Inhibition of prostate cancer via inhibition of peptidylglycine α-amidating monooxygenase (PAM)

Nicole R. Bearss
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A Thesis

Entitled

Inhibition of Prostate Cancer via Inhibition of Peptidylglycine α-Amidating Monooxygenase (PAM)

by

Nicole Renee Bearss

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Biology

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Prostate cancer (CaP) is a widespread and costly disease. Hormone-dependent CaP can be treated with hormone-deprivation therapy, often resulting in the development of a hormone-independent stage via neuroendocrine differentiation, which currently has no effective treatments. Differentiated hormone-independent CaP also can express peptidylglycine α-amidating monooxygenase (PAM), which amidates peptides that have Gly as their C-terminus resulting in a much more potent activity of the peptide. Several PAM substrates are cellular growth signals. Because hormone-independent CaP often express PAM, and PAM activates many growth factors, inhibiting PAM could result in decreased cell growth and therefore PAM inhibitors may be effective treatments for hormone-independent CaP.

A series of PAM substrate-like inhibitors was synthesized. The potencies of these agents were determined by in vitro tests against PAM from cell culture media and cellular extracts. These inhibitors were also tested against PAM-dependent DU145 CaP cell growth, and one of these inhibitors, CD3-246, emerged as our lead PAM inhibiting agent. In vivo studies determined that CD3-246 inhibited the growth of implanted CaP
xenographs in mice. In conclusion, CD3-246 appears to represent a potential novel
treatment for hormone-independent CaP.
For my mother, who is the strongest woman I know. Thank you for your many sacrifices to give me all that I have ever wanted.
Acknowledgments

This thesis would not have been possible without the love, support, understanding, and encouragement I received from my husband, parents, sisters, and my stepson, Dean. I am beginning to realize the sacrifices that you all have made to allow my education to come first.

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List of Abbreviations

AM ..................... Adrenomedullin
CaP .................... Cancer of the Prostate
CD3-246 .............. Designation for the compound Thba-(D)Tyr-Met-Gly
cp,max ................ Maximum Plasma Concentration
DDC .................... Diethylidithiocarbamate
FBS ..................... Fetal Bovine Serum
F ......................... Bioavailability
fu ......................... Fraction of Dose Excreted Unchanged in the Urine
GI50 ..................... Concentration of Agent that causes a 50% Reduction in Growth
                      compared to Control Samples
[I] ...................... Concentration of Inhibitor
iAM ..................... Intermediate Glycine-Extended Adrenomedullin
IC50 ..................... Concentration of Agent that causes 50% Inhibition compared to
                      Control Samples
ip ........................ Intraperitoneal
Ki ....................... Inhibitor Constant
Km ....................... Michaelis-Menten Constant
L ........................ Length of the Longest Dimension of the Tumor in mm
LD50 .................... Lethal Dose in 50% of the Subjects
LOQ ..................... Limit of Quantification
n ......................... Number of Repeated Samples
NCI .................... National Cancer Institute
NDA ..................... No Detectable Activity
NS ....................... NuSerum IV
PAL ..................... Peptidlyglycine α-Amidating Lyase
PAM ..................... Peptidylglycine α-Amidating Monooxygenase
PBA ..................... 4-Phenyl-3-Butenoic Acid
PBS ..................... Phosphate Buffered Saline
PHM ..................... Peptidylglycine α-Hydroxylating Monooxygenase
PK ................... Pharmacokinetic
PMSF ................... Phenylmethylsulfonyl Fluoride
po ................... oral
PSA ................... Penicillin, Streptomycin, and Amphotericin

[S] ................... Concentration of Substrate
SAR ................... Structure-Activity Relationship
sc ................... Subcutaneous
sd ................... Standard Deviation
SD-FBS ............... Dextran-coated Charcoal Steroid-Depleted Fetal Bovine Serum

t_{1/2} ................... Terminal Plasma Elimination Half-Life
TES ................... N-Tris[Hydroxymethyl]Methy-2-Aminoethane-Sulfonic Acid
Thba ................. 4-(2-Theinyl)butyric Acid
TIS ................... Transferrin, Insulin, and Selenium
t_{max} ................... Time of Maximum Plasma Concentration
TNP ................... Trinitrophenyl
TNP Intermediate ... TNP-(D)Tyr-Val-COOH
TNP Product ........... TNP-(D)Tyr-Val-NH₂
TNP Substrate ....... TNP-(D)Tyr-Val-Gly
tox .................... Toxicity
Tris Base ................ Tris(hydroxymethyl)aminomethane

v ................ Velocity
V .................. Apparent Volume of Distribution
v_{max} ............. Maximum Velocity

w .................. Width of the Tumor Perpendicular to the Longest Dimension of the Tumor in mm
Chapter 1

