FPP. The competitiveness of the analogs with functional groups larger than fluorine was highly dependent on the position of the aniline ring that was functionalized. The *meta*- substituted analogs were the most competitive followed by the *ortho-* and *para-* substituted analogs. Several of the functionalized AGPP analogs were even more competitive than AGPP including all three of the fluorine substituted analogs. The structure dependence of analog competition with FPP appeared to correlate with the structure dependence on peptide reactivity.
Figure 4-3 Farnesylation Inhibition
N-dansyl-GCVLS was mixed with FPP and an equimolar amount of the analog. FTase was added and the percent inhibition was calculated by comparing the rate of reactions containing the analog and FPP to the reaction rate with FPP alone. Note that only the ortho- substituted analogs inhibit greater than 50%.
Table 4-2 Analog Ki

Analog Ki values were calculated from reaction rates of FPP/analog competition reactions for an N-dansyl-GCVLS peptide, where the analog concentration was varied and the FPP concentration was held at 6.7 µM. Note that the Ki of the ortho- substituted AGPP analogs is lower than the $K_m^{FPP}$ which is 40 nM.

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<th>$K_i^{XPP}$ (nM)</th>
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<td>oCF$_3$O</td>
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<td>3.0 ± 0.3</td>
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</table>
Figure 4-4 Analog/FPP competition for CVLS
Ratios of products, analog modified CVLS:farnesylated CVLS were found from reactions set up with 3µM peptide and 6 µM of the analog and FPP. Reactions were allowed to continue until all peptide was consumed and were then analyzed by RP-HPLC. Fluorescence detection indicated the retention time of each of the products and integration of peaks at 340 nm was used to quantify products. Analogs pNO2-AGPP 2b and mCN-AGPP 2i increased absorption at 340 nm so product from reactions containing these analogs was calculated based on input peptide relative to peptide unmodified. * indicates that the ortho-analogue was not available.
Discussion

FPP Analog Reactivity and Peptide Substrate Inhibition

We found that a number of the AGPP analogs were FTase transferable substrates with transfer properties that could not have been predicted by the analysis presented in chapter 2. In chapter 2, I described the reactivity of the analogs based on reaction rates with high concentrations of peptide and analog. However, the concentration of peptide that would be required to be saturating would differ depending on the analog and at concentrations that are too high the analog reaction may be inhibited by the peptide. When using only the reaction rate to study the analog reactivity analogs that induce a low $K_m^{\text{peptide}}$ will also increase the ability of the peptide to inhibit the reaction with that analog. In other words analogs that appeared in the earlier analysis to be poor substrates, in fact increased the reactivity of the peptide. The effects of peptide inhibition therefore make it very difficult to study the FTase reaction without a steady-state kinetic evaluation with variable peptide concentrations.

Cellular protein Incorporation

A number of the analogs that were rapidly transferred in the FTase reaction were not competitive with FPP. In chapter 2 the $p$CN-AGPP analog was utilized in the Xenopus studies and was a Ras function inhibitor. However, the analysis in this chapter indicates that the $p$CN-AGPP analog would be a poor choice to study analog incorporation into cells that contained endogenous FPP due to the extremely poor ability to compete with FPP. Instead, the equally hydrophilic $m$CN-AGPP analog may be a much more competitive and therefore potent analog to use for cell studies. Several of the analogs were more competitive than AGPP which is competitively incorporated into cellular proteins. This indicates that numerous analogs could be used for cellular protein incorporation.

Reactivity and Hydrophobicity

Previous studies have suggested that reactive FPP analogs must be hydrophobic to bind the active site of the FTase enzyme. However, we found that the relatively hydrophilic analogs containing a nitrile group in both the para- and meta- positions of the AGPP aniline ring were highly reactive in the FTase transfer reaction. In particular,
the position of a nitrile group in the aniline ring of AGPP had a major affect on the ability of the analog to compete with FPP. The hydrophobicity of the \( mCN-AGPP \) 2i and \( pCN-AGPP \) 2j analogs based on RP-HPLC retention times of the parent alcohols are much lower than farnesol. Surprisingly, the \( mCN-AGPP \) 2i analog reaction with CVLS was as efficient as the reaction with FPP and the analog competed with FPP giving nearly 1:1 product ratios. However, the \( pCN-AGPP \) 2j analog was unable to effectively compete with FPP. These results indicated that the structure of the AGPP analogs was much more significant than the hydrophobicity of the analogs on FTase transferability and competitiveness.

**Competition with FPP Occurs at Free Enzyme**

The transferable analogs may compete with FPP for either the E•product complex or the free enzyme. Since AGPP does not compete with FPP as well as would be expected based on the catalytic efficiency of the analog, it is likely that the analog is competing primarily for the free enzyme. This suggests that the ability of the analogs to compete with FPP may also be dependent on the identity of the target Ca\( _1\)a\( _2\)X sequence. Different functional groups in the aniline ring of AGPP may more readily interact with particular product amino acid residues and therefore enhance competitiveness depending on the identity of those residues. Peptide, FPP and the analog may stimulate product release and the ability of the analog to compete would be highly dependent on the mechanism of product release. Under competition conditions the \( k_{cat}/K_m^{\text{peptide}} \) would likely predict selectivity assuming only isoprenoid or only peptide stimulated product release. However, with both mechanisms of product release it is possible that AGPP poorly stimulates release of the isoprenylated target yet can effectively compete for free enzyme under peptide stimulated release conditions.

**Relationships of Analog Structure on Transfer and Competition**

As described earlier the rate limiting step of the FTase reaction is product release from the enzyme active site. The \( k_{cat} \) of the reaction represents the rate of product release. An increase in \( k_{cat} \) and \( K_m^{\text{peptide}} \) suggests that the product from a reaction is rapidly released from the enzyme active site, while a decrease in the \( k_{cat} \) indicates that the reaction product is slowly released from the enzyme active site. The larger ortho-
substituted analogs reacted very slowly with the enzyme, but were potent inhibitors indicating that they most likely bind, but the product of the reaction is not rapidly released. However, the para- substituted analog products were released rapidly as indicated by the high $k_{cat}$ and $K_m^{peptide}$ values for the reactions. The meta- substituted analogs were most like the parent molecule AGPP and the natural substrate FPP. In several cases the efficiency of the FTase reaction was even increased with by the meta-substituted AGPP analogs. These results suggest that by simply moving the functional group from one position to another in the AGPP aniline ring there were drastic effects on the reactivity of the FTase enzyme. These changes in reactivity are likely a direct result of changing the interactions between the isoprenoid, the target peptide and the enzyme itself.

**Implications**

In this chapter, I have shown that the FTase enzyme can accept analogs of AGPP with a wide variety of functional groups in the $ω$-terminal isoprene position of FPP. The analogs affect the reactivity of a CVLS target peptide depending on both the size and position of the functional group in the AGPP aniline moiety. Analogs with ortho-substituted anilines were potent inhibitors of the FTase reaction, while analogs with para-substituents were rapidly transferred yet unable to effectively compete with FPP. The AGPP analogs that contained meta-substitutions often enhanced the catalytic efficiency of the FTase reaction with a CVLS peptide and effectively competed with FPP for the modification of the CVLS peptide. This chapter demonstrates that subtle alterations in the structure of a transferable analog of FPP can drastically alter FTase activity towards a peptide substrate. Such analogs will be important for understanding and exploiting the activity of the FTase enzyme for the design of selective FTase inhibitors or the design of unnatural probes to alter FTase protein target activity.

**Experimental**

**General**

Analogs and FPP were synthesized as described previously. All RP-HPLC was performed on an Agilent 1100 HPLC system equipped with a microplate autosampler,
diode array and fluorescence detector. The HPLC analysis was performed with a microsorb C18 column with 0.01% TFA in water (A) and 0.01% TFA CH₃CN (B) as the mobile phase. N-dansyl-GCVLS peptide was purchased from peptidogenics. Spectrofluorometric analyses were performed in a 96-well flat bottom, non-binding surface, black polystyrene plate (Excitation wavelength, 340 nm; emission wavelength 505 nm with a 10 nm cutoff). The plate reader was a SpectraMax GEMINI XPS fluorescence well-plate reader. Absorbance readings were determined using a Cary UV/Vis spectrophotometer.

**Steady-state peptide kinetics**

The kinetic constants $K_m$ and $k_{cat}$ with FPP, and the analogs with the N-dansyl-GCVLS peptide were determined in triplicate measurements using a continuous spectrofluorometric assay adapted for a 96-well plate. The following assay components were assembled in individual wells of a 96-well plate and incubated at 30° C for 20 minutes: 180 µL of assay buffer (52 mM Tris-HCl, 12 mM MgCl₂, 12 µM ZnCl₂ pH 7.4), 40 µL of detergent (0.125% n-dodecyl-β-D-maltoside in assay buffer), 40 µL of reducing agent (50 mM DTT in assay buffer) 20uL of FPP or AGPP (200 µM in 25 mM NH₄HCO₃) and 20 µL of N-dansyl-GCaax peptide (variable concentration). Fluorescence was detected using a time based scan at 30° C for 120 minutes. The velocity of each reaction was determined by converting the rate of increase in fluorescence intensity units (FLU/s) to µM/s with equation 4-1.

**Equation 5-1:** $v_i = \frac{R \cdot P}{(F_{MAX} - F_{MIN})}$

Where $v_i$ is the velocity of the reaction in µM/s, $R$ is the rate of the reaction in FLU/s, $P$ is equal to the concentration of modified product in µM (see below). $F_{MAX}$ is the fluorescence intensity of fully modified product. $F_{MIN}$ is the fluorescence of a reaction mixture that contained 20 µL of assay buffer in the place of FTase. Complete modification of peptides was assumed when fluorescence stabilized for more than 10 minutes and was confirmed by RP-HPLC analysis of the reaction mixture.

Final analysis peptide concentrations were chosen based on preliminary determination of the $K_m$ with 0.5, 1, 5 and 10 µM dansyl-GCaaX concentrations (see below). The final analysis used 8 peptide concentrations which were: 1/6, 1/5, 1/4, 1/2, 3/4 1, 2, and 3 times the estimated $K_m$ value except with the CVIM peptide.
The reaction was then initiated by the addition of 20 µL of FTase (final concentration 5 nM). Fluorescence enhancement was determined as described above.

Complete modification of peptides was assumed when fluorescence stabilized for more than 10 minutes. If the reactions that contained higher concentrations of peptides did not go to completion the Fmax-Fmin value was extrapolated using a linear plot of the Fmax-Fmin for each of the lower concentration reactions that did go to completion. Alternatively the extent of input peptide modified was determined by RP-HPLC analysis of the dansyl moiety peak absorbance corresponding to the unmodified and modified peptides. The percent of modified peptide relative to the total input peptide was used to calculate the concentration of product in the mixture.

The velocities of the reactions were plotted against the concentration of peptide and were fit to the Michaelis-Menten equation (Equation 4-2) to give the apparent $k_{cat}$ (app$k_{cat}$) and $K_m^{peptide}$ (app$K_m^{peptide}$) values. Where $e_t$ was the total enzyme concentration and [Pep] was the total input peptide. The $k_{cat}/K_m^{peptide}$ value was derived from a Lineweaver-Burke plot of the same data points.

**Equation 4-2:** $v_i/e_t=(appk_{cat}[Pep])/(appK_m^{peptide}+[Pep])$

**FPP competition**

Competition reactions were prepared with the same components given for the $k_{cat}/K_m^{peptide}$ analysis except two analogs were added to the mixture and the peptide concentration was held constant. FPP and the analog were diluted to 200 µM in 25 mM NH$_4$HCO$_3$. 10 µL of FPP and 10 µL of analog were then added to a 280 µL solution of peptide, DTT and detergent in 52 mM Tris-HCl buffer pH=7.4 (final concentrations DTT=6.7 mM, DM=0.033%, Peptide=3µM, analog= 6.7 µM and FPP=6.7 µM). The concentration of peptide was determined by absorbance of the dansyl group and isoprenoid diphosphate concentration was determined by consumption of isoprenoid in FTase reactions with the CVLS peptide where the isoprenoid was limiting. The reactions were initiated with the addition of FTase and analyzed spectrofluorometrically (20 nM final FTase concentration). The reactions were stopped after 2 hours with a mixture of acetic acid and iPrOH. HPLC analysis was then performed with 100 µL of the reaction mixture loaded onto C18 column and eluted with a linear gradient of 0-30 min 10% B to 100% B at a flow rate of 1 mL/min. Peaks on the 340 nm trace chromatogram that
corresponded to standard reaction fluorescent peaks were then integrated. The ratios of products were calculated using \( I_{\text{analog}}/I_{\text{FPP}} \), where \( I_{\text{analog}} \) is the integral of the peptide modified with the analog and \( I_{\text{FPP}} \) is the integral of the peptide modified with FPP.

**Inhibition**

**Inhibition Screen**

The analog ability to inhibit FTase transfer of FPP was initially determined by measuring the percent inhibition in farnesylation rate in the presence of equimolar amounts of FPP and the analog. Reactions were prepared as described for the FPP competition analysis and the rate of FPP modification was determined based on the rate of fluorescence increase over time with or without the analog present. The % inhibition was determined using equation 4-3.

**Equation 4-3:** \( \% \text{ inhibition} = \left[1 - \left( \frac{\text{rate}_{\text{FPP}}}{\text{rate}_I} \right) \right] \times 100 \)

Where the rate\(_{\text{FPP}}\) was the rate of the CVLS reaction without the analog present and the rate\(_I\) was the rate of the reaction with the analog present.

**Ki determination**

The Ki of the analogs was determined for analogs which showed no product formation with 3 µM peptide. The Ki was determined from reactions containing 3 µM peptide 16 µM FPP and varying concentrations of the analogs (0.016-16 µM). The data was fit to the 3 parameter hill function in equation 4-4 and the Ki was calculated from equation 4-5.

**Equation 4-4:** \( \frac{v}{v_0} = \left( a \cdot I^b \right) / \left( IC50^b + [I]^b \right) \)

Where I is the concentration of the inhibitor, IC50 is the IC50 of the inhibitor, \( \frac{v}{v_0} \) is the rate of the inhibited reaction divided by the rate of the uninhibited reaction and a and b are coefficients for Hill function fitting.

**Equation 4-5:** \( Ki = IC50 / \left( 1 + \left( \frac{[\text{FPP}]}{Km_{\text{FPP}}} \right) \right) \)

Where [FPP] is the concentration of FPP and \( Km_{\text{FPP}} \) is the known Km for FPP.

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CHAPTER 5 ORTHO SUBSTITUTION AND THE SELECTIVE MODIFICATION OF A K-RAS PEPTIDE

Introduction

The oncprotein Ras must be prenylated for both membrane localization and to participate in cellular transformation [57]. Constitutive activation of Ras is transforming in a wide variety of cell types and mutations that activate Ras have been found in 30% of all cancers [26, 108, 210]. A wide variety of other proteins with C-terminal Ca\textsubscript{1}a\textsubscript{2}X boxes have also been implicated in oncogenesis and tumor progression [18, 211]. Consequently, development of inhibitors of both FTase and GGTaseI are subjects of major drug discovery programs [212, 213]. Several FTase inhibitors (FTIs) are currently in phase I, II and III clinical trials for the treatment of cancer [15, 17, 92, 94]. However, K-Ras is the most prevalent mutated Ras isoform found in human cancers, and cells transformed by K-Ras are often resistant to FTI treatment. The K-Ras Ca\textsubscript{1}a\textsubscript{2}X sequence (CVIM) is normally farnesylated, but in the presence of an FTI it can be alternatively prenylated by GGTaseI [107]. Alternative prenylation of K-Ras is thought to contribute to K-Ras FTI evasion.

Over 30 proteins are known FTase substrates [49] and as many as 700 proteins in the human genome contain a C-terminal Ca\textsubscript{1}a\textsubscript{2}X box [56]. The Ca\textsubscript{1}a\textsubscript{2}X sequence alone is all that is required for FTase recognition and small peptides corresponding to the Ca\textsubscript{1}a\textsubscript{2}X sequence often mimic the reactivity of full length proteins [204, 214]. The Ca\textsubscript{1}a\textsubscript{2}X sequence of a protein is often unique to a single protein or to subsets of proteins [56]. An alternative mechanism to inhibit the function of prenylated proteins is to selectively modify particular Ca\textsubscript{1}a\textsubscript{2}X sequences with lipid groups that do not support normal prenyl group function [141]. Numerous analogs of FPP such as 8-anilinogeranyl diphosphate (AGPP, 2a) are utilized by farnesyl transferase to modify Ca\textsubscript{1}a\textsubscript{2}X proteins both in vitro and in cell culture [159]. The aniline moiety of AGPP appears to act as an isostere for the \(\omega\)-isoprene unit of FPP and reacts with various Ca\textsubscript{1}a\textsubscript{2}X peptides with steady-state kinetics nearly identical to FPP [161]. However, subtle changes in the structure of AGPP drastically alter FTase activity [161].
Figure 5-1 Analog Structures
Structure of farnesyl diphosphate (1, FPP) 8-anilinogeranyl diphosphate (2a, AGPP)

1: FPP

2a: R=H AGPP
2b-p see table 1

3: BenzophenoneGPP
The reaction mechanism of FTase is unexpectedly complex (Figure 5-2) [49, 82]. Substrate association is assumed to proceed through a functionally ordered mechanism where FPP (1) binds first, giving the enzyme-FPP complex (E•FPP) followed by Ca₁a₂X substrate association forming the enzyme-FPP-Ca₁a₂X peptide complex (E•FPP•CaaX) [72, 201]. FPP binding to free enzyme enhances association of the Ca₁a₂X substrate up to 70-fold. After thioether formation, a modified peptide remains associated with FTase in an enzyme-product complex (E•product) [72, 74]. Product release is the rate determining step (k_{cat}) for the FTase reaction mechanism, and must be stimulated by the binding of either a new FPP or Ca₁a₂X peptide substrate to the E•product complex. Another interesting feature of the FTase reaction mechanism is the close association between the isoprenoid lipid and the target Ca₁a₂X sequence in the E•product•FPP and E•FPP•CaaX complexes. In both complexes, FPP binds at the same hydrophobic surface and the ω-isoprene of FPP associates directly with the C-terminal amino acids of the Ca₁a₂X sequence. The interaction between the target Ca₁a₂X motif and the isoprenoid indicates that changes in the structure of FPP may be analogous to amino acid mutations in the active site of the enzyme.
Figure 5-2  FTase reaction mechanism
Extended mechanism incorporating peptide stimulated product release. The mechanism is split into two pathways path A representing FPP stimulated product release and path B representing peptide stimulated product release. E is the FTase enzyme, E-FPP is the FTase-FPP complex, E-FPP-CaaX is the FTase-FPP-CaaX peptide complex, E-Product is the FTase bound product complex, E-Product-FPP is the FTase bound to both FPP and the reaction product, and E-CaaX is the peptide bound FTase inhibitory complex, E-Product-CaaX is the new peptide bound enzyme product complex
Since the isoprenoid donor of the FTase reaction interacts directly with the target Ca$_1$a$_2$X substrate then the activity of an analog may be highly dependent upon the sequence of the isoprenylation target [80, 154]. In this chapter, to determine the effects from altering the acceptor peptide sequence and the isoprenoid structure we have tested the ability of ortho- substituted AGPP analogs to act as substrates with multiple Ca$_1$a$_2$X peptides. Several analogs that were potent FTIs with a H-Ras based CVLS peptide as the acceptor were excellent substrates when the isoprenylation target was a K-Ras4B based CVIM peptide. Several of the analogs enhanced the selectivity of FTase for CVIM over several other important Ca$_1$a$_2$X sequences. These results suggest that analogs of the transferable FTase substrate AGPP can convert FTase from a relatively promiscuous enzyme to an enzyme that selects for specific Ca$_1$a$_2$X substrates. Selective modification of FTase target substrates is the first step to designing Ca$_1$a$_2$X sequence selective molecules that can be used to inhibit the function of specific cellular proteins.