Introduction

Cancer is a wide-spread disease causing over 500,000 deaths in the United States annually. Cancer of the prostate (CaP) is the second most common cancer among males, following skin cancer. Prostate cancer is ranked second in cancer-related deaths in males in the United States, following lung cancer (American Cancer Society, 2011). There are 180,000 new cases of CaP diagnosed (Denmeade and Isaacs, 2002) and 32,000 deaths due to CaP in the United States annually. The United States spends $9.9 billion on treatment of CaP each year (American Cancer Society, 2011).

Prostate cancer generally begins as a hormone-dependent form for which there are several options for treatment. Historically, types of treatment included brachytherapy, implantation of radioactive pellets to kill surrounding prostate tissue; cryotherapy, freezing of the prostate tissue; radical prostatectomy, surgical removal of the prostate; orchiectomy, surgical removal of the testicles; radiation therapy, external radiation treatment; and chemotherapy, treatment with a non-specific chemical to kill fast growing cells (National Cancer Institute, 2011; Abrahamsson, 2003). Recently, hormone therapy, which blocks testosterone binding to the prostate cells that express the androgen receptor, has been the therapy of choice as it is non-invasive and provides more specific targeting
of the cancer cells. However, anti-androgen treatments, which initially lead to a reduction in CaP growth (Denmeade and Isaacs, 2002), appear to contribute to the progression of CaP into a hormone-independent stage (Rau et al. 2005) where androgen receptor expression is decreased (Jiborn et al. 1998) and hormone therapy becomes ineffective.

The development of hormone-independent CaP is often coupled with neuroendocrine differentiation (Jiborn et al. 1998; Hansson and Abrahamsson, 2001) by aiding in the selection of neuroendocrine cells present in the prostate rather than other cell types (Jiborn et al. 1998). With few therapeutic options available for hormone-independent CaP, it remains difficult to treat (Hansson and Abrahamsson, 2001; American Cancer Society, 2011). One characteristic of the neuroendocrine phenotype is the expression of an enzyme named peptidylglycine α-amidating monooxygenase (PAM, EC1.14.17.3). This may be an important step in the progression from hormone-dependent to hormone-independent CaP (Prigge et al. 2000). Small cell lung cancers (SCLC) are also neuroendocrine tumors (Moody and Cuttitta, 1993) and several SCLC cell lines are known to produce PAM (Vos et al. 1996).

PAM amidates peptides having glycine at the carboxy-terminus and is the only enzyme known to catalyze this reaction. Many of these peptides do not exhibit full activity until the carboxy-terminus is amidated (Prigge et al. 2000). PAM can also amidate fatty acyl glycines, forming fatty acyl amines (Mueller and Driscoll, 2009).

PAM is a bifunctional, monomeric protein encoded by a single gene that is expressed by many tissues (Prigge et al. 2000), including the pituitary gland (Eipper et al. 1992) and heart atrium at high levels (Eipper et al. 1992, Thiele et al. 1989). In other tissues including the lungs (Saldise et al. 1996), smooth muscle, astrocytes, and olfactory...
epithelium (Prigge et al. 2000) PAM is found in lower levels. Although PAM is encoded by a single gene, it is expressed in different alternatively-spliced isomers. At least seven variants have been identified and include the exclusion of part or all of exon 15 that contains the linker sequence for the two active sites, and the trans-membrane domain. Deletion of exon 15 limits PAM’s susceptibility to proteases, keeping PAM in its bifunctional form, whereas deletion of the trans-membrane domain results in a soluble form of PAM (Prigge et al. 2000). PAM can be proteolytically cleaved between the active sites and the active sites can also be expressed independent of each other. The processed forms of rat PAM are expressed in a tissue-specific manner, as seen in the variant forms expressed in pituitary (Eipper et al. 1992; El Meskini et al. 2000) and atrium (Stoffers et al. 1991) tissues.