**Results**

**AGPP analog reactivity is dependent on Ca$_1$a$_2$X acceptor sequence**

In order to investigate the relationship of the isoprenoid donor and peptide target structure on the FTase reaction we measured the steady-state transfer kinetics of a K-Ras4B based CVIM peptide and 15 ortho- substituted AGPP analogs 2a-p. The reaction kinetics were measured utilizing a continuous fluorescence assay with peptides N-linked through a glycine linker to a fluorescent dansyl reporter group. We then compared the catalytic efficiency of these reaction with those previously published with a H-Ras based CVLS peptide. The CVIM (K-Ras4B) and CVLS (H-Ras) Ca$_1$a$_2$X sequences are two of the most extensively characterized Ca$_1$a$_2$X substrates in the literature and we found $k_{cat}/K_m^{peptide}$ values with FPP identical to those previously published (Figure 5-3) [154]. We found that the $k_{cat}/K_m^{peptide}$ with the two Ca$_1$a$_2$X peptides differed depending on both the Ca$_1$a$_2$X substrate sequence and the isoprenoid analog structure. With FPP and AGPP as the isoprenoid donors the catalytic efficiency with CVLS was nearly two-fold higher than the catalytic efficiency with CVIM. However, with the oCl-AGPP 2c, oCF$_3$-
AGPP 2e, oEt-AGPP 2h, oI-AGPP 2i, oCF3O-AGPP 2j, and oSMe-AGPP 2k analogs
the catalytic efficiency of the reaction with CVIM was much higher than the efficiency
with CVLS. Surprisingly, CVIM was efficiently utilized with the oCF3O-AGPP 2j analog
although previously we observed that this analog was not a substrate with CVLS and
was a potent inhibitor of CVLS farnesylation. (Table 5-1) These results indicated that the
reactivity of the analogs was highly dependent on the identity of the Ca1a2X target
sequence.

In chapter 4, I showed that the ortho- substituted AGPP analogs were unable to
effectively compete with FPP for CVLS modification. The ability of the analogs to
compete with FPP may also be dependent on the target Ca1a2X sequence. To test the
ability of the analogs to compete with FPP for the CVIM modification we mixed each
analog and FPP 1:1 in excess of the CVIM peptide and analyzed the resulting product
by HPLC and integration of the dansyl peak absorbance. We found that AGPP
competed with similar effectiveness when CVLS and CVIM were the isoprenylation
targets (Figure 5-4). However, the oCF3O-AGPP 2j, oMe-AGPP 2d, and oMeO-AGPP
2f analog competition with FPP was enhanced when CVIM was the target Ca1a2X motif.
Interestingly, the ability of the oF-AGPP 2b analog to compete was decreased with
CVIM. These results suggested major differences in the activity and competitiveness of
the AGPP analogs depending on the amino acid sequence of the target peptide Ca1a2X
motif.
Figure 5-3 Peptide transfer efficiency

Steady-state $k_{cat}/K_m^{peptide}$ values from each of the ortho- substituted AGPP analogs with the CVLS and CVIM peptides. Kinetics were measured with varying concentrations of peptide and holding the analog concentration constant at 6.7 µM. All reactions were performed in Tris-HCl (pH=7.4) buffer containing reducing agent and detergent. Note that the efficiency of the reactions with CVIM and CVLS change with the different analogs and that several analogs are effective substrates with CVIM but not CVLS.
Figure 5-4 Analog Competitiveness with FPP

Ratios of products analog modified Ca₁₂X:farnesylated Ca₁₂X were found from reactions set up with 3µM peptide and 6 µM of the analog and FPP. Reactions were allowed to continue until all peptide was consumed and were then analyzed by HPLC. Fluorescence detection indicated the retention time of each of the products and integration of peaks at 340 nm were used to quantify products. Note that the ability of the analogs to compete is also dependent on the identity of the peptide.
AGPP analog substituent size affects Ca1a2X reactivity

The peptide target selectivity of the FTase enzyme can be predicted by the ratio of the Ca1a2X $K_m^{peptide}$ (see chapter 3). The $K_m^{peptide}$ values of the different Ca1a2X sequences suggest that the analogs may increase the selectivity of the enzyme for particular Ca1a2X peptides. To investigate the structural properties of the ortho-substituted AGPP analogs that affected FTase target selectivity we determined the steady-state $K_m^{peptide}$ of four Ca1a2X sequences with all 15 ortho- substituted AGPP analogs. (Table 5-1) The peptide targets included the CVLS and CVIM Ca1a2X sequences as well as the Ca1a2X motifs of the DNAJ homologue RDJ-2 (CAHQ) and the centromere protein, CenpF-f (CKVQ). The analogs that were not substrates were further screened for their ability to act as a FTI. (Figure 5-5)

The ortho- substituted analog reactivity fell into four general categories of substrate/inhibition activity that appeared to be dependent on the size of the ortho-substituent. The largest analogs (class 4, 2m-p) were not substrates with any of the peptides, and three of these analogs (oPh-AGPP 2n, oiPr-AGPP 2m, and oPhCH$_2$-AGPP 2p) were able to inhibit the farnesylation of the CVLS, CVIM and CAHQ peptides. (Figure 5-5) All of the other AGPP analogs tested (Class 1-3, 2a-l) were substrates, but the activity depended on the target Ca1a2X sequence. The $K_m^{peptide}$ in reactions where the analogue functional group substitutions were the smallest were similar to those with FPP and AGPP (class 1, 2b-c, 2f-g). The medium sized analogs (class 2, 2d-e, 2h-i and class 3, 2j-l) were selective for CVIM, based on the apparent $K_m^{peptide}$. The class 2 analogs showed some reactivity with the CVLS peptide while the class 3 analogs reacted very poorly with all of the peptides other than CVIM. The class 3 analogs also potently inhibited the farnesylation of CVLS. These results indicated that the selectivity of the FTase enzyme for the different Ca1a2X substrates is highly dependent on the size of the ω-aniline moiety.
Table 5-1 $K_m^{\text{peptide}}$ for ortho substituted analogs

$K_m^{\text{peptide}}$ kinetics were measured with varying concentrations of peptide and with analog concentrations constant at 6.7 µM. All reactions were performed in Tris-HCl (pH=7.4) buffer containing reducing agent and detergent. The size of each of the aniline units was estimated using the PreADME online molecular descriptor software. The analog class is based on the ability of each analog to inhibit and act as a substrate for the FTase enzyme as discussed in the text. Note that the analogs fall into four general categories of reactivity.

<table>
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<th>class</th>
<th>R</th>
<th>#</th>
<th>Size*</th>
<th>$K_m^{\text{CVLS}}$ (µM)</th>
<th>$K_m^{\text{CAHQ}}$ (µM)</th>
<th>$K_m^{\text{CKVQ}}$ (µM)</th>
<th>$K_m^{\text{CVIM}}$ (µM)</th>
<th>$K_i^{\text{PP}}$</th>
<th>CVLS (nM)</th>
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<td>2l</td>
<td>155</td>
<td>NR***</td>
<td>NR***</td>
<td>&gt;10</td>
<td>4.0 ± 0.6</td>
<td>12 ± 1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>oIPr</td>
<td>2m</td>
<td>159</td>
<td>NR***</td>
<td>NR***</td>
<td>NR***</td>
<td>&gt;10</td>
<td>4.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>oPh</td>
<td>2n</td>
<td>187</td>
<td>NR***</td>
<td>NR***</td>
<td>NR***</td>
<td>NR***</td>
<td>3.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>oPhO</td>
<td>2o</td>
<td>198</td>
<td>NR***</td>
<td>NR***</td>
<td>NR***</td>
<td>NR***</td>
<td>&gt; 40</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>oPhCH$_2$</td>
<td>2p</td>
<td>202</td>
<td>NR***</td>
<td>NR***</td>
<td>NR***</td>
<td>NR***</td>
<td>18 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

* NR indicates no reaction was observed
Figure 5-5: Percent inhibition of FTase farnesylation
N-dansyl-GCaaX a) CVIM or b) CAHQ was mixed with FPP and an equimolar amount of the analog. FTase was added and the percent inhibition was calculated by comparing the initial rate of reactions containing the analog and FPP to the reaction rate with FPP alone. Note that the largest analogs inhibit both CVIM and CVLS and that CAHQ farnesylation is more easily inhibited than CVIM.

A) CVIM

B) CAHQ
Analogs with similar structures to $\text{oCF}_3\text{O-AGPP}$ are highly selective for CVIM peptide

The third class of molecules was made up of analogs that only reacted with the CVIM peptide and inhibited farnesylation of the other peptides. In order to determine whether or not the analogs were selective for only CVIM we measured the competition products from reactions containing the class 3 analogs $\text{oCF}_3\text{O-AGPP 2j}$ or $\text{oSMe-AGPP 2k}$ (Figure 5-6) with competing CVIM and CVLS or CVIM and CAHQ peptide targets. We found with both analogs only the CVIM peptide was modified at the detection limits of the assay. Interestingly, even after the CVIM peptide had been completely consumed with the $\text{oCF}_3\text{O-AGPP 2j}$ analog, very little if any modified CVLS peptide was detected. (Figure 5-5a)

The reaction rate of the CVIM/CVLS competition reaction with $\text{oCF}_3\text{O-AGPP 2j}$ appeared to be the same as the reaction rate with only CVIM present. This indicated that the presence of CVLS had no affect on the modification of CVIM. To determine if higher concentrations of CVLS could effectively inhibit the modification of CVIM, we measured the reaction rate with increasing CVLS concentrations. At CVLS concentrations as much as five times the concentrations of CVIM no inhibition was observed. Taken together these results suggest that the class 3 analogs convert FTase from a relatively promiscuous enzyme to one that selects for only the CVIM peptide.
Figure 5-6 Competition oCF3O and oSMe plus two peptides
Reactions containing two peptides mixed at equimolar concentrations with excess oCF3O-AGPP or oSMe-AGPP were initiated by the addition of FTase. After several hours the reaction was stopped and analyzed by HPLC. Below are the fluorescence chromatograms from each reaction. a) oCF3O-AGPP, N-dansyl-GCVIM, N-dansyl-GCVLS b) oCF3O-AGPP, N-dansyl-GCVIM, N-dansyl-GCAHQ c) oSMe-AGPP, N-dansyl-GCVIM, N-dansyl-GCVLS d) oSMe-AGPP, N-dansyl-GCVIM, N-dansyl-GCAHQ. Note that the CVIM peptide is the only peptide modified even after the majority of it has been consumed.

a) oCF3O-AG-CVIM
   CVLS
   CVIM
   oCF3O-AG-CVLS

b) oCF3O-AG-CVIM
   CAHQ
   CVIM
oCF₃O-AGPP inhibits CVLS farnesylation only when CVIM is not present

Since the oCF₃O-AGPP 2j analog was an inhibitor of CVLS farnesylation yet a competitive substrate when CVIM was the target Ca₁a₂X sequence we examined the affect of CVIM on the CVLS farnesylation inhibition reaction. To do this we prepared a CVIM spike experiment where CVLS, FPP and oCF₃O-AGPP 2j were mixed and allowed to react for 10 minutes. After the ten minute period CVIM peptide was added to the reaction mixture and the rate of the reaction was measured. Upon CVIM addition the reaction rate immediately increased to the modification rate of CVIM with the oCF₃O-AGPP 2j analog. These results indicated that the presence of the CVIM peptide relieved FTase inhibition. However, it was not clear whether the CVLS peptide farnesylation was still inhibited after the addition of the CVIM peptide. We prepared a separate reaction containing FPP, oCF₃O-AGPP 2j, CVLS and CVIM, and allowed the reaction to progress for several hours, then analyzed the products by HPLC. (Figure 5-7) Surprisingly, we found that CVLS was farnesylated when CVIM was present, yet without CVIM in the mixture CVLS was modified to a much lesser extent. Previously we have shown that the ratio of products from a CVIM/CVLS competition reaction with FPP was 3:1 f-CVIM:f-CVLS. However, in the presence of oCF₃O-AGPP 2j the ratio of f-CVIM to f-CVLS was changed to nearly 1:1. The prenylated CVIM peptide was split between the oCF₃O-AGPP 2j analog and FPP, while the CVLS peptide was only modified with a farnesyl group. These results indicated that oCF₃O-AGPP 2j was an inhibitor of the farnesylation of CVLS only when the catalytically competent CVIM peptide was not present.

Selectivity is dependent on Ca₁a₂X motif X residue

The ω-terminal isoprene unit of FPP is thought to directly interact with the a₂ residue of a target Ca₁a₂X box; however, it was not clear whether the peptide selectivity of the class 2 and 3 analogs was due to an interaction with the X residue or other Ca₁a₂X sequence residues. To determine what residue affected the selectivity induced by the analogs we switched the a2 residues of CVLS and CVIM to give CVIS and CVLS and determined the \( K_m^{peptide} \) of each with the analogs. (Table 5-2) The activity of the ortho- substituted analogs changed with the X residue not the Ca₁a₂X sequence a₂
residue. In the case of the class 2 and 3 molecules there was a productive interaction with the CVIM and CVLM peptides, but not with the CVIS and CVLS peptides. These results suggested that the selectivity of the analogs is dependent on the identity of the X residue of the target Ca₁a₂X sequence.

Discussion

Chemical Genetics of FTase

Previous work with the FTase enzyme has demonstrated that the enzyme catalyzes the modification of a wide variety of Ca₁a₂X substrates with varying steady-state kinetics. This chapter has shown that by altering the isoprenoid donor of the FTase reaction the ability of FTase to catalyze the modification of these different Ca₁a₂X substrates is changed. Several of the analogs that were effective inhibitors of the farnesylation of a CVLS peptide were excellent substrates for the enzyme, and induced unnatural selectivity for peptide with a methionine residue in the Ca₁a₂X motif X position. This chapter demonstrates that changing the structure of the isoprenoid alters the activity of the enzyme in a manner analogous with amino acid mutations in the active site of the enzyme.
Figure 5-7 Peptide and FPP competition with oCF3OAGPP

Quaternary competition reactions between two peptides an analog and FPP. Reactions containing two peptides mixed at equimolar concentrations with excess oCF$_3$O-AGPP equimolar to FPP were initiated by the addition of FTase. After several hours the reaction was stopped and analyzed by HPLC. Below are the chromatograms from fluorescence detection of the mixtures. a) FPP, oCF3OAGPP, N-dansyl-GCVIM, N-dansyl-GCVLS b) FPP, oCF3OAGPP, N-dansyl-GCVIM, N-dansyl-GCAHQ. Note that the CVIM peptide is modified by both the analog and FPP while the competing peptide is only modified with FPP.

a)

b)
Table 5-2 $K_m^{\text{peptide}}$ values with CVIS and CVLM peptides

Kinetic parameter $K_m^{\text{peptide}}$ for N-dansyl-GCVIS and N-dansyl-GCVLM for comparison with N-dansyl-GCVIM and N-dansyl-GCVLS. $K_m^{\text{peptide}}$ was measured with varying concentrations of peptide and with analog concentrations constant at 6.7 µM. All reactions were performed in Tris-HCl (pH=7.4) buffer containing reducing agent and detergent. Note that the $K_m^{\text{peptide}}$ follows the X residue not the a$_2$ residue.

<table>
<thead>
<tr>
<th>R</th>
<th>#</th>
<th>$K_m^{\text{CVLS}}$</th>
<th>$K_m^{\text{CVIM}}$</th>
<th>$K_m^{\text{CVIS}}$</th>
<th>$K_m^{\text{CVLM}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPP</td>
<td>1</td>
<td>0.8 ± 0.1</td>
<td>0.30 ± 0.06</td>
<td>0.22 ± 0.04</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>oEt</td>
<td>2x</td>
<td>7 ± 1</td>
<td>3.6 ± 0.9</td>
<td>5 ± 1</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>oI</td>
<td>2x</td>
<td>8 ± 3</td>
<td>1.1 ± 0.1</td>
<td>&gt;10</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>oCF$_3$O</td>
<td>2j</td>
<td>&gt;10</td>
<td>2.76 ± 0.08</td>
<td>&gt;10</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>oSMe</td>
<td>2x</td>
<td>&gt;10</td>
<td>4.4 ± 0.6</td>
<td>&gt;10</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>
Active Site Discrimination of Peptides

There are two possibilities that explain the ability of oCF₃O-AGPP 2j to inhibit CVLS farnesylation, yet act as a substrate with CVIM. The first of these possibilities is that the product from the reaction of oCF₃O-AGPP and CVLS cannot be dissociated by the CVLS peptide, FPP or oCF₃O-AGPP 2j. The second possibility is that the chemical step of the reaction is compromised with the oCF₃O-AGPP analog and CVLS peptide. Both mechanisms of inhibition could be relieved by the addition of a more competent peptide to either stimulate product release or react with oCF₃O-AGPP 2j. If there was no discrimination between CVIM and CVLS prior to the chemical step of the reaction, CVIM relief of product inhibition would lead to an increase in oCF₃O-AG-CVLS formation in the CVIM, CVLS, FPP and oCF₃O-AGPP 2j quaternary competition experiment. However, the amount of CVLS modified with oCF₃O-AGPP 2j in the quaternary competition experiment was the same as when CVIM was not present. Interestingly, the amount of CVLS modified by FPP relative to CVIM increased in the presence of oCF₃O-AGPP 2j. The most likely cause of this increase is the selective depletion of the CVIM peptide by the E•oCF₃O-AGPP complex. The ability of oCF₃O-AGPP 2j to inhibit the modification of the CVLS and CAHQ peptides, yet competitively react with the CVIM peptide is most likely due to a pre-chemical step discrimination between the CVIM and CVLS peptides by the E•oCF₃O-AGPP complex. A selective target peptide exclusion from the FTase active site demonstrates that changing the structure of the isoprenoid alters the activity of the enzyme in a manner analogous with amino acid mutations in the active site of the enzyme.

The exclusion of peptide by an FPP analog has been described previously. Turek-Etienne et al described a benzophenone based FPP (benzophenone-GPP 3) analog that inhibits FTase by exclusion of a target peptide from the active site of the enzyme [215]. Support for this mechanism of inhibition was achieved from a crystal structure of the analog bound to FTase where the benzophenone portion of the molecule (3) protruded into the X residue binding site, presumably excluding peptide from the active site. In this chapter, I have described a similar mechanism except that differences in the structure of the target peptide appear to affect the ability of the analog
to exclude particular peptides. Interestingly, we have found that the peptide selectivity of the molecules reported were dependent upon the X residue of the target Ca$_{1}$a$_{2}$X sequence. These observations are consistent with the peptide exclusion model of inhibition.

Other Regions of Protein may Affect Reactivity

This chapter was designed to analyze the Ca$_{1}$a$_{2}$X selective properties of the enzyme with respect to interactions in the active site of the enzyme. It is not yet clear if the peptide selectivity of these analogs with FTase will translate directly to selectivity for full length protein targets. In particular, the K-Ras4B protein contains an upstream poly-basic region that enhances affinity for the enzyme through interactions outside the enzyme active site. However, H-Ras proteins do not contain an upstream poly-basic region and the steady-state transfer kinetics are nearly identical to those of the Ca$_{1}$a$_{2}$X peptide alone. We have shown that several of the analogs enhance FTase selectivity for the K-Ras4B Ca$_{1}$a$_{2}$X peptide. Increased affinity by the upstream poly-basic region would likely only further enhance this selectivity. The ability to target K-Ras4B will be useful for Ca$_{1}$a$_{2}$X selective protein function inhibition.

Experimental

General

AGPP analogs and FPP were synthesized as described in Chapter 2. All RP-HPLC was performed on an Agilent 1100 HPLC system equipped with a microplate autosampler, diode array and fluorescence detector. The HPLC analysis was performed with a microsorb C18 column with 0.01% TFA in water (A) and 0.01% TFA CH$_3$CN (B) as the mobile phase. Peptides were purchased from peptidogenics and each contained a dansyl group N-linked to a glycine linker followed by a C residue then a variable aaX sequence (VLS, VIM, AHQ, or KVQ). Spectrofluorometric analyses were performed in a 96-well flat bottom, non-binding surface, black polystyrene plate (Excitation wavelength, 340 nm; emission wavelength 505 nm with a 10 nm cutoff). The plate reader was a SpectraMax GEMINI XPS fluorescence well-plate reader. Absorbance readings were determined using a Cary UV/Vis spectrophotometer.
Steady-state peptide kinetics

The kinetic constants $K_{m}^{\text{peptide}}$ and $k_{cat}$ with FPP, and the analogs with the each peptide were determined in triplicate measurements using a continuous spectrofluorometric assay adapted for a 96-well plate. The following assay components were assembled in individual wells of a 96-well plate and incubated at 30° C for 20 minutes: 180 µL of assay buffer (52 mM Tris-HCl, 12 mM MgCl₂, 12 µM ZnCl₂ pH 7.4), 40 µL of detergent (0.125% n-dodecyl- β-D-maltoside in assay buffer), 40 µL of reducing agent (50 mM DTT in assay buffer), 20 µL of FPP or AGPP (200 µM in 25 mM NH₄HCO₃) and 20 µL of N-dansyl-GCaaX peptide (variable concentration). Fluorescence was detected using a time based scan at 30° C for 120 minutes. The velocity of each reaction was determined by converting the rate of increase in fluorescence intensity units (FLU/s) to µM/s with equation 5-1.

Equation 6-1: $v_{i} = \frac{R \cdot P}{F_{\text{MAX}} - F_{\text{MIN}}}$

Where $v_{i}$ is the velocity of the reaction in µM/s. R is the rate of the reaction in FLU/s. P is equal to the concentration of modified product in µM (see below). $F_{\text{MAX}}$ is the fluorescence intensity of fully modified product. $F_{\text{MIN}}$ is the fluorescence of a reaction mixture that contained 20 µL of assay buffer in the place of FTase. Complete modification of peptides was assumed when fluorescence stabilized for more than 10 minutes and was confirmed by RP-HPLC analysis of the reaction mixture.

Final analysis peptide concentrations were chosen based on preliminary determination of the $K_{m}^{\text{peptide}}$ with 0.5, 1, 5 and 10 µM dansyl-GCaaX concentrations (see below). The final analysis used 8 peptide concentrations which were: 1/6, 1/5, 1/4, 1/2, 3/4, 1, 2, and 3 times the estimated $K_{m}^{\text{peptide}}$ value except with the CVIM peptide. The reaction was then initiated by the addition of 20 µL of FTase (final concentration 10 nM with the CLVS, CAHQ, CVIL and CRPQ peptide, 20nM with the CVIM peptide). Fluorescence enhancement was determined as described above. Complete modification of peptides was assumed when fluorescence stabilized for more than 10 minutes. Samples in which the reactions that contained higher concentrations of peptides did not go to completion the Fmax-Fmin value was extrapolated using a linear plot of the Fmax-Fmin for each of the lower concentration reactions that did go to completion.
The velocities of the reactions were plotted against the concentration of peptide and were fit to the Michaelis-Menten equation (Equation 5-2) to give the apparent $k_{cat}$ (app$k_{cat}$) and $K_m^{peptide}$ (app$K_m^{peptide}$) values. Where $e_t$ was the total enzyme concentration and [Pep] was the total input peptide. The $k_{cat}/K_m^{peptide}$ value was derived from a Lineweaver-Burke plot of the same data points.

**Equation 5-2:** $v_i/e_t = (appk_{cat}*[Pep])/(appK_m^{peptide}+[Pep])$

**Peptide Competition**

Competition reactions were prepared with the same components given for the $k_{cat}/K_m^{peptide}$ analysis except two peptides were added to the mixture at a single concentration. Peptides A and B were diluted to 45 µM in Tris-HCl buffer (pH=7.4) and the concentration checked by reading the Abs of the solution at 340 nm ($\varepsilon_{\text{dansyl}}=4250$ Abs·M$^{-1}$·cm$^{-1}$). 20 µL of peptide A and 20 µL of peptide B were then added to reaction mixtures as described above except only 160 µL of assay buffer was added. Separate reactions were prepared as a standard with FPP or the analog and only one peptide as described above. The reactions were then initiated with the addition of FTase and analyzed spectrofluorometrically (Reactions containing CVIM peptide used 20 nM final FTase concentration all other used 10 nM). The reactions were stopped prior to consumption of more than 50% of either peptide by adding 20 µL of a stop solution (isopropyl alcohol and acetic acid 8:2). HPLC analysis was then performed with 100 µL of the reaction mixture loaded onto C18 column and eluted with a linear gradient of 0-30 min 10% B to 100% B at a flow rate of 1 mL/min. Peaks on the 340 nm trace chromatogram that corresponded to both the standard reactions and fluorescent peaks were then integrated. The ratios of products were calculated according to equation 5-3.

**Equation 6-3:** $(I_{A_{\text{mod}}}/I_{A_{\text{tot}}})/(I_{B_{\text{mod}}}/I_{B_{\text{tot}}})$

Where $I_{A_{\text{mod}}}$ is the integral of the modified peptide A, $I_{A_{\text{tot}}}$ is the integral of the modified peptide A plus the unmodified peptide A, $I_{B_{\text{mod}}}$ is the integral of the modified peptide B and $I_{B_{\text{tot}}}$ was the integral of the modified peptide B plus the unmodified peptide B. Alternatively, the ratios were calculated using the integrals of the standard peptides fluorescence relative to competition product fluorescence giving less than a 5% difference in the calculated ratio.
FPP competition

Competition reactions were prepared with the same components given for the $k_{cat}/K_m^{peptide}$ analysis except two analogs were added to the mixture and the peptide concentration was held constant. FPP and the analog were diluted to 200 µM in 25 mM NH$_4$HCO$_3$. 10 µL of FPP and 10 µL of analog were then added to a 280 µL solution of peptide, DTT and detergent in 52 mM Tris-HCl buffer pH=7.4 (final concentrations DTT=6.7 mM, DM=0.033%, Peptide=3µM, analog= 6.7 µM and FPP=6.7 µM). The concentration of peptide was determined by absorbance of the dansyl group and isoprenoid diphosphate concentration was determined by consumption of isoprenoid in FTase reactions with the CVLS peptide where the isoprenoid was limiting. The reactions were initiated with the addition of FTase and analyzed spectrofluorometrically (20 nM final FTase concentration). The reactions were stopped after 2 hours with a mixture of acetic acid and iPrOH. HPLC analysis was then performed with 100 µL of the reaction mixture loaded onto C18 column and eluted with a linear gradient of 0-30 min 10% B to 100% B at a flow rate of 1 mL/min. Peaks on the 340 nm trace chromatogram that corresponded to standard reaction fluorescent peaks were then integrated. The ratios of products were calculated using $I_{analog}/I_{FPP}$, where $I_{analog}$ is the integral of the peptide modified with the analog and $I_{FPP}$ is the integral of the peptide modified with FPP.

Inhibition Screen

The analog ability to inhibit FTase transfer of FPP was initially determined by measuring the percent inhibition in farnesylation rate in the presence of equimolar amounts of FPP and the analog. Reactions were prepared as described for the FPP competition analysis and the rate of FPP modification was determined based on the rate of fluorescence increase over time with or without the analog present. The % inhibition was determined using equation 5-4.

**Equation 5-4:** % inhibition=\left [ 1- \frac{\text{rate}_{FPP}}{\text{rate}_i} \right ]*100

Where the rate$_{FPP}$ was the rate of the CVLS reaction without the analog present and the rate$_i$ was the rate of the reaction with the analog present.
**FPP and peptide competition**

Peptides A and B were diluted to 45 µM in Tris-HCl buffer (pH=7.6) and the concentration checked by reading the Abs of the solution at 340 nm ($\varepsilon_{\text{dansyl}}=4250 \text{ Abs} \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$). FPP and oCF3O-AGPP were diluted to 200 µM in 25 mM NH$_4$HCO$_3$. 20 µL of peptide A and 20 µL of peptide B were then added to reaction mixtures along with 10 µL of FPP and 10 µL of analog were added to reaction mixtures containing 140 µL of assay buffer (52 mM Tris-HCl, 12 mM MgCl$_2$, 12 µM ZnCl$_2$ pH 7.4), 40 µL of detergent (0.125% n-dodecyl- β-D-maltoside in assay buffer), 40 µL of reducing agent (50 mM DTT in assay buffer) Separate reactions were prepared as a standard with FPP or the analog and only one peptide as described above. The reactions were then initiated with the addition of FTase (Final concentration 20 nM) and analyzed spectrofluorometrically. The reactions were stopped prior to consumption of more than 50% of either peptide by adding 20 µL of a stop solution (isopropyl alcohol and acetic acid 8:2). RP-HPLC analysis was then performed with 100 µL of the reaction mixture loaded onto C18 column and eluted with a linear gradient of 0-30 min 10% B to 100% B at a flow rate of 1 mL/min. Peaks on the 340 nm trace chromatogram that corresponded to both the standard reactions and fluorescent peaks were then integrated.
CHAPTER 6 CELLULAR ACTIVITY OF ISOPRENOID DIPHOSPHATES

Introduction

Monitoring the prenylation state of proteins in cells is a challenging undertaking. Initially, prenylation of proteins was discovered by metabolic labeling with $[^3H]$mevalonolactone [216]. In these experiments, some of the tritium label was incorporated into both farnesylated and geranylgeranylated proteins. Subsequently, a salvage pathway was discovered where radiolabeled farnesol 1a (FOH) and geranylgeraniol 2a (GGOH), precursors of FPP 1b and GGPP 2b, were selectively incorporated into their respective farnesylated or geranylgeranylated proteins [217]. A significant drawback of these approaches is the inherently low sensitivity of autoradiographic detection of the weak tritium $\beta$-emission. In fact, it can take up to four weeks to visualize proteins extracted from cells labeled with tritiated mevalonate or farnesol by autoradiography [141]. Also, the incorporation of tritiated prenyl groups does not provide a convenient method for isolation of these modified proteins.
Figure 6-1 FPP Analogs

1a: \( R_1 = \text{OH} \)
1b: \( R_1 = \text{P}_2\text{O}_7^{3-} \)

2a: \( R_1 = \text{OH} \)
2b: \( R_1 = \text{P}_2\text{O}_7^{3-} \)

3a: \( R_1 = \text{OH} \)

4: \( R_1 = \text{OH} \)

5a: \( R_1 = \text{OH} \)
5b: \( R_1 = \text{P}_2\text{O}_7^{3-} \)

6a: \( R_1 = \text{OH} \)
6b: \( R_1 = \text{P}_2\text{O}_7^{3-} \)
The facile detection, isolation and purification of farnesylated proteins based solely on their post-translational modification status is essential to understanding the mechanism of cellular growth inhibition by FTIs. Antibodies are useful both in the routine detection and immunoprecipitation of proteins with other post-translational modifications, such as phosphorylation [218]. However, reports of previous attempts to produce antibodies to detect farnesylated proteins have had mixed results. Two of these reports described non-specific antibodies that could not differentiate between proteins modified by farnesylation, geranylgeranylation or other lipids [113, 219]. The other report from Baron et al does describe the production of anti-farnesyl antibodies specific for farnesylation, but the analysis for specificity was limited [220]. These antibodies are apparently not available commercially and all of the commercially available sources for anti-farnesyl antibodies cross-react with geranylgeranylated proteins.

An attractive alternative to targeting the natural farnesyl moiety is to detect proteins modified with unnatural farnesyl analogs. Several unnatural analogs of FPP appear to be utilized by cells and incorporated into the proteins by FTase, including the unnatural FPP analog 3-vinyl-farenosol 3a, a pro-drug of the FTase transferable 3-vinyl-farnesyl diphosphate (3vFPP) 3b, and 8-azido-farnesol 4 [141]. A tagging-via-substrate (TAS) approach to the detection and isolation of farnesylated proteins involving the incorporation of 4 into cellular proteins has been developed by Kho et al. [168]. Modified proteins were isolated from cell lysates using a biotinylated phosphine capture reagent and subsequently identified by mass spectrometry. However, the sensitivity is relatively low and the technique does not lend itself to the routine detection of modified proteins.

A more convenient method to simplify the detection of such proteins would be to use antibodies specific for the unnatural modification.

A variety of FPP analogs that may be useful in implementing this strategy have been prepared by our group and others [149, 157, 193, 221]. Anilinogeranyl diphosphate (AGPP) 5b is transferred by FTase to protein substrates with kinetics similar to those of FPP and does not resemble any known natural protein modification [159, 161]. Antibodies against an anilinogeranyl (AG) epitope may circumvent the problems associated with tritium labeling and the preparation of anti-farnesyl antibodies, and provide a facile way to detect and isolate prenylated proteins.
We report the preparation of anti-anilinogeranyl antibodies (anti-AG and anti-NAG) raised against haptens 12a and 12b that detect proteins modified with anilinogeranyl or $p$NO$_2$-anilinogernayl moieties derived from AGPP 5b and NAGPP 6b. Activated ester haptens 9a-b were synthesized and coupled to immunogenic carrier proteins and used to raise antibodies directed to the anilinogeranyl modification. The primary antibodies were able to bind proteins with an intact analog modified C-terminal Ca$_1$a$_2$X motif. We demonstrate that anilinogeraniol 5a, a prodrug form of AGPP 5b, is incorporated into cellular proteins in an FTase dependent manner, and that endogenous protein labeling was competitive with endogenous FPP 1b. Combining the unnatural FPP analog AGPP with anti-anilinogeranyl antibodies provides a useful reagent system to monitor the prenylation status and effects of FTIs in cells.