The two active sites of PAM include a hydroxylase domain, peptidylglycine α-hydroxylating monooxygenase (PHM), and a lyase domain, peptidyl-α-hydroxyglycine α-amidating lyase (PAL), that function independently (Merkler et al. 1992; Moore and May, 1999). PHM catalyzes the formation of a peptidyl-α-hydroxyglycine from a glycine-extended precursor through an alkyl radical intermediate. PHM is oxidized in its resting state; however, PHM must be reduced to become active. Reduction occurs via electron transfers from two ascorbic acid molecules and this electron transfer can only proceed in the presence of bound substrate. PHM is copper-dependent and the hydroxyl group that is added to the glycine residue must come from molecular oxygen. To form the hydroxyglycine product, two bound Cu\textsuperscript{2+} molecules, CuA and CuB, are reduced to Cu\textsuperscript{+}, and molecular oxygen binds to CuB. One electron from oxygen is transferred from the oxygen to CuB, which passes the electron along PHM to oxidize CuA, leaving a
radical oxygen molecule. The oxygen radical group then attacks the α-carbon of glycine, producing peptidyl-α-hydroxyglycine (Prigge et al. 2000).

PAL then catalyzes the formation of the amidated peptide from peptidyl-α-hydroxyglycine. Interestingly, amidation by PAL is not a transamidation. Rather, PAL removes the carboxy-terminal carbon, leaving the amine group from the glycine residue. Conversion from a terminal carboxyl group to an amide makes these peptides more hydrophobic, enabling them to bind to their receptor with higher affinity. There are no known homologs to any part of PAL and the mechanism of PAL is not completely understood (Prigge et al. 2000).

Endogenous PAM substrates include Substance P, adrenomedullin, vasopressin, oxytocin, gastrin, calcitonin, and neuropeptide Y, all of which are involved in intracellular signaling (Prigge et al. 2000). Interestingly, Substance P up-regulates the inflammatory response (Jeng et al. 1997) and is involved in pain regulation (Barak et al. 1999). Neuropeptide Y (Jolicoeur et al. 1991; Jolicoeur et al. 1994) and vasopressin (Murphy et al. 1994) are involved in regulating body temperature, and altering neuropeptide Y levels in the brain can cause anxiety-like behaviors in a mouse (Heilig et al. 1989; Wahlestedt et al. 1993).

Adrenomedullin (AM) is a PAM substrate of key interest in this study. AM is generated by PAM from preproadrenomedullin, which is first translated to an intermediate glycine-extended AM (iAM). The glycine-extended AM is then converted to AM by PAM. AM may be involved in controlling blood pressure (Kitamura et al. 1993). AM increases the cAMP concentration in cells (Ishizaka et al. 1994; Hinsen et al. 2000) and stimulates cell growth in vitro. Miller et al. (1996) have shown that treating
cells with the AM-neutralizing antibody MoAb-G6 caused a significant decrease in cell proliferation in the cells lines H157 (adenosquamous), H720 (lung carcinoid), MCF7 (breast carcinoma), and OVCAR-3 (ovarian carcinoma), but not in SNUC-1 (colon carcinoma). Adding exogenous AM to these cell lines led to almost complete recovery of growth. Likewise, exogenous AM has been shown to increase DU145 CaP cell growth. However, exogenous AM did not stimulate growth of the prostate carcinoma cell line PC3. This may indicate that PC3 cells achieve maximum proliferation from endogenous AM levels (Rocchi et al. 2001) or that these PC3 cells were not expressing sufficient AM receptor to stimulate cellular reproduction.

Because AM is a PAM-activated growth signal and some prostate cancers express PAM, CaP cells may rely on PAM for autocrine growth stimulating signals. Therefore, the inhibition of PAM might decrease prostate cancer growth via depletion of growth signals. Notably, the tissue-specific variations in PAM expression due to alternative-splicing may permit tissue-specific PAM inhibition. If a PAM inhibitor can be identified that acts specifically on one PAM isoform, it may be useful in the treatment of hormone-independent CaP. Alternatively, if a PAM inhibitor that is not variant specific is identified, it may be useful in demonstrating that targeting PAM is a valid method of CaP treatment.

Due to the copper molecules required for PHM activity, metal ion chelators such as Disulfiram (Anabuse®) and DDC (diethyldithiocarbamate) have been tested as PAM inhibitors. Disulfiram causes an irreversible PAM inhibition by making a covalent modification within the PAM enzyme (Rahman et al, 1997). DDC completely inhibited activity of purified PAM, but this activity was partially restored by adding cobalt or
copper and completely restored by adding nickel (Kizer et al. 1986). Other PHM inhibitors include N-formyl amides (Klinge et al. 1994) and α-unsaturated thioacetic acid (Casara et al. 1996), whereas 2,4-diketo-5-acetamido-6-phenyl-hexanoic acid selectively inhibits the PAL domain (Mounier et al. 1997).

Substrate-like inhibitors including PBA (4-phenyl