**Results**

**Synthesis of Analog modified Antigen**

The preparation of the prenyl analog carrier protein conjugates 12a-b and 13a-b is outlined in Figure 6-2. The haptens were designed to mimic the C-terminal peptide of a fully processed prenylated protein that had undergone both endoproteolysis and carboxyl group methylation. Thioether 8a-b was obtained in 60-74% yield by addition of a solution of l-cysteine methyl ester HCl in NH$_3$/MeOH to the previously described chloride 7a-b [161]. The farnesyl thioether 6b was prepared by addition of farnesyl bromide 6b to l-cysteine methyl ester HCl in NH$_3$/MeOH. Condensation of amines 8a-c with succinic anhydride in THF:DCM afforded succinates 9a-c in 57%-85% yield after chromatography [222]. Activated esters 10a-c were prepared from carboxylates 9a-c in 58%-85% yield by reaction with CDI followed by condensation with N-OH phthalimide [223]. The $N$-hydroxysuccimidyl (NHS) ester of a similar farnesyl hapten is reported to undergo an undesired intramolecular cyclization to an unreactive succinimide [219]. In contrast to the NHS ester, phthalimides 10a-b were stable and showed no evidence of intramolecular cyclization. The suitability of phthalimides 10a-b to form conjugates with carrier protein was modeled under conditions typical for coupling activated esters to lysine residues. Condensation of benzylamine with phthalimides 10a-b in 1:5 Acetonitrile:5% NaHCO$_3$ gave model amides 11a-b in 51%-76% yield.
With activated haptens in hand, we proceeded to generate anilinogeranyl, \( p\text{NO}_2\)anilinogeranyl and farnesyl immunoconjugates. Phthalimides 10a-c were reacted with carrier proteins KLH and BSA to provide hapten conjugated proteins 12a-c (AG-KLH, NAG-KLH and F-KLH) and 13a-c (AG-BSA, NAG-BSA and F-BSA). BSA has approximately 32 reactive lysine residues and the extent of covalent modification of immunoconjugates 13a-c was quantified by measuring the remaining free amino groups relative to unmodified BSA. BSA, and each of the BSA conjugates 13a-b were reacted with trinitrobenzene sulfonate and the ratio of free amines in unmodified BSA to modified BSA were determined by UV absorbance at 335 nm [224]. We found that 50% of the lysine residues in immunoconjugates 13a-c were modified by the activated esters 10a-c. As KLH is a heterogeneous protein, similar assays to quantify the coupling efficiency were not performed. By analogy with the BSA results, we assumed a similar coupling efficiency for the KLH bioconjugates.
Figure 6-2 Synthesis of analog modified haptens
Reagents: (a) Cysteine methyl ester HCl, NH₃/MeOH, 0° C (%). (b) Succinic anhydride, THF/DCM(%). (c) N-Hydroxyphthalimide, CDI, MeCN (%). (d) R², NaHCO₃(aq)/MeCN

7a-b

7a: R¹=aniline-CH₂C(CH₃)CHCH₂; X=Cl
7b: R¹=pNO₂-aniline-CH₂C(CH₃)CHCH₂; X=Cl
7c: R¹=geranyl; X=Br

8a-c

9a-c

10a-c

11a-c: R²=benzylamine
12a-c: R²=KLH
13a-c: R²=BSA
Generation of anti-anilinogeranyl antibodies

Polyclonal antibodies to the anilinogeranyl modification were prepared by immunizing rabbits with AG-KLH (12a) and NAG-KLH (12b). In our hands, F-KLH (12c) elicited no immunogenic response. This is in agreement with previous reports in which attempts to prepare anti-farnesyl antibodies have resulted in weakly binding reagents that recognize both farnesyl and geranylgeranyl isoprenoid lipids [113, 219, 220]. Due to the poor immune response to the F-KLH protein, (12c) we abandoned attempts to prepare an anti-farnesyl antibody. Polyclonal immune sera collected from the rabbits were screened using an enzyme linked immunosorbent assay (ELISA). No ELISA response was detected to unmodified BSA, AG-BSA (13a), or NAG-BSA (13b) in pre-immune sera. Gratifyingly, sera antibodies raised against AG-KLH 12a (anti-AG) and NAG-KLH 12b (anti-NAG) were able to bind to AG-BSA (13a) and NAG-BSA (13b) respectively (Figure 6-3). Interestingly, there was considerable cross-reactivity using the anti-AG antibody with the anilinogeranyl and pNO$_2$anilinogeranyl modified BSA proteins.
Figure 6-3  ELISA Assay of Anilinogeranylated BSA Constructs

a) anti-AG and b) anti-NAG ELISA response to AG-BSA (closed squares), NAG-BSA (open circles) and unmodified BSA (triangles) (0.1µg BSA/well). Note that the anti-AG antibody cross reacts with NAG-BSA and AG-BSA, yet the anti-NAG antibody only detects NAG-BSA.

a) Anti-AG ELISA

b) Anti-NAG ELISA
Specificity and Analysis of anti-anilinogeranyl antibodies

The antibodies generated in this study were directed to proteins modified with cysteine methyl ester analogs of FPP. However, the actual antigen that is presented to the immune system is unknown as the ester may be hydrolyzed by enzymes in the whole animal. Additionally, it is possible that in vivo incorporation of unnatural analogs into proteins will alter the normal Ca₁a₂X endoproteolysis and carboxyl group methylation steps. The utility of the polyclonal anilinogeranyl antibodies might well be limited if they only recognized the cysteine methyl ester form of modified proteins. Therefore, it was essential to test the ability of these antibodies to recognize proteins modified by FTase with the anilinogeranyl moiety without subsequent endoproteolysis and carboxyl group methylation. Bacterially expressed DNAJ-like RDJ2[200] and Q61L oncogenic H-Ras proteins contain the un-farnesylated and unprocessed CAHQ and CVLS Ca₁a₂X motifs respectively. FTase catalyzed in vitro modification of these proteins with FPP 1b, AGPP 5b, and NAGPP 6b provided lipid thioether linked proteins with intact Ca₁a₂X motifs 14a-c and 15a-c (Figure 6-4). These reaction mixtures were then analyzed by western blot with anti-AG and anti-NAG antibody sera. Figure 6-5 shows that the anti-AG was able to bind to AG-BSA 13a and NAG-BSA 13b derivatized by the methyl ester haptens as well as the AG-RDJ2 14c and NAG-RDJ2 14d, but, showed no reactivity to the unmodified RDJ2 14a protein. Identical results were found when AG-Ras 14c was examined. Additionally, the antibodies were also unreactive towards farnesylated F-RDJ2 (13b) and F-Ras (14b). Similar results were found with the anti-NAG antibody except the anti-NAG antibody was significantly more specific for the pNO₂-anilinogeranylated proteins over the anilinogeranylated.
Figure 6-4 Ca_{1:a_{2}}X modification

C-terminal structures of *in vitro* modified RDJ2 and H-Ras proteins

14a: RDJ2, $R_1=H$
14b: RDJ2, $R_1=farnesyl$
14c: RDJ2, $R_1=8$-anilinogeranyl
14d: RDJ2, $R_1=pNO_2$anilinogeranyl

15a: H-Ras, $R_1=H$
15b: H-RAS, $R_1=farnesyl$
15c: H-RAS, $R_1=8$-anilinogeranyl
15d: H-RAS, $R_1=pNO_2$anilinogeranyl
Figure 6-5 Western Analysis of Anilinogeranylated RDJ2
Western Blot analysis of unmodified (RDJ2-ve control), anilinogeranyl modified and Nitroanilinogeranyl modified RDJ2 (AG-RDJ2, NAGRDJ2) and BSA (AG-BSA, NAG-BSA) proteins. Probed with anti-AG (AG-Ab) and anti-NAG (NAG-Ab) antibodies. Note that as in the ELISA assay the anti-AG antibody detects both AG and NAG modified protein.
Since there was cross reactivity with the anti-AG antibody we tested the ability of the anti-AG and anti-NAG antibodies to recognize other aniline analogs incorporated into proteins by FTase. We reacted H-Ras with the analogs $p$CN-AGPP 16, $m$CN-AGPP 17, $o$CF$_3$O-AGPP 18, $m$CF$_3$O-AGPP 19, $p$CF$_3$O-AGPP 20, $o$F-AGPP 21, $m$F-AGPP 22, $p$F-AGPP 23, $o$l-AGPP 24, $m$l-AGPP 25, $p$l-AGPP 26, $o$Br-AGPP 27, $m$Br-AGPP 28, $p$Br-AGPP 29, $o$MeO-AGPP 30, $m$MeO-AGPP 31, $m$CF$_3$-AGPP 32, $m$Me-AGPP 33, and $p$Me-AGPP 34. As expected we found that the anti-AG antibody was less specific for the aniline substitution and was able to cross-react with the analogs containing small substitutions in the aniline ring of AGPP (Figure 6-6). This included the $m$CN-AGPP 17, $o$F-AGPP 21, $m$F-AGPP 22, $p$F-AGPP 23, $m$Br-AGPP 28, $p$Br-AGPP 29, $m$Me-AGPP 33, and $p$Me-AGPP 34 modified H-Ras proteins. The anti-NAG antibodies recognized the $m$CN-AGPP 16, $p$CN-AGPP 17, and $p$Br-AGPP 29 modified H-Ras proteins. These results suggested that the antibodies could be useful for many different anilinogeranyl diphosphate analogs not just the ones they were designed to recognize.
Figure 6-6 Cross Reactivity
Western blot analysis of Ras proteins modified with various AGPP analogs as indicated, and probed with anti-AG and anti-NAG polyclonal antibodies. Each well contains equal amounts of protein. However, extent of modification was not analyzed. Note that both antibodies recognize more aniline structure than they were designed to detect, yet they are specific for the aniline modified proteins and do not detect unmodified.

\[
\text{H} \quad \text{N} \quad R_1 \quad 16-34: \text{R indicated below}
\]

(a)

(b)
FTase dependent Anilinogeraniol incorporation into cellular proteins

Farnesol 1a, geranylgeraniol 2a and a number of farnesol analogs become incorporated into cellular proteins when added to the culture media of growing cells [141, 170, 217, 225]. These compounds are thought to traverse the plasma membrane and act as substrates for sequential kinase reactions to give FPP 1b, GGPP 2b or the diphosphate analog which can then be utilized by FTase or GGTaseI to appropriately modify cellular proteins [170, 226]. The kinases involved in the phosphorylation reactions have been characterized in plants, but not in mammalian cells. In vitro, both FPP 1b and AGPP 5b are transferable by FTase to a CVLS substrate with similar $k_{cat}$ and $K_m$ values [159]. Therefore, we speculated that anilinogeraniol might also cross the plasma membrane of cells, be converted to the diphosphate 5b and become incorporated into proteins by FTase. However, it was also possible that anilinogeraniol 5a might be toxic to mammalian cells. Accordingly, the acute cytotoxicity of anilinogeraniol 5a was examined by incubating human embryonic kidney (HEK-293) cells with up to 100 µM anilinogeraniol 5a for three days. Somewhat surprisingly, we found that cells treated with anilinogeraniol showed no morphological changes and no decrease in cell growth relative to controls at any of the concentrations tested.

The anti-AG antibodies were used to probe extracts of HEK-293 cells incubated in the presence of AGOH 5a to determine if the anilinogeranyl group was incorporated into cellular proteins. These cells were initially blocked with lovastatin an upstream inhibitor of endogenous FPP and GGPP formation and treated with GGHOH for the geranylgeranylation of proteins critical to cell survival [227]. Figure 6-7 shows that there are numerous anilinogeranyl modified proteins in cells treated with AGOH 5a, while no proteins were detected in the absence of the unnatural FPP analog. In order to determine whether the protein modification was FTase dependent, cells were incubated in the presence of AGOH 5a and either the farnesyl transferase inhibitor FTI-277 [93] or the geranylgeranyl transferase I inhibitor GGTI-2147 [228]. Lane 3 of Figure 6-7 shows that no anilinogeranyl modified proteins were detected in the presence of the FTI. However, in the presence of the geranylgeranyl transferase I inhibitor, the pattern of proteins modified with anilinogeranyl moiety was very similar to that without any inhibitor present. Interestingly, some additional, lower molecular weight anti-AG reactive bands
appear when GGTasel is inhibited. FTase catalyzed transfer of the anilinogeranyl lipid to protein is the most consistent explanation for these observations. These results also suggest that FTase may be able to utilize AGPP to modify some normally geranylgeranylated proteins when GGTasel is inhibited. This is not unreasonable, as the normally farnesylated K-Ras4B becomes geranylgeranylated in the presence FTIs [229, 230].

The AGOH analog must undergo diphosphorylation to be incorporated into cellular proteins. This diphosphorylation may not be on time-scale that allows competitive modification of proteins in the presence of FPP. Cells were incubated without a lovastatin blockade on endogenous FPP formation with 30 µM AGOH and sampled every few hours. Figure 6-8 shows that anilinogeranyl incorporation into proteins begins within two hours of incubation with AGOH and is therefore competitive with the endogenous pools of FPP. Importantly, these experiments demonstrate that intracellular concentrations of AGPP 5b can be achieved that are high enough to compete with the endogenous pool of FPP 1b for FTase dependent transfer to proteins.
Figure 6-7 FTase Dependent Incorporation of Anilinogeranyl Label in Cells

Incorporation and detection of anilinogeranyl modified endogenous proteins. HEK-293 cells were incubated with 30 µM lovastatin and 10 µM GGOH for one day then media was poured off and the cells were incubated with or without 100 µM AGOH 3a (lane 1-2), in the presence and absence of an FTI or a GGTI (lanes 2-4). The cells were then analyzed by western blot with the anti-AG sera. Note the difference in FTI + and – cells, and the 31 kDa band in the GGTI treated cells.

<table>
<thead>
<tr>
<th>AGOH</th>
<th>FTI</th>
<th>GGTI</th>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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[Western blot image with molecular weight markers: 97kDa, 66kDa, 45kDa, 31kDa, 22kDa, 14kDa]
Figure 6-8 Endogenous Competition
Cells with no lovastatin blockade were incubated with 30 µM AGOH for the given time points then were harvested and lysed. 50 µg of total protein was loaded onto an SDS-PAGE gel transferred to nitrocellulose and immunodetected using the anti-AG. Note the time dependent increase in anilinogeranylated proteins.
**Alternative Pro-drug strategy**

The labile phosphoanhydride bond and the multiple negative charges of FPP analogs likely prevent easy diffusion across cell membranes although naturally occurring isoprenoids such as FPP and geranylgeranyl diphosphate are apparently taken up through an active transport system [231]. The isoprenoids farnesol, geranylgeraniol and the unnatural analog 3-vinyl-farnesol (3a) are converted in mammalian cells to the corresponding pyrophosphates [141, 226, 232]. 3-vinyl-farnesyl diphosphate is an alternative substrate for FTase and the prodrug 3-vinyl-farnesol is able to block anchorage-independent growth of ras-transformed cells. Presumably, this effect is due to FTase catalyzed modification of proteins with the 3-vinyl-farnesyl group. These observations suggest that alcohol precursors of other FPP analogs may also be useful prodrugs.

However, it is far from clear that arbitrary farnesol analogs will be efficiently converted to the corresponding pyrophosphates in cells. An alternative strategy for creating membrane permeant molecules is to reduce the overall charge and increase the lipophilicity of the analog. The bioavailability of membrane impermeant phosphate and phosphonate containing drug molecules has been dramatically increased by masking the charged groups with acyloxymethyl esters [233-235]. The acyloxymethyl ester allows the drugs to diffuse across the cellular membrane into the cytoplasm where general esterases cleave the acyloxymethyl groups revealing the active, charged species (Figure 6-10) [195, 236]. However, this strategy is unlikely to succeed for uncharged pyrophosphate tetra-esters, as the anhydride linkage is rapidly hydrolyzed at physiological pH. Methylene diphosphonate (MDP) is a structural analog of pyrophosphate, which does not contain a labile anhydride linkage. Replacing the labile pyrophosphate with MDP and masking the negative charges with acyloxymethyl esters may result in membrane permeant FPP analogs. We found no description of mono-allylic triacyloxymethyl methylenediphosphonate tetraesters in the literature. We report the preparation of three such isoprenoids where the pyrophosphate has been replaced by MDP and the negative charges are masked by frangible pivaloyloxymethyl (POM) esters.
**Figure 6-9 Proposed Mechanism of In Vivo Prodrug Activation**

- **Cellular Esterases**
  - $R = \text{CH}_2\text{OCO}-\text{Bu}$
  - $n = 1-3$

- **Spontaneous**
Farnesyl methylene diphosphonate is transferable.

The strategy outlined above requires that the substitution of MDP for the pyrophosphate in FPP and other analogs results in molecules that are substrates for FTase. Farnesyl methylene diphosphonate (FMDP) 2 has previously been prepared and was shown by Eummer et al. to be a competitive inhibitor (IC$_{50}$=160 nM) of FTase [185]. However, the assay used in that study was unable to distinguish whether FMDP was an alternative substrate for FTase or an FTI. To resolve this question, FMDP, 36 was synthesized as previously described, and was shown to be a substrate for FTase in an in vitro fluorescence assay varying isoprenoid concentration [194]. The catalytic efficiency for transfer of the farnesyl group from FMDP 36 to a dansyl-GCVLS peptide substrate by FTase was found to be an order of magnitude lower than that for FPP 1 (Table 6-1). The previous study also reported that treatment of NIH3T3 cells with 36 had no effect on their growth. Eummer at al. suggested that this might be due to FMDP 36 acting as an alternative substrate for FTase, leading to normal farnesylation of Ras. The results of the in vitro fluorescent assay are consistent with this observation.
Table 6-1 Kinetics of FMDP

Steady-state transfer kinetics of FPP and FMDP with 1 μM peptide concentration and varying FPP and analog concentrations. Rates were measured in a single cuvette fluorescence assay using N-dansyl-GCVLS as the peptide substrate and FTase as the catalyst in Tris-HCl (pH=7.4) with reducing agent and detergent. Note the order of magnitude difference in catalytic efficiency.

\[
\text{O} \quad \text{P} \quad \text{X} \quad \text{P} \quad \text{O}^- \quad \text{O}^- \quad \text{O}^- \\
\text{O} \quad \text{O}^- \quad \text{P} \quad \text{O}^- \quad \text{O}^- 
\]

1. FPP \hspace{1cm} X=O
36. FMDP \hspace{1cm} X=CH₂

<table>
<thead>
<tr>
<th>Analog</th>
<th>Km(nM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPP (1)</td>
<td>40</td>
<td>0.09</td>
<td>2 x 10⁶</td>
</tr>
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<td>FMDP (36)</td>
<td>180</td>
<td>0.06</td>
<td>3 x 10⁵</td>
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Synthesis of masked methylene diphosphonates

The synthesis of tri-POM esters of isoprenoid diphosphonates is summarized in Figure 6-11. Tetrapivaloyloxymethyl methylenediphosphonate (38) was prepared from tetramethyl methylenediphosphonate (37) and pivaloyloxymethyl chloride (POM-Cl) in 47% yield as previously described [237]. In analogy to previously reported methods, treatment of 38 with DABCO provided the tripivaloyloxymethyl methylenediphosphonate salt 39 in 80-95% yield. The crude DABCO salt 39, was converted to the corresponding chloride 41 with oxalyl chloride and catalytic DMF in toluene in 90-95% yield. The purity of the air and moisture sensitive chloride 41 was established by examining the $^1$H and $^{31}$P NMR spectra of the crude filtrate. The $^1$H NMR spectrum of 41 showed no trace of the quaternary DABCO cation 40. Tri-POM-methylenediphosphonate isoprenoids 42a-c were prepared by combining the corresponding allylic alcohols with chloride 41 in either dilute toluene or methylene chloride solution containing a three fold excess of DBU and catalytic DMAP. The farnesyl, geranylgeranyl and geranyl isoprenoids 42a-c were isolated in 40-50% yield after purification by silica gel flash chromatography using 1% TEA added to a hexane:ethyl acetate mobile phase. Significantly lower yields of the isoprenoids 42a-c were obtained if TEA was omitted from the eluent. Isoprenoids 42a-c were quite labile and no product was recovered from attempts to purify them using alumina and fluorosil. Purification by RP-HPLC was unsuccessful due to progressive decomposition of the tetraesters 42a-c in water or buffer (see below).
Figure 6-10 Synthesis of POM protected methylene diphosphonates

Reagents and Yields: (a) POMCl, NaI, acetonitrile, reflux (47%). (b) DABCO, acetonitrile, reflux (90%). (c) (COCl)2, DMF(cat.), rt (96%). (d) ROH, DBU, DMAP(cat.) Toluene/methylene chloride (40-50%)

R= -CH₂OCOt-Bu

(1a) Farnesol, n=2 to give 42a
(2a) Geranylgeraniol, n=3 to give 42b
(43c) Geraniol, n=1 to give 42c
**Tetraester stability**

Tetraesters 42a-c were water sensitive and can only be stored for a few days at –20°C without significant decomposition. There is evidence that indicates acyloxyethyl group deprotection occurs chemically at the phosphorous atom in H$_2^{18}$O [238]. If the chemical hydrolysis occurs at the allylic alcohol linkage rather than the acyloxyethyl group linkage then these compounds may not be able to traverse a cell membrane as the MDP analog, but rather the parent alcohol. Tetraester stability was monitored by RP-HPLC for the disappearance of methylene diphosphonates 42a-b and the concomitant appearance of the parent alcohols (Figure 6-12). (Due to low aqueous solubility of the tetraesters, decomposition was measured in mixed aqueous/organic solution. 50 % of tetraester 42a had decomposed after 200 min in water:acetonitrile (99:1) and 42b decomposed after 500 min in pH=7.4 buffered cell culture media:acetonitrile (99:1). Under these conditions only 10% of the tetraester 42b was converted to the parent alcohol 2a after 500 min. $^{31}$P NMR spectra of the time dependent breakdown of methylenediphosphonate 42a in 9:1 water:acetonitrile is consistent with the formation of the mono-allylic diPOM salt (Figure 6-13). Niemi et al. prepared and tested the aqueous stability of tetra-, tri- and P,P'-diPOM esters of dichloromethylenediphosphonate [198]. They found that the tri-POM ester had a half-life of 78 min in serum and that the insoluble tetra-POM was converted to the P,P'-di-POM and tri-POM esters in pH 7.4 serum. Together these data suggest that the charged mono-allylic diPOM salts are the primary decomposition products of tetraesters 42a-c. Niemi et al have also shown that pivaloyloxymethyl esters of prodrug bisphosphonates are fully deprotected in the presence of 10% rabbit liver homogenates.
Figure 6-11 Prodrug Decomposition HPLC

All RP-HPLC data calculated based on relative peak integrals of initial tetraester verses tetraester or alcohol produced at each time point and fit to exponential curve. a) 42a decomposition in water b) 42b decomposition in cell media. Note that the rate of decomposition does not match the rate of alcohol formation.
Figure 6-12 Time dependent decomposition of FTriPOM 9a analyzed by 31P NMR

73 mg (0.1 mmols) of FTriPOM 42a was dissolved in 2 mL of 10% acetonitrile/water. A 31P NMR spectrum was taken at time 0 then again 24 hrs later. Note that the Tripivaloyloxymethyl ester decomposes into what appears to be the dipivaloyloxy ester, but from the previous decomposition data in Figure 6-11 it is not from isoprenoid loss.
Discussion

Unnatural analogs as cellular FTase substrate probes
This chapter outlines a general strategy for detecting prenylated proteins using antibodies directed towards unnatural analogs of FPP 1b bearing unique epitopes that can be transferred to proteins by FTase. We have found that the farnesol 1a analog AGOH 5a can be utilized by a cell to modify proteins in an FTase dependent manner and is not particularly toxic. The structure of the anilinogeranyl moiety is also dissimilar to any other known cellular components or protein modifications making it an excellent epitope for selective antibody recognition. In this chapter I have shown that the AGPP prodrug anilinogeraniol is incorporated into cellular protein in the presence of the endogenous FPP pool. Previous reports of FPP analog incorporation into cellular proteins have required lovastatin to block endogenous FPP formation in order to detect modified proteins. These reagents may be useful for the analysis of FTase mediated protein modification and cellular events further downstream. The lack of acute cellular toxicity of AGOH 5a may allow for long-term analysis of proteins modified by FTase by immunohistochemical methods. These antibodies could be especially useful for screening the activity and protein targeting of farnesyl transferase inhibitors helping to identify the targets of these reagents to better understand the role of farnesylated proteins in cancer biology.

Alternative pro-drugs for FPP analogs
This chapter also described a synthetic methodology to prepare mono-allylic tripivaloyloxyethyl tetraesters of methylene diphosphonic acid and has shown that farnesyl methylenediphosphonate is a substrate for protein farnesyl transferase. The lipophilicity of isoprenoid methylenediphosphonates is significantly increased by masking the phosphonic acid groups with acyloxymethyl esters. As we increase the structural diversity of the FTase transferable substrates it is likely that analogs will eventually be developed that cannot be endogenously diphosphorylated. In the event that this occurs the tripivaloyloxymethyl methylene diphosphonate masking strategy may provide a new efficient delivery mechanism for the isoprenoid analogs. Interestingly, bisphosphonates have proved to be very useful for the treatment of bone
diseases. The methylene diphosphonate moiety readily chelates Ca\textsuperscript{2+} ions, and is directed by the body to bone. It is possible that the isoprenoid methylene diphosphonates could be directed to bone with a similar mechanism and effect delivery of such molecules in a whole animal system. However, the stability of the protected methylene diphosphonates may present a problem for such molecules, and may need to be improved using alternative cell labile masking approaches.

**Experimental**

**General**

All reactions were conducted under dry argon and stirred magnetically, except as noted. Reaction temperatures refer to the external bath temperatures. Analytical TLC was performed on pre-coated (0.25 mm) silica gel 60F-245 (Merck) plates and developed with given solvent conditions. Visualization was achieved by UV irradiation, iodine or by subjecting the plates to a 15% sulfuric acid, 1% ceric ammonium sulfate, 2% Molybdic anhydride solution followed by heating. Flash chromatography was performed on Merck silica gel 60 (230-400 mesh ASTM). All chromatography solvents were purchased from VWR (EM Science-Omnisolv high purity) and used as received. All reagents were purchased from Aldrich or Alfa Aesar. NMR Spectra were obtained in CDCl\textsubscript{3} (unless otherwise noted) at 200 or 400 MHz. Chemical shifts for the following deuterated solvents are reported in ppm downfield using the indicated reference peaks: CDCl\textsubscript{3} (CDCl\textsubscript{3} internal peak 7.27 ppm for \textsuperscript{1}H, 77.4 ppm for \textsuperscript{13}C). ESI-MS were performed at the University of Kentucky Mass Spectra Facility.

**Synthesis**

**Cysteine Methyl Ester Synthesis (8a-c)**

***Anilinogeranyl Cysteine Methyl Ester***

2-amino-3-(3,7-dimethyl-8-phenylamino-octa-2,6-dienylsulfanyl)-propionic acid methyl ester 8a

Chloride 7a was prepared from the corresponding alcohol (734mg, 3.0 mmols) in 10 mL of acetonitrile as previously described [159]. A solution of L-cysteine methyl ester hydrochloride (1.13g, 6.50 mmols) in 20 mL of 7M NH\textsubscript{3}/MeOH was added to the cooled
solution (-20º C) of chloride 7a. The reaction initially formed a white precipitate which quickly disappeared. After 3 hours, the reaction mixture was allowed to warm to room temperature over one hour. The reaction mixture was partitioned between ether and water and extracted with ether (3X). The combined organic extracts were then dried over MgSO₄ and solvent was evaporated. Purification by flash chromatography (1:2:8) i-Pr-OH:EtOAc:Hexane yielded 625 mg (60%) of a yellow oil. Rf=0.32 in 1:2:5 i-Pr-OH: EtOAc: Hexane. 

1H NMR (CDCl₃, 200 MHz) 7.10 (t, 2H, J=8.0 Hz), 6.63 (t, 1H, J=7.2 Hz), 6.55 (d, 2H, J=8.0 Hz) 5.39 (t, 1H, J=8.0 Hz), 5.23 (t, 1H, J=8Hz), 3.75 (s, 3H), 3.59 (m, 3H), 3.13 (m, 2H), 2.86-2.95 (2d, 1H), 2.65-2.76 (2d, 1H), 2.06-2.19 (m, 4H), 1.63 (s, 3H), 1.62 (s, 3H) 13C NMR (CDCl₃, 50 MHz) 174.58, 148.77, 139.42, 132.96, 129.38, 125.75, 120.53, 117.39, 113.08, 54.51, 52.49, 51.96, 39.50, 36.14, 30.14, 26.30, 16.33, 14.98; MS: (ESI, +) (M-H+) 363

Nitroanilinogeranyl cysteine methyl ester
2-amino-3-[3,7-dimethyl-8-(4-nitro-phenylamino)-octa-2,6-dienylsulfanyl]-propionic acid methyl ester (8b)

Purification by flash chromatography (3:2:5 i-Pr-OH: EtOAc: Hexane) yielded 276 mg (60%) of a yellow/brown oil. Rf=0.28 (3:2:5 i-Pr-OH: EtOAc: Hexane) 1H NMR (CDCl₃, 400 MHz) 8.65 (d, 2H, J=8.0 Hz), 6.52 (d, 2H, J=8.0 Hz), 5.34 (t, 1H, J=12 Hz), 5.20 (t, 1H, J=16 Hz), 3.75 (s, 3H), 3.71 (s, 2H), 3.65 (m, 1H), 3.17 (d, 2H, J=16 Hz), 2.84-2.93 (2d, 1H), 2.64-2.75 (2d, 1H) 2.18 (t, 2H), 2.02(t, 2H) 1.22 (s, 3H), 1.19 (s, 3H) 13C NMR (CDCl₃, 50 MHz) 174.80, 153.92, 139.04, 131.14, 126.60, 120.79, 111.40, 54.48, 52.52, 50.99, 39.25, 36.79, 30.25, 25.98, 25.61, 16.26, 14.92; MS: (ESI, +) (M-H+) 408

Farnesyl Cysteine Methyl Ester
2-amino-3-(3,7,11-trimethyl-dodeca-2,6,10-trienylsulfanyl)-propionic acid methyl ester (8c)

Into a 100 mL round bottomed flask at 0 ºC was added L-Cysteine methyl ester (1.38 g, 10.2 mmols) in 60 mL of 7M NH₃/MeOH. Neat farnesyl bromide (7c) (1.00 g 3.52 mmols) was then added and the solution was stirred at 0º C for four hours. The reaction mixture was partitioned between ether and water and extracted 3X with ether, extracts were combined, dried over MgSO₄ filtered and evaporated. Purification by flash
chromatography (1% MeOH:CHCl₃) yielded 881 mg (74%) of a clear oil. Rf=0.22 in 5% MeOH in CHCl₃. ¹H NMR (CDCl₃, 400 MHz): 5.24 (t, 1H, J=8Hz), 5.10 (m, 2H), 3.75 (s, 3H), 3.64 (q, 1H), 3.10-3.25 (m, 2H), 2.86-2.91 (2d, 1H), 2.65-2.71 (2d, 1H), 2.04-2.10 (m, 6H), 2.04(m, 6H) 2.0 (t, 2H), 1.69 (s, 3H), 1.68 (s, 3H), 1.60 (s, 6H); ¹³C NMR (CDCl₃, 50 MHz) 174.93, 139.91, 135.71, 131.62, 124.69, 124.11, 120.35, 54.64, 52.35, 39.93, 36.77, 30.15, 26.98, 26.67, 25.86, 17.86, 16.33, 16.21 MS: (ESI, +) (M-H+) 340

**General Conditions for Succinylation (9a-c)**

Cysteine methyl ester 8c (881 mg, 2.6 mmols) was diluted in 20 mL of 1:1 THF:CH₂Cl₂ in a 100mL round bottomed flask. Succinic anhydride (280 mg, 2.8 mmols) was then added and the mixture stirred for 4 hours, followed by evaporation of solvent. The residue was diluted in ether, washed sequentially with water and brine and then dried over sodium sulfate and concentrated in vacuo.

**Anilinogeranyl cysteine succinate**

N-[2-(3,7-dimethyl-8-phenylamino-octa-2,6-dienylsulfanyl)-1-methoxycarbonyl-ethyl]-succinamic acid (9a)

Cysteine methyl ester 8a (302 mg, 0.83 mmols) provided 250 mg (65%) of succinate 9a as a white solid. Purification by flash chromatography (1:4:5: 0.1 i-Pr-OH: EtOAc: hexane: acetic acid) Rf=0.32 (1:4:5 i-Pr-OH: EtOAc: Hexane.) ¹H NMR (CDCl₃, 400 MHz) 7.12 (t, 2H, J=8.0 Hz), 6.65 (t, 2H, J=8.0 Hz), 6.58 (t, 2H), 5.34 (t, 1H, J=8.0 Hz) 5.14 (t, 1H, J=8.0 Hz), 4.75 (m, 1H), 3.72 (s, 3H), 3.60 (s, 2H), 3.11 (m, 2H), 2.79-2.93 (m, 2H), 2.65 (t, 2H, J=8.0 Hz), 2.52 (t, 2H, J=8 Hz), 2.11 (t, 2H, J=8 Hz) 2.02 (t, 2H, J=8 Hz), 1.63 (s, 3H), 1.62 (s, 3H); ¹³C NMR (CDCl₃, 50 MHz) 176.95, 171.84, 171.55, 148.48, 139.80, 132.79, 129.34, 125.86, 120.06, 117.68, 113.36, 52.91, 52.13, 39.42, 33.55, 30.69, 30.19, 29.53, 28.92, 26.22, 16.25, 14.96 MS: (ESI, +) (M-H+) 463

**Nitroanilinogeranyl cysteine succinate**

N-[2-[3,7-dimethyl-8(4-nitro-phenylamino)-octa-2,6-dienylsulfanyl]-1-methoxycarbonyl-ethyl]-succinamic acid (9b)

Cysteine methyl ester 8b (339 mg, 0.83 mmols) provided 204 mg (57%) of succinate 9b as a yellow solid. Purification by flash chromatography (1:4:5:0.1) i-Pr-OH: EtOAc: hexane: acetic acid) Rf=0.3 (3:2:5) i-Pr-OH: EtOAc: hexane 1HNMR (CDCl₃, 200 MHz):
8.03 (d, 2H, J=8.0 Hz), 6.49 (d, 3H), 5.30 (t, 1H, J=8 Hz), 5.11 (t, 1H, J=8 Hz), 4.75 (m, 1H), 3.77 (s, 3H), 3.74 (s, 2H), 3.10 (m, 2H), 2.81-3.02 (m, 2H), 2.57-2.76 (m, 4H), 2.06-2.19 (m, 4H), 1.67 (s, 3H), 1.64 (s, 3H); 13C NMR (CDCl3, 50 MHz): 176.20, 172.00, 171.50, 153.91, 139.60, 131.17, 126.83, 126.61, 120.39, 111.60, 52.92, 52.33, 51.14, 39.20, 33.65, 30.70, 30.28, 29.42, 26.01, 16.18, 14.80 MS: (ESI, +) (M-H+) 508

**Farnesyl cysteine succinate**

N-[1-methoxycarbonyl-2-(3,7,11-trimethyl-dodeca-2,6,10-trienylsulfanyl)-ethyl]-succinamic acid (9c)

Cysteine methyl ester 8c (881 mg, 2.6 mmols) provided 93 7 mg (82%) of succinate 9c as a white solid. Purification by flash chromatography (1:2:7 i-Pr-OH: EtOAc: hexane) ¹H NMR (CDCl₃, 400 MHz) 10.55 (bs, 1H), 6.72 (d, 1H, J=8 Hz), 5.17 (t, 1H, J=8 Hz), 5.07 (m, 2H), 4.75-4.80 (q, 1H), 3.73-3.73 (s, 3H), 3.13 (m, 2H), 2.90-2.94 (m, 1H), 2.80-2.85 (m, 1H), 2.69 (t, 2H), 2.57 (t, 2H), 1.92-2.08 (m, 8H), 1.65 (s, 3H), 1.64 (s, 3H), 1.57 (s, 6H); ¹³C NMR (CDCl₃, 100 MHz) 177.40, 171.90, 171.64, 140.17, 135.44, 131.38, 124.44, 124.38, n123.85, 123.78, 119.60, 119.55, 52.80, 52.75, 52.01, 51.97, 39.79, 39.72, 33.28, 31.67, 30.55, 29.96, 29.39, 26.80, 26.51, 25.79, 22.74, 17.78, 16.19, 16.10; MS: (ESI, +) (M-H+) 438

**General Conditions for Activated Ester Formation (10a-c)**

Succinamic acid 8c (59.6 mg, 0.126 mmols) was dissolved in 10mL of dry acetonitrile in a 100 mL round-bottomed flask. Carbonyldiimidazole (26.4 mg 0.163 mmols) was then added and the mixture was allowed to stir for 5 minutes. N-hydroxyphthalimide (26.4 mg, 0.163 mmols) was added and the solution was stirred for 1.5 hours. The solvent was then evaporated.

**Anilinogeranyl cysteine succinyl phthalimide**

N-[2-(3,7-dimethyl-8-phenylamino-octa-2,6-diensulfanyl)-1-methoxycarbonyl-ethyl]-succinamic acid 1,3-dioxo-1,2-;dihydro-isoindol-2-yl ester (10a)

Succinate 9a (30 mg, 0.07 mmols) provided 25 mg (63%) of phthalimide 10a as a yellow/brown oil. Purified by column chromatography (CHCl₃). Rf=0.28 (1:1)
Hexane:EtOAc $^1$H NMR (CDCl$_3$, 400 MHz): 7.88 (q, 2H), 7.78 (q, 2H), 7.16 (t, 2H), 6.68 (t, 1H), 6.60 (d, 2H), 6.37 (d, 1H), 5.37 (t, 1H), 5.17 (t, 1H), 4.83 (m, 1H), 3.76 (s, 3H), 3.63 (s, 2H), 3.15 (m, 2H), 3.06 (t, 2H), 2.92 (m, 2H), 2.71 (t, 2H), 2.03-2.18 (m, 4H), 1.67 (s, 3H), 1.65 (s, 3H); $^{13}$C NMR (CDCl$_3$, 50 MHz): 171.43, 169.94, 169.12, 161.93, 148.70, 139.79, 134.06, 132.89, 129.34, 125.72, 124.19, 120.08, 117.38, 113.07, 52.86, 52.10, 51.97, 39.43, 33.64, 30.63, 30.21, 26.78, 26.25, 16.26, 14.94 MS: (ESI, +) (M-H+), 608

**Nitroanilinogeranyl cysteine succinyl phthalimide**

N-[1-[3,7-Dimethyl-8-(4-nitro-phenylamino)-octa-2,6-diensulfanyl]-1-methoxycarbonyl-ethyl]-succinamic acid 1,3-dioxo-1,3-dihydro-isoindol-2-yl ester (10b)

Succinate 9b (38 mg, 0.08 mmols) provided 41 mg (85%) of phthalimide 10b as a yellow oil. Purified by column chromatography (CHCl$_3$). Rf=0.12 (1:1) Hexane:EtOAc

$^1$H NMR (CDCl$_3$, 400 MHz) 8.02 (d, 2H), 7.84 (q, 2H), 7.75 (q, 2H), 6.54 (d, 2H), 6.36 (d, 1H), 5.30 (t, 1H), 5.11 (t, 1H), 4.78 (q, 1H), 3.73 (s, 3H), 3.70 (s, 2H), 3.10 (t, 2H), 3.02 (t, 2H), 2.80-2.98 (m, 2H), 2.69 (t, 2H), 2.11 (m, 2H), 2.00 (t, 2H), 1.63 (s, 3H), 1.59 (s, 3H); $^{13}$C NMR (CDCl$_3$ 100 MHz) 171.36, 169.17, 161.94, 153.85, 146.06, 139.44, 138.02, 135.02, 131.06, 129.03, 126.60, 126.56, 124.21, 120.32, 111.40, 52.95, 52.24, 51.02, 39.21, 33.60, 30.62, 30.24, 27.02, 26.75, 26.00, 16.22, 14.87

**Farnesyl cysteine succinyl phthalimide**

N-[1-methoxycarbonyl-2-(3,7,11-trimethyl-dodeca-2,6,10-trienylsulfanyl)-ethyl]-succinamic acid 1,3-dioxo-1,2-dihydro-isoindol-2-yl ester (10c)

Succinate 9c (60 mg, 0.14 mmols) provided 80 mg (58%) of phthalimide 10c as a brown oil. Purified by column chromatography (CHCl$_3$). Rf=0.3 (1:1) Hexane:EtOAc

$^1$H NMR (CDCl$_3$, 400 MHz) 7.88 (q, 2H), 7.79 (q, 2H), 6.38 (d, 1H), 5.20(t, 1H), 5.09 (t, 2H), 4.82 (m, 1H), 3.76 (s, 3H), 3.74 (s, 2H), 3.11-3.22 (m, 2H), 3.07 (t, 2H), 2.86-3.00 (m, 2H), 2.74 (t, 2H), 1.95-2.10 (m, 8H), 1.68 (s, 3H), 1.66 (s, 3H), 1.60 (s, 6H); $^{13}$C NMR (CDCl$_3$, 100 MHz) 171.44, 169.90, 169.93, 161.91, 140.29, 135.57, 134.96, 131.53, 129.06, 124.50, 124.19, 123.90, 119.64, 52.85, 52.00, 39.88, 39.81, 33.54, 30.62, 30.14, 29.03, 28.23, 26.90, 26.75, 26.59, 25.90, 17.89, 16.31, 16.21.
General Conditions for Benzyl amine Coupling (11a-c)

Phthalimide 10c (47.2 mg, 0.08 mmols) was dissolved in 1 mL of acetonitrile and 5 mL of 5% NaHCO₃ buffer pH=9 in a 25 mL RB flask. Benzylamine (17.3 mg, 0.16 mmols) was then added and the reaction was allowed to stir for 1.5 hours at room temperature. 10 mL of water was added and organics were extracted with ether (3X), dried over MgSO₄, and organic solvent evaporated.

Anilinogeranyl benzyl amine

2-(3-Benzylcarbamoyl-propionylamino)-3-(3,7-dimethyl-8-phenylamino-octa-2,6-Dienylsulfanyl)-propionic acid methyl ester (11a)

Phthalimide 10a (133 mg, 0.22 mmols) provided 59 mg (48%) of amide 11a as a white solid. Purification by column chromatography (3:7) Hexane: EtOAc. ¹H NMR (CDCl₃, 400 MHz) 7.21-7.31 (m, 5H), 7.12 (t, 2H), 6.55-6.66 (m, 4H), 6.23 (bt, 1H), 5.34 (t, 1H), 5.14 (t, 1H), 4.71 (q, 1H), 4.38 (d, 2H, J=6 Hz), 3.70 (s, 3H), 3.59 (bs, 2H), 3.10 (m, 2H), 2.76-2.91 (m, 2H), 2.50-2.61 (m, 4H), 1.99-2.13 (m, 4H), 1.63 (s, 3H), 1.62 (s, 3H) ¹³C NMR (CDCl₃, 50 MHz): 172.23, 171.94, 171.46, 148.63, 139.77, 138.41, 132.84, 129.42, 129.34, 129.19, 128.94, 128.87, 128.08, 127.93, 127.73, 125.74, 120.08, 117.411, 113.10, 52.82, 52.09, 51.963, 43.85, 39.45, 33.50, 31.71, 31.66, 30.12, 26.25, 16.28, 14.95 MS: (ESI, +) (M-H⁺) 552

Nitroanilinogeranyl benzyl amine

2-(3-Benzylcarbamoyl-propionylamino)-3-[3,7-dimethyl-8(4-nitro-phenylamino)-octa-2,6-Dienylsulfanyl]-propionic acid methyl ester (11b)

Phthalimide 10b (13 mg, 0.02mmols) provided 9 mg (76%) of amide 11b as a yellow solid. Purification by column chromatography (3:4) Hexane: EtOAc. 1HNMR (CDCl₃, 400 MHz) 8.01 (d, 2H, J=9.2 Hz), 7.20-7.29 (m, 5H), 6.69 (d, 1H, J=7.6 Hz), 6.48 (d, 2H, J=9.2 Hz), 6.26 (t, 1H), 5.30 (t, 1H), 5.12 (t, 1H), 4.96 (t, 1H), 4.6-4.72 (m, 1H), 4.38 (d, 2H, J=5.6 Hz), 3.70 (s, 3H), 3.68 (d, 2H, J=5.6 Hz), 3.10 (m, 2H), 2.77-2.91 (m, 2H), 2.51-2.62 (m, 4H), 2.10-2.16 (t, 2H), 2.00-2.03 (t, 2H), 1.62 (s, 3H), 1.60 (s, 3H); ¹³C NMR (CDCl₃, 50 MHz): 172.22, 171.92, 171.41, 153.89, 139.39, 139.39, 138.33, 131.04,
Farnesyl Benzyl amine

2-(3-Benzylcarbamoyl-propionylamino)-3-(3,7,11-trimethyl-dodeca-2,6,10-trienylsulfanyl)propionic acid methyl ester (11c)

Phthalimide 11c (47 mg, 0.08mmols) provided 42 mg (67%) of amide 11c as a white solid. Purification by column chromatography (4:6) Hexane: EtOAc. $^1$H NMR (CDCl$_3$, 400 MHz) 7.25-7.34 (m, 5H), 6.623 (d, 1H), 6.28 (t, 1H), 5.20 (t, 1H), 5.09 (t, 2H), 4.75 (q, 1H), 4.44 (d, 2H), 3.75 (s, 3H), 3.09-3.22 (m, 2H), 2.81-2.96 (m, 2H), 2.55-2.67 (m, 4H), 1.96-2.13 (m, 8H), 1.68 (s, 3H), 1.67 (s, 3H), 1.60 (s, 6H); $^{13}$C NMR (CDCl$_3$, 50 MHz): 172.21, 171.94, 171.47, 140.29, 138.44, 135.60, 131.54, 128.87, 127.94, 127.64, 124.53, 123.93, 119.72, 52.79, 52.07, 43.89, 39.92, 39.84, 33.47, 31.77, 31.73, 30.12, 26.95, 26.66, 25.91, 17.90, 16.36, 16.23; MS: (ESI, +) (M-H+) 529

General Conditions for Protein Coupling (12a-c and 13a-c)

Phthalimido esters (10a-c) were dissolved in 6 mL of 1:5 acetonitrile and 5% NaHCO$_3$ in a 100 mL pear-shaped flask. BSA (13) (0.1 mL of 10 mg/mL solution in phosphate buffered saline (PBS) pH 7.2) or KLH protein (12) (3 mL of 10 mg/mL solution in PBS pH 7.2) was added and stirred vigorously for 2 hours or until analysis by TLC in 1:1 hexane:EtOAc showed no residual activated phthalimide ester spot. The solution was dialyzed overnight in 20% DMSO, PBS pH 7.5 then PBS (3 X 1L, 6 hrs each).

Tetra(pivaloyloxymethyl) Methylene-diphosphonate (38)

A mixture of tetramethyl methylenediphosphonate 37 (3.77g, 17.2 mmols) sodium iodide (11.1g, 73.7 mmols) and pivaloyloxymethyl chloride (16.1g, 106 mmols) were heated at reflux overnight in acetonitrile. Solvents were evaporated under reduced pressure and the residue was partitioned between 100 mL of ether and water. The organics were washed with water (2X) and 5% NaHCO$_3$ (2X) and dried over Na$_2$SO$_4$. The ether was evaporated under reduced pressure and the residue dissolved in a minimal volume of hexanes and placed at 0° C overnight. Crystals were collected and
dried to constant weight yielding 4.68g (46%) of 38. $^1$HNMR 5.67 (m, 8H), 2.57 (t, 2H, J=21.6 Hz), 1.20 (s, 36H) $^{13}$CNMR 177.04, 82.18, 38.93, 27.08 (t, J=143 Hz) $^{31}$PNMR 19.13 (ESI-MS)M-Na+ 655

**Tri(pivaloyloxymethyl) Methylene(diphosphonate)**

1,4-(N-(pivaloyloxymethyl)) diazonium-bicyclo[2.2.2]-octane salt (39)

TetraPOM 38 (10.0g, 15.8 mmols) and DABCO (1.70g, 15.2 mmols) in 200 mL of acetonitrile were heated at reflux overnight after which solvent was evaporated under reduced pressure. The residue was diluted with 100 mL of ether and washed 4X with water. The collected aqueous extracts were evaporated under reduced pressure and the residue taken up in dry toluene and the solvent evaporated under reduced pressure (3X) leaving 9.59g of triPOM salt 39 as a white oily solid (84.2% yield relative to DABCO). $^1$H NMR 5.34 (m, 8H), 3.37 (t, 6H, J=7.2 Hz) 3.00 (t, 6H, J=7.2 Hz), 2.17 (t, 2H, J=19.8 Hz) 1.00 (s, 9H), 0.94 (s, 18H), 0.92 (s, 9H) $^{13}$C NMR 177.71, 177.09, 175.76, 83.34, 82.11, 80.90, 50.67, 45.17, 44.94, 39.27, 38.85, 27.54, 27.22, 22.81 $^{31}$P NMR 26.77 (d, 1P, J=10.95 Hz), 15.74 (d, 1P, J=10.95Hz) (ESI-MS)M- 517

**Tri(pivaloyloxymethyl) methylenediphosphonyl Chloride (41)**

TriPOM salt 39 (546 mg, 0.73 mmols) was dissolved in 20 mL of dry acetonitrile and the solvent evaporated under reduced pressure (3X) followed by the addition of 10 mL dry toluene, 40 µL anhydrous DMF and 0.3 mL oxalyl chloride (3.3 mmols). A white precipitate formed immediately with evolution of heat and the reaction was stirred at rt for 45 min. Solids were removed by filtration through a pad of Na$_2$SO$_4$ which was washed with 5 mL of dry toluene. The combined organics were immediately evaporated under reduced pressure to give a pale yellow oil used without further purification (377 mg, 96%). $^1$H NMR 5.64-5.85 (m, 6H) 3.03 (t, 2H, J=20 Hz) 1.23 (s, 27H) $^{31}$P NMR 28.74 (d, 1P, J=10.95 Hz), 15.74 (d, 1P, J=10.95Hz)

**General synthesis of Tri-POM esters (42a-c)**

**Farnesyl tri(pivaloyloxymethyl) methylenediphosphonate (42a)**

Chloride 41 (697 mg, 1.3 mmols) diluted in 5 ml of dry toluene or anhydrous methylene chloride was added drop wise to a cooled (-20° C), stirred solution of
farnesol 1a (94mg, 0.42 mmols) DBU (198 mg, 1.3 mmols) and DMAP (5 mg, 0.04 mmols) in 10 mL of dry toluene. Stirring was continued for 2 hours followed by the addition of 50 ml of ether. The organic layer was washed 2X each with 10 ml of 0.1N HCl, water and freshly prepared 5% NaHCO₃, then dried over Na₂SO₄ and evaporated under reduced pressure. Farnesyl ester 42a was obtained in 40% yield (121 mg) after silica gel flash chromatography with 7:3 Hexane:EtOAc + 1% triethylamine. ¹H NMR 5.61-5.71 (m, 6H) 5.35 (t, 1H), 5.04 (t, 2H), 4.62 (m, 2H), 2.57 (t, 2H, J=21.6 Hz), 1.90-2.08 (m, 8H), 1.67 (s, 3H), 1.63 (s, 3H), 1.55 (s, 6H), 1.16 (s, 27H) ¹³C NMR 177.09, 177.01, 143.80, 135.80, 124.55, 124.36, 123.74, 123.55, 118.57, 82.40, 63.59, 39.87, 38.91, 27.02 ³¹P NMR 20.02 (d, 1P, J=7.29 Hz), 19.30 (d, 1P, J=6.16 Hz).

**Geranylgeranyl tri(pivaloyloxymethyl) methylenediphosphonate (42b)**

Geranylgeraniol 2a (93 mg, 0.32 mmols) provided 124 mg of 9b (48%). ¹H NMR 5.69-5.75 (m, 6H), 5.33 (t, 1H, J=7.2 Hz), 5.01-5.05 (t, 3H), 4.60 (m, 2H), 2.55 (t, 2H, J=21.6 Hz), 1.90-2.03 (m, 12H), 1.65 (s, 3H), 1.61 (s, 3H), 1.53 (s, 9H), 1.17 (s, 27H) ¹³C NMR 177.08, 177.01, 143.81, 135.83, 135.18, 131.16, 124.14, 124.24, 123.73, 123.55, 118.54, 82.46, 82.13, 63.65, 39.90, 38.91, 27.07, 16.17; ³¹P NMR 20.04 (d, 1P, J=7.29 Hz), 19.31 (d, 1P, J=7.45 Hz)

**Geranyl tri(pivaloyloxymethyl) methylenediphosphonate (42c)**

Geraniol (28 mg, 0.18 mmols) provided 62 mg of 42c (55%). ¹H NMR 5.61-5.71 (m, 6H), 5.35 (t, 1H, J=7.2 Hz) 5.03 (t, 1H, J=6.8 Hz), 4.60 (m, 2H), 2.57 (t, 2H, J=22 Hz), 1.98-2.06 (m, 4H), 1.67 (s, 3H), 1.64 (s, 3H), 1.56 (s, 3H), 1.19 (s, 27H); ¹³C NMR 177.06, 176.99, 143.65, 132.14, 124.03, 123.48, 118.65, 82.21, 63.68, 39.70, 38.91, 26.97, 16.74; ³¹P NMR 20.00 (d, 1P, J=7.29 Hz), 19.27 (d, 1P, J=8.43 Hz)

**Antibody Generation and Characterization**

**Polyclonal antibody production**

Hapten protein conjugates 12a-c were used to immunize rabbits subcutaneously by emulsifying 1.5 mg of each conjugate with Freund’s complete adjuvant. This was followed by six secondary boosters of the same dose with incomplete Freund adjuvant at intervals of 14 days. The rabbits were bled 10 days after each boost. Blood was
allowed to coagulate overnight in 50 mL centrifuge tubes and sera was collected and stored at 0 °C.

**ELISA Screening**

ELISA plates (96-well polystyrene) were coated with 100 up per well of BSA conjugates or BSA (1 ug/mL) in PBS (pH=7.6) and incubated for 1 hr at 37 °C. The plates were then washed thoroughly with 10% Tween in PBS buffer (PT) (pH=7.4). 150 up of serum diluted 1:100 in PT buffer containing casein (PCT) was added and diluted 1:3 down the plate, then incubated 1 hr at 37 °C. The plates were washed with PT buffer and 100 up of secondary antibody (1:10,000) was added to each well. The plates were incubated 1 hr at 37 °C and again washed. 100 up of p-nitrophenylphosphate was added to each well (2 pills in 10 ml of Tris buffer ph=9.5). After 20 minutes color began to develop and absorbance was read at 405 nm.

**FTase Protein Substrate Modification**

2 µg of recombinant bacterially expressed RDJ2 [200] was incubated with 20 µM AGPP 5b or FPP 1b or the other analogs and 5 µg FTase in 50 µL of Tris-HCl buffer (pH=7.4) The reactions were incubated for 30 min at 37 °C, boiled with SDS and PAGE performed using the protein concentrations indicated on the blots.

**Cell Based Antibody Analysis**

**AGOH Labeling and FTI inhibition of label in HEK-293 cells**

Plates were coated with poly-L-Lysine and seeded with HEK-cells in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum. Cells were incubated in medium containing 30 µM lovastatin and 10 µM GGOH for 24 hrs. The media was poured off then replenished with media without lovastatin and GGOH and were treated with 3 µM FTI-277 (calbiochem), 20 µM GGTI-2147 (calbiochem) or no inhibitor and incubated at 37° C for 1 hour. DMSO as a vehicle, or 100 µM AGOH 5a was then added and incubated at 37° C for two hours. Medium was then removed and cells were extracted into 300 µL PBS containing Protease Inhibitor cocktail set 1 (Calbiochem). The samples were sonicated and centrifuged for 5 minutes. The supernatant was removed and used for western blotting.
AGOH incorporation into unblocked cells

Plates were coated with poly-L-Lysine and seeded with HEK-cells in Dulbecco’s modified Eagle's medium with 5% fetal bovine serum. The cells were then treated with DMSO as a vehicle, or 30 µM AGOH 5a and incubated at 37°C for the indicated time points. Medium was then removed and cells were extracted into 300 µL PBS containing Protease Inhibitor cocktail set 1 (Calbiochem). The samples were sonicated and centrifuged for 5 minutes. The supernatant was removed and used for western blotting.

Western Blot Analysis

Cells were harvested by scraping into 300 µl phosphate buffered saline containing protease inhibitor cocktail set 1 (calbiochem), followed by sonication on ice. Protein concentration was determined using a Bradford reagent kit (Pierce). The proteins from the FTase reaction mixtures or cell lysates were then separated using 12% SDS-polyacrylamide gels followed by transfer to nitrocellulose membranes. The membranes were incubated in blocking buffer PCT (see ELISA) for one hour at room temperature, after which the following sequential steps were used: incubation with anti-AG 1:1000 to 1:10,000 in PCT for one hour; 3X 15 minute washes with vigorous shaking in PT buffer (see ELISA); incubation at room temperature one hour with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Zymed) at a dilution of 1:20,000 in PCT buffer; and four 15 minute washes with PT buffer. The membranes were then subjected to enhanced chemiluminescence detection by incubation in 20 mL of detection buffer (Pierce Super Signal) for one minute.

Cell Permeant analog Stability

The time dependent decomposition of 9a and 9b was carried out by analyzing the disappearance of the tetraesters and the appearance of the parent alcohols 8a and 8b by RP-HPLC analysis. An appropriate amount of either 9a (7 mg, 0.01 mmols) or 9b (8 mg, 0.01 mmols) was dissolved in 10 µL of acetonitrile and diluted into 990 µL water or 990 µL serum (DMEM + 10% fetal calf serum) adjusted to pH 7.4. The solutions were kept at room temperature. At suitable intervals 10 µL aliquots were withdrawn with the autoinjector and analyzed by RP-HPLC. Each sample was eluted with a gradient of
water to acetonitrile (9a 70% acetonitrile/water → 100% acetonitrile over 20 min and 9b 80% acetonitrile/water → 100% acetonitrile over 20 min.) Alternatively the analog was placed in dissolved in D$_2$O and the decomposition was monitored by $^{31}$P-NMR.

This work has been published under the following citations.

Credits:
1. Post-doctoral researcher Michael J. Roberts was responsible for cell labeling studies.
2. Former graduate student Kareem A. H. Chehade was responsible for measuring the farnesyl methylene diphosphonate kinetics
Introduction

The major mechanism by which GGTaseI differentiates between a canonical FTase target Ca1a2X box and a canonical GGTaseI substrate Ca1a2X sequence is the composition of the a2-X binding site of the enzyme [49]. In GGTaseI the a2 binding site is made up of Thr49β, Phe53β, Leu320β, the third and forth GGPP 1 isoprenes and the target X residue. The X residue binding site is made up of the fourth GGPP isoprene the a2 residue, Thr49β, His121β, Ala123β, and Phe174β. Interestingly, in the FTase Ca1a2X binding site the third (terminal) isoprene of FPP 2 interacts directly with only the a2 residue of the target Ca1a2X motif.
Figure 7-1 Analogs

1: GGPP

2: FPP

3: AGPP

4a: AFPP

4b: NAFPP
Analogs of FPP with an aniline moiety replacing the third isoprene of FPP have been extremely useful in probing the endogenous activity of proteins modified by FTase and in understanding the selectivity of the FTase enzyme. 8-anilinogeranyl diphosphate (AGPP 3) is transferable by FTase with kinetics nearly identical to those of FPP [159, 161]. FTase reactions with AGPP are also highly selective for canonical FTase substrates over canonical GGTasel substrates.(see Chapter 3) Often analogs as substrates for FTase versus GGTaseI are designed by extending the FPP analog isoprene chain by one isoprenoid to make GGTasel substrates analogs [166, 239]. In this chapter, I will describe how we have utilized this strategy with AGPP 3 and a pNO2 aniline analog of AGPP in an attempt to design new transferable GGTasel substrate analogs. We have found that anilinofarnesyl diphosphate (AFPP 4a) and pNO2 anilinofarnesyl diphosphate (NAFPP 4b) analogs can be prepared in a manner analogous to the method for preparing the AGPP series of analogs. Both of these analogs are substrates for GGTasel and a CVIL GGTasel Ca1a2X peptide, and AFPP appears to compete very well with the natural substrate of GGTasel, GGPP. We have also found that antibodies designed to detect anilinogeranylated proteins are also useful for the detection of anilinofarnesylated proteins. These results suggest that the AFPP analogs are substrates and can act as specific probes for the activity of GGTasel in a way analogous to AGPP for FTase.

Results

Synthesis of geranylgeranyl diphosphate analogs with substituted aniline moiety

The synthetic route shown in figure 7-2 is analogous to the synthesis of AGPP with farnesyl acetate 5 as the starting material rather than geranyl acetate. The SeO2 oxidation of farnesyl acetate 5 was significantly less efficient than geranyl acetate oxidation giving maximum yields of 20%. The yield of the reaction was increased slightly (30%) using ethereal co-solvents apparently stabilizing the transition state with SeO2. Altering the hydroxyl protecting group had no effect on the overall yield of the reaction and no further attempts to optimize the reaction were attempted. Following farnesyl
acetate oxidation to the α,β-unsaturated aldehyde 6 the aniline moiety was incorporated by reductive amination followed by saponification to give the alcohols 9a (AFOH) and 9b (NAFOH). The alcohols were then halogenated and converted to diphosphate as previously described for the AGPP series of FPP analogs giving AFPP 4a and NAFPP 4b.

Affects of altering Analogue Structure on Peptide reactivity

In order to determine if the anilinofarnesyl diphosphate analogs were substrates for GGTaseI we screened the transfer rate of the analogues with two dansylated-GCaaX peptide substrates utilizing a continuous fluorescence assay. The peptides corresponded to a canonical GGTaseI substrate peptide (CVIL) and a peptide that is a substrate for both FTase and GGTaseI (CVIM). We confirmed that GGPP was a substrate with the CVIM and CVIL peptides (Figure 7-3). We found that AFPP was a substrate for GGTaseI and reacted with a rate similar to that of GGPP with both the CVIL and CVIM peptides. However, NAFPP was a substrate only with the CVIL peptide and no reaction was observed with the CVIM peptide. In addition, AFPP and NAFPP were not substrates for FTase with an N-dansyl-GCVIM peptide as the isoprenoid acceptor.

We have previously observed in the FTase reaction that there are three peptide concentration dependent binding states in the steady-state mechanism of FTase. To determine if the same was true for GGTaseI we measured initial velocities of a wide range of CVIL peptide concentrations with each of the analogues. We found that the third peptide binding state was somewhat apparent in the GGPP reaction. However, we were unable to reach peptide concentrations sufficient to observe a third peptide concentration dependent binding state with the AFPP and NAFPP analogues (Figure 7-4). In addition, we did not observe peptide inhibition with the NAFPP analogue at the concentrations of peptide analyzed.
Figure 7-2 synthesis of AFPP and NAFPP
Reagents: (a) SeO₂ DCM 2hrs 0° C; (b) aniline 8a-b HOAc, NaBH(OAc)₃, DCE, rt; (c) K₂CO₃ MeOH/Water; (d) PPh₃Cl₂, MeCN, rt; 10 minutes ((nBu)₄N)₃HP₂O₇, MeCN overnight.
Figure 7-3 Analog reactivity with CVIL and CVIM peptides and GGTagel catalyst

Reactions were initiated by addition of GGTagel. Only the compounds shown below could be transferred to the peptides. No reaction was detected with any of the analogs with CVLS, CVIM or CAHQ peptides as the isoprenoid acceptor and GGTagel as the catalyst. Rates of the reactions are reported in pmols/sec and are based on the fluorescence enhancement and RP-HPLC analysis of reactions containing 3μM peptide, 12 μM analog and 10 nM GGTagel. Reactions were run in 96 well plate format. All reactions were performed in Tris-HCl buffer (pH=7.4) plus detergent and reducing agent. Note that AFPP is the only analog that also reacts with the CVIM peptide.
Figure 7-4 GGTasel Steady-State reaction kinetics

GGTasel Steady-State reaction with a) GGPP b) AFPP c) NAFPP and CVIL. Rates are reported as rate per unit enzyme. Note the increase in apparent Ki with each analogue.
In order to determine if the AFPP analogues altered the CVIL peptide reactivity in the GGTSasel reaction we measured the apparent $k_{cat}$, $Km^{peptide}$ and $k_{cat}/Km^{peptide}$ with the analogs, GGPP and FPP (Table 7-1). We found that the catalytic efficiency of the CVIL peptide GGTSasel reactions was lowest with FPP and highest with the natural substrate GGPP. The NAFPP analogue increased the $Km^{peptide}$ and $k_{cat}$ with the CVIL peptide. Interestingly, the catalytic efficiency with NAFPP was five times and three times lower than the efficiency with GGPP and AFPP respectively. However, the efficiency of the GGTSasel reaction with NAFPP and the CVIL peptide was four times higher than the efficiency of GGTSasel with FPP as the donor substrate. Because there was only a small contribution of a third peptide binding state we also fit the data to the substrate inhibition equation given in equation 1. We found that the Ki of the CaaX peptide was significantly higher with AFPP as the substrate therefore peptide inhibition was much more potent with GGPP as the substrate (Table 7-2).

When the GGTSasel target peptide was replaced with a CVIM peptide we found that only GGPP and AFPP were reacted efficiently. Both AFPP and GGPP catalytic efficiency were on the same order as with the CVIL peptide, but the value was reduced 3.6 and 3.2 fold respectively. Surprisingly, no reaction was observed with the NAFPP analogue, yet even FPP was utilized to some extent by the enzyme. These results suggest that the NAFPP analogue reduces the ability of GGTSasel to catalyze the modification of alternative peptide substrates. Interestingly, the reactivity of AFPP with the different peptide substrates was similar to the reactivity of GGPP only the catalytic efficiency was slightly reduced. In the case of CVIL as the isoprenoid acceptor the catalytic efficiency was reduced by an increase in the $Km^{peptide}$ while with CVIM both the $k_{cat}$ and $Km^{peptide}$ were increased.
Table 7-1 GGTaseI reaction kinetics
Steady-state kinetics with the GGTaseI enzyme and substrate analogs.
All reactions were performed in Tris-HCl buffer (pH=7.4) plus detergent and reducing agent with varying concentrations of the peptide, 12 µM analog, and 10 nM GGTaseI. Note that the transfer kinetics of AFPP are similar to GGPP with CVIL but not CVIM

<table>
<thead>
<tr>
<th>cmpd</th>
<th>#</th>
<th>peptide</th>
<th>Km&lt;sub&gt;CaaX&lt;/sub&gt; (µM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/Km&lt;sub&gt;CaaX&lt;/sub&gt; (µM&lt;sup&gt;-1&lt;/sup&gt;•s&lt;sup&gt;-1&lt;/sup&gt;)</th>
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</thead>
<tbody>
<tr>
<td>F</td>
<td>2</td>
<td>CVIL</td>
<td>6 ± 1</td>
<td>0.034 ± 0.005</td>
<td>0.0053 ± 0.0002</td>
</tr>
<tr>
<td>GG</td>
<td>1</td>
<td>CVIL</td>
<td>0.31 ± 0.05</td>
<td>0.044 ± 0.002</td>
<td>0.091 ± 0.005</td>
</tr>
<tr>
<td>AF</td>
<td>4a</td>
<td>CVIL</td>
<td>0.65 ± 0.06</td>
<td>0.043 ± 0.002</td>
<td>0.054 ± 0.002</td>
</tr>
<tr>
<td>NAF</td>
<td>4b</td>
<td>CVIL</td>
<td>30 ± 5</td>
<td>0.64 ± 0.08</td>
<td>0.020 ± 0.001</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>CVIM</td>
<td>4 ± 2</td>
<td>&lt;0.001</td>
<td>&lt;0.0003</td>
</tr>
<tr>
<td>GG</td>
<td>1</td>
<td>CVIM</td>
<td>2.7 ± 0.2</td>
<td>0.086 ± 0.002</td>
<td>0.025 ± 0.001</td>
</tr>
<tr>
<td>AF</td>
<td>4a</td>
<td>CVIM</td>
<td>6.4 ± 0.4</td>
<td>0.127 ± 0.004</td>
<td>0.017 ± 0.001</td>
</tr>
<tr>
<td>NAF</td>
<td>4b</td>
<td>CVIM</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>NAF</td>
<td>4b</td>
<td>CVIM</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>
Table 7-2 Substrate inhibition data fit with AFPP and GGPP

<table>
<thead>
<tr>
<th>cmpd</th>
<th>#</th>
<th>peptide</th>
<th>$K_m^{CaaX}$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_i^{CaaX}$ (µM)</th>
<th>$k_{cat}/K_m^{CaaX}$ (µM⁻¹ • s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>1</td>
<td>CVIL</td>
<td>2.4 ± 0.9</td>
<td>0.19 ± 0.06</td>
<td>0.5 ± 0.2</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>AF</td>
<td>4a</td>
<td>CVIL</td>
<td>1.2 ± 0.2</td>
<td>0.066 ± 0.008</td>
<td>5 ± 1</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>
The steady-state kinetic results suggest that the CVIL peptide is much more readily modified than the CVIM peptide. To test this we mixed the CVIL and CVIM peptides 1:1 in competition reactions with excess GGPP or AFPP, and analyzed the resulting products by HPLC. We found that the CVIL to CVIM product ratios were 6:1 and 5:1 with AFPP and GGPP respectively (Figure 7-5). The CVIL to CVIM product ratio was slightly higher than expected based on the selectivity factor $k_{cat} / K_{m}^{\text{peptide}}$ and significantly lower than the expected ratio based on the $K_{m}^{\text{peptide}}$ alone. These results were consistent with the model proposed previously for the substrate association pathway in FTase giving figure 7-6 as the likely kinetic mechanism for GGTagel.

**AFPP competes effectively for CVIL modification**

AGPP is competitive with FPP for FTase catalyzed modification of proteins both *in vitro* and in cell culture [159, 209]. The relative efficiency of peptide modification by an alternative isoprenoid substrate over the natural substrate can be determined from the ratio of products formed in competition experiments between the transferable analogues, the natural substrate and peptide. We measured products ratios from competition reactions between transferable AFPP analogues and GGPP for modification of the Dns-GCVIL peptide by HPLC separation of the products and integration of the dansyl peak absorbance. Reaction of 1:1 ratios of AFPP and GGPP with limiting CVIL peptide resulted in a 2:1 ratio of geranylgeranyl to anilinofarnesyl modified CVIL. This was an identical ratio to that found for AGPP with FTase and a CVLS peptide (Figure 7-7). Surprisingly, when the peptide target was changed to CVIM, AFPP was not able to compete with GGPP nearly as well. The product ratio from reactions containing 1:1 AFPP:GGPP in excess of the CVIM peptide was 8:1 geranylgeranyl to anilinofarnesylated peptide. These results suggested that the identity of the ability of the analogue to compete with the natural substrate was completely dependent on the identity of the peptide target. We also found that the NAFPP analogue was not competitive with GGPP for the modification of either GGTagel substrate peptide.
**Figure 7-5 Peptide competition between CVIL and CVIM peptides with a GGTagse catalyst**

Peptide competition between CVIL and CVIM with GGPP or AFPP as the isoprenoid donor. Each competition reaction was prepared with a) GGPP or b) AFPP at 6.7 µM and 3 µM CVIL and CVIM peptide. Reactions were stopped using a solution of acetic acid and isopropanol then analyzed by HPLC. Note that the CVIL outcompetes CVIM with both GGPP and AFPP as the isoprenoid donor.
Figure 7-6 GGTasel proposed reaction mechanism
Proposed mechanism for the GGTasel reaction. The mechanism is split into two pathways path A representing GGPP stimulated product release and path B representing peptide stimulated product release. E is the GGTasel enzyme, E-GGPP is the GGTasel•GGPP complex, E•GGPP•CaaX is the GGTasel•GGPP•CaaX peptide complex, E-Product is the GGTasel bound product complex, E-Product-GGPP is the GGTasel bound to both GGPP and the reaction product, and E-CaaX is the peptide bound GGTasel inhibitory complex, E-Product-CaaX is the peptide bound enzyme product complex.

Path A

E-GGPP ⇌ E-GGPP-CaaX ⇌ E-Product

Path B

E ⇌ E-CaaX ⇌ E-Product-CaaX

[GGPP] ⇌ [CaaX]

[Product] ⇌ E-Product-GGPP ⇌ [GGPP]
Figure 7-7 GGPP competition for GGTaseI depends on target peptide. Fluorescence HPLC chromatograms of competition reactions between AFPP and GGPP for the modification of a CVIL or CVIM peptide. Each competition reaction was prepared with equimolar GGPP and AFPP at 6.7 µM each and 3 µM CVIL or CVIM peptide. Reactions were stopped using a solution of acetic acid and isopropanol then analyzed by HPLC. Note that AFPP competes much more effectively for CVIL than CVIM.

A) AFPP + GGPP + CVIL

B) AFPP + GGPP + CVIM
AFPP and NAFPP are recognized by anti-AG and anti-NAG antibodies
AGPP as an unnatural probe for the modification of proteins has been extremely useful to determine the cellular prenylation status of proteins and for confirming the \textit{in vitro} modification of proteins by FTase. In chapter 6, I described the development of antibodies specific for the anilinogeranyl and \textit{pNO}_2anilinogeranyl modification of proteins. The antibodies are specific for the aniline isoprenoid of the modified protein. In order to determine whether the antibodies could detect anilinofarnesylated or \textit{pNO}_2anilinofarnesylated proteins we prepared proteins modified with the AFPP analogs. Using a large amount of GGTasel we were able to obtain bacterially expressed H-Ras proteins modified \textit{in vitro} with the AFPP and NAFPP analogs. Western blot analysis of the reaction mixtures indicated that the analog was incorporated into the protein and that the antibodies specifically detected the anilinofarnesylated and \textit{pNO}_2anilinofarnesylated proteins. (Figure 7-8a) Interestingly, the polyclonal anti anilinogeranyl antibody was unable to detect the anilinofarnesylated protein. However, a mouse monoclonal antibody raised against the same hapten was able to detect the modified protein.
Figure 7-8 NAF and AF detection with anti-AG and anti-NAG antibodies

a) Western blot analysis of anilinofarnesylated and pNO2-anilinofarnesylated Ras proteins with anti-AG and anti-NAG polyclonal antibodies and anti-AG monoclonal antibody detection (mAG). Equal protein was loaded into each well but the extent of modification was not analyzed. b) Western blot analysis of H460 and MCF7 cells labeled with 30 µM AFOH overnight and lysed then run on SDS-PAGE gel. AG-Ras control is an in vitro AGPP modified Ras protein. Note that the monoclonal but not the polyclonal anti-AG antibody can detect the AF modified Ras proteins and that some AF modified proteins appear to be labeled in the cell lysates.

a)
Cell labeling with AFOH

Since the anilinofarnesylated proteins could be detected by the anti-AG antibodies we were then interested in whether they could be used to label cellular proteins. To test this we treated cells H460 (lung cancer cell line) and MCF7 (breast cancer cell line) cells with the AFOH analog or DMSO, lysed the cells and analyzed for AFOH incorporation into proteins (Figure 7-8b). The anti-AG antibody did not appear to label a large number of proteins, but AFOH did appear to be incorporated into proteins with a molecular weight around that of the small molecular weight GTPases. Small molecular weight GTPases are most commonly geranylgeranylated which is consistent with these results. Low detection of labeled proteins may have been due to poor detection by the anti-AG antibody for AF modified proteins, or decreased ability of the AFOH due be converted to diphosphate and compete with endogenous GGPP.

Discussion

In this chapter we have found that extending the isoprenoid chain of the FTase transferable analogues AGPP and $p$NO$_2$-AGPP by one isoprenoid unit gives selective transferable substrates for the related enzyme GGTasel. Surprisingly, we found that peptide substrate inhibition appeared to be significantly less potent for GGTasel than has previously been observed with FTase, and with NAFPP as the isoprenoid donor peptide substrate inhibition was not detected. In addition, the selectivity of the enzyme for the different GGTasel substrate peptides appeared to correlate more closely with $k_{cat}/K_{mpeptide}$ than was observed previously with FTase. These results suggested that peptide stimulated release in the GGTasel mechanism is not nearly as effective as it is in the FTase reaction mechanism.

AFPP reactivity with CVIL and CVIM peptides were similar to GGPP while the addition of the $p$NO$_2$ group to the aniline moiety resulted in a molecule that only reacted with the CVIL peptide. In addition, AFPP did not compete effectively with GGPP when the CVIM peptide was the isoprenylation target. The terminal isoprene of GGPP is known to interact with more of the surface of a peptide substrate than in the FTase reaction. Therefore, GGTasel substrate analogues may be particularly sensitive to the structure of an incoming peptide. Changes in the structure of the isoprenoid result in
changes in the kinetics of peptide substrates reactivity and may provides an exogenous mechanism for altering the selectivity of the GGTaseI enzyme.

**Experimental**

**General**

All RP-HPLC was performed on an Agilent 1100 HPLC system equipped with a microplate autosampler, diode array and fluorescence detector. The HPLC analysis was performed with a vydac C4 column with 0.01% TFA in water (A) and 0.01% TFA CH$_3$CN (B) as the mobile phase. Peptides were purchased from peptidogenics and each contained a dansyl group N linked to a glycine linker followed by a C residue then a variable aaX sequence (VIL or VIM). Spectrofluorometric analyses were performed in a 96-well flat bottom, non-binding surface, black polystyrene plate (Excitation wavelength, 340 nm; emission wavelength 505 nm with a 10 nm cutoff). The plate reader was a SpectraMax GEMINI XPS fluorescence well-plate reader. Absorbance readings were determined using a Cary UV/Vis spectrophotometer.

**Steady-state peptide kinetics**

The kinetic constants $K_m$ of peptide and $k_{cat}$ with GGPP, and the analogs with each peptide were determined in triplicate measurements using a continuous spectrofluorometric assay adapted for a 96-well plate. The following assay components were assembled in individual wells of a 96-well plate and incubated at 30° C for 20 minutes: 180 µL of assay buffer (52 mM Tris-HCl, 12 mM MgCl$_2$, 12 µM ZnCl$_2$ pH 7.4), 40 µL of detergent (0.125% n-dodecyl-β-D-maltoside in assay buffer), 40 µL of reducing agent (50 mM DTT in assay buffer) 20uL of GGPP or analog (200 µM in 25 mM NH$_4$HCO$_3$) and 20 µL of N-dansyl-GCaaX peptide (variable concentration). Fluorescence was detected using a time based scan at 30° C for 120 minutes. The velocity of each reaction was determined by converting the rate of increase in fluorescence intensity units (FLU/s) to µM/s with equation 7-1.

**Equation 7-1:** $v_i = \frac{(R \cdot P)}{(F_{MAX} - F_{MIN})}$

Where $v_i$ is the velocity of the reaction in µM/s. R is the rate of the reaction in FLU/s. P is equal to the concentration of modified product in µM (see below). $F_{MAX}$ is the fluorescence intensity of fully modified product. $F_{MIN}$ is the fluorescence of a reaction
mixture that contained 20 µL of assay buffer in the place of GGTaseI. Complete modification of peptides was assumed when fluorescence stabilized for more than 10 minutes and was confirmed by RP-HPLC analysis of the reaction mixture.

Final analysis peptide concentrations were chosen based on preliminary determination of the $K_m^{peptide}$ with 0.5, 1, 5 and 10 µM dansyl-GCaaX concentrations (see below). The final analysis used 8 peptide concentrations which were: 1/6, 1/5, ¼, ½, ¾, 1, 2, and 3 times the estimated $K_m^{peptide}$. The reaction was then initiated by the addition of 20 µL of GGTaseI (final 10 nM). Fluorescence enhancement was determined as described above.

Complete modification of peptides was assumed when fluorescence stabilized for more than 10 minutes and was confirmed by RP-HPLC analysis of the reaction mixture. If the reactions that contained higher concentrations of peptides did not go to completion the Fmax-Fmin value was extrapolated using a linear plot of the Fmax-Fmin for each of the lower concentration reactions that did go to completion. Alternatively the extent of input peptide modified was determined by RP-HPLC analysis of the dansyl moiety peak absorbance corresponding to the unmodified and modified peptides. The percent of modified peptide relative to the total input peptide was used to calculate the concentration of product in the mixture.

The velocities of the reactions were plotted against the concentration of peptide and were fit to the Michaelis-Menten equation (Equation 7-2) to give the apparent $k_{cat}^{app}$ ($k_{cat}^{app}$) and $K_m^{peptide}^{app}$ ($K_m^{peptide}^{app}$) values. Where $e_t$ was the total enzyme concentration and [Pep] was the total input peptide. The $k_{cat}^{app}/K_m^{peptide}^{app}$ value was derived from a Lineweaver-Burke plot of the same data points.

Equation 7-2: $v/e_t = (k_{cat}^{app}[Pep])/(K_m^{peptide}^{app}+[Pep])$

**Peptide competition**

Competition reactions were prepared with the same components given for the $k_{cat}^{app}/K_m^{peptide}^{app}$ analysis except two peptides were added to the mixture at a single concentration. Peptides A and B were diluted to 45 µM in Tris-HCl buffer (pH=7.4) and the concentration checked by reading the Abs of the solution at 340 nm ($\varepsilon_{dansyl}^{4250}=4250$ Abs·M⁻¹·cm⁻¹). 20 µL of peptide A and 20 µL of peptide B were then added to reaction
mixtures as described above except only 160 µL of assay buffer was added. Separate reactions were prepared as a standard with GGPP or the analogs and only one peptide as described above. The reactions were then initiated with the addition of GGTaseI (10 nM final concentration) and analyzed spectrofluorometrically. The reactions were stopped prior to consumption of more than 50% of either peptide by adding 20 µL of a stop solution (isopropyl alcohol and acetic acid 8:2). HPLC analysis was then performed with 100 µL of the reaction mixture loaded onto C18 column and eluted with a linear gradient of 0-20 min 50% B to 100% B at a flow rate of 1 mL/min. Peaks on the 340 nm trace chromatogram that corresponded to both the standard reactions and fluorescent peaks were then integrated. The ratios of products were calculated according to equation 7-3.

**Equation 7-3**: \( \frac{I_{A\text{mod}}}{I_{A\text{tot}}} \left/ \frac{I_{B\text{mod}}}{I_{B\text{tot}}} \right. \)

Where \( I_{A\text{mod}} \) is the integral of the modified peptide A, \( I_{A\text{tot}} \) is the integral of the modified peptide A plus the unmodified peptide A, \( I_{B\text{mod}} \) is the integral of the modified peptide B and \( I_{B\text{tot}} \) was the integral of the modified peptide B plus the unmodified peptide B. Alternatively, the ratios were calculated using the integrals of the standard peptides fluorescence relative to competition product fluorescence giving less than a 5% difference in the calculated ratio.

**GGPP competition**

Competition reactions were prepared with the same components given for the \( k_{cat}/K_m \) peptide analysis except two isoprenoids were added to the mixture and the peptide concentration was held constant. GGPP and the analog were diluted to 200 µM in 25 mM NH₄HCO₃. 20 µL of GGPP and 20 µL of analog were then added to a 260 µL solution of peptide, DTT and detergent in 52 mM Tris-HCl buffer pH=7.4 (final concentrations DTT=6.7 mM, DM=0.033%, Peptide=3µM, analog= 13 µM and FPP=13 µM). The concentration of peptide was determined by absorbance of the dansyl group and isoprenoid diphosphate concentrations were determined using absorbance and the extinction coefficient of N-Methyl-aniline (9120 Abs·M⁻¹·cm⁻¹) and N-methyl-p-NO₂aniline (18,430 Abs·M⁻¹·cm⁻¹). The reactions were initiated with the addition of FTase and analyzed spectrofluorometrically (20 nM final GGTaseI concentration). The reactions
were stopped after 2 hours with a mixture of acetic acid and iPrOH. HPLC analysis was then performed with 100 µL of the reaction mixture loaded onto C18 column and eluted with a linear gradient of 0-20 min 50% B to 100% B at a flow rate of 1 mL/min. Peaks on the 340 nm trace chromatogram that corresponded to standard reaction fluorescent peaks were then integrated. The ratios of products were calculated using $I_{\text{analog}}/I_{\text{FPP}}$, where $I_{\text{analog}}$ is the integral of the peptide modified with the analog and $I_{\text{FPP}}$ is the integral of the peptide modified with FPP.

**GGTasel Protein Substrate Modification**

3 µM recombinant bacterially expressed H-Ras [200] was incubated with 10 µM AFPP or NAFPP and 1µM FTase in 300 µL of Tris-HCl buffer (pH=7.4) The reactions were incubated for 30 min at 37 °C, boiled with SDS and PAGE performed using 10 µL of the reaction mixtures.

**AFOH incorporation into unblocked cells**

Plates were coated with poly-L-Lysine and seeded with in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum. The cells were then treated with DMSO as a vehicle, or 30 µM AFOH was added and incubated at 37° C overnight. Medium was then removed and cells were extracted into 500 µL of RIPA buffer containing Protease Inhibitor cocktail I (calbiochem). The supernatant was removed and used for western blotting.

**Western Blot Analysis**

Protein concentration was determined using a Bradford reagent kit (Pierce) with 1:100 dilutions of the cell lysates. The proteins from the GGTasel reaction mixtures or cell lysates were then separated using 12% SDS-polyacrylamide gels followed by transfer to nitrocellulose membranes. The membranes were incubated in blocking buffer PBS Casein and Tween buffer for one hour at room temperature, after which the following sequential steps were used: incubation with anti-AG or anti-NAG to 1:10,000 in PCT for one hour; 3 X 15 minute washes with vigorous shaking in PBS Tween buffer; incubation at room temperature one hour with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Zymed) at a dilution of 1:20,000 in PCT buffer; and four
15 minute washes with PT buffer. The membranes were then subjected to enhanced chemiluminescence detection by incubation in 20 mL of detection buffer (Pierce Super Signal) for one minute.

Credits:
Post-doctoral researcher Zhongwen Wang was responsible for the synthesis of AFPP and NAFPP
Post-doctoral researcher Thangiah Subramanian was responsible for finding that ethereal solvents improved $\alpha,\beta$-unsaturated aldehyde formation
Post-doctoral researcher David Dremmer in the department of pharmacy practice and science laboratory of Val Adams was responsible for cell treatments with AFOH.
CHAPTER 8 SUMMARY, DISCUSSION AND FUTURE DIRECTIONS

The results presented in this dissertation describe the development of and biochemical analysis of lipid analogs of isoprenoid diphosphate prenyltransferase substrates. In chapter 2, I described the synthesis of a small library and a few exploratory FPP analogs and the finding that FTase accepts a wide variety of ω-isoprene substituted analogs as substrates, and that many of these substrates disrupt the function of oncogenic H-Ras in a whole cell model system. The structural parameters of the analogs that affected the ability of the analog to transfer to protein targets did not appear to be the same structural parameters important for the function of the lipid group on the protein target. The results strongly support the hypothesis that the relationship of lipid structure of FTase transfer is orthogonal to that of lipid structure on function. Specifically, the hydrophobicity of the analog did not appear to affect analog transferability, but was the central property important for the function of the lipid.

The major limitation with using the isoprenoid analogs was the fact that there were no efficient methods to observe cellular prenylation of proteins with the analogs. It was possible that the highly charged diphosphate anion of the lipid diphosphate molecules would prevent cellular uptake of the farnesyl diphosphate analogs. The Xenopus studies avoided this problem because the analogs were directly injected into the relatively large oocyte cells. However, such microinjection would not be feasible for cell culture studies or in vivo studies with the analogs. In chapter 6, I have described the development of antibodies directed towards the modification of proteins with two of the unnatural prenyl lipid analogs. Utilizing these antibodies we found that the parent alcohol of AGPP could traverse a cell membrane and apparently was endogenous converted to diphosphate and utilized by cellular FTase. AGPP was even competitive with endogenous pools of FPP for the modification of proteins. In chapter 4 I described the factors of the analogs affecting FPP competitiveness and found that numerous transferable analogs were even more competitive that AGPP in vitro. It would be interesting to see if this was the case in whole cells. It would be possible to test the competitiveness of a number of the analogs in cells using the reagents developed for this dissertation since numerous analogs cross-react with the anti-anilinogeranyl
antibodies. In addition, the antibodies could be very useful for the study of protein prenylation in multi-cellular organisms and potentially provide a method for studying the affect of FTIs in such organisms. Collaborators in the laboratory of Professor Daret St. Clair at the University of Kentucky have treated mice with the anilinogeranil and $p\text{NO}_2\text{anilinogeranil}$ compounds. However, western blot analysis of tissue samples from these animals have been inconclusive. Importantly, we are also developing methods to utilize the anti-anilinogeranyl antibodies to isolate prenylated proteins from cell lysates. The anti-anilinogeranyl antibodies have proved useful for our laboratory as well as a number of others around the world. Dr. Carol Fierke’s laboratory at the University of Michigan has made use of them for the purposes of studying the in vitro prenylation of unusual $\text{Ca}_1\text{a}_2\text{X}$ containing proteins. The laboratory of Dr. Michael Morgan at Hanover Medical School in Germany has also made use of these antibodies in studying the targets of FTIs in hematologic malignancies.

Chapters 3 through 5 are extremely important for understanding the mechanism of the FTase enzyme and how the kinetic mechanism can be exploited with analogs of the natural substrate of FTase. In chapter 3, I describe the kinetic mechanism by which FTase chooses between different $\text{Ca}_1\text{a}_2\text{X}$ sequence targets. We found that unfarnesylated $\text{Ca}_1\text{a}_2\text{X}$ peptide binding to the E•product complex had a major affect on the selectivity of the enzyme for different $\text{Ca}_1\text{a}_2\text{X}$ substrates. One of the most important results of this chapter with regards to future studies was the fact that the selectivity of the enzyme could not be predicted by the classic Michaelis-Menten “selectivity constant” $k_{\text{cat}}/K_m^{\text{peptide}}$. Instead FTase selectivity was much more dependent on the $K_m^{\text{peptide}}$ alone. This result gives a key parameter of the steady-state FTase reaction that is central to predicting the $\text{Ca}_1\text{a}_2\text{X}$ selectivity of the enzyme with different isoprenoid donors. In addition, I described the ability of the analog to alter the $\text{Ca}_1\text{a}_2\text{X}$ selectivity of the FTase enzyme. Later in chapter 5, I described how we could take advantage of the lipid interaction between the isoprenoid and peptide target of the FTase reaction to create $\text{Ca}_1\text{a}_2\text{X}$ selective analogs that converted FTase from a relatively promiscuous enzyme to one with much more limited target selectivity. The next logical extension of this work would be to determine if these factors were equally important in a whole cell system. Unfortunately, the antibodies development thus far cannot detect the
modification of proteins with the selective analogs. However, the methods used for developing the anti-anilinogeranyl antibodies could easily be adapted to other lipid modifications.

Taken together the results I have described in this dissertation indicate that it will be possible to develop selective, FPP or GGPP competitive, prenyl function inhibitors of proteins. Such prenyl function inhibitors would be a major step forward for the development of new therapeutics for diseases ranging from cancer to hepatitis delta infections. Selectivity would allow specific targeting of only certain proteins for inhibition of function and likely greatly reduce toxicity associated with the use of a FTI. Selectivity would be especially important for diseases like hepatitis delta infection. The large antigen of the hepatitis delta protein that must be farnesylated for the virus to function has a highly unusual C-terminal Ca_{1:a_{2}}X sequence, CRPQ. Analogs designed to only target that particular sequence would likely not affect the prenylation of any proteins other than the hepatitis delta protein. In conclusion, there is still a great deal of work that needs to be done to reach the development of Ca_{1:a_{2}}X protein selective prenyl function inhibitors. The work in this dissertation however lays significant ground work for understanding the properties important for such an inhibitor and the properties that are important for Ca_{1:a_{2}}X selectivity. This work also describes the development of an important novel reagent that could be used to study prenylation in cells and possibly large organisms.
REFERENCES


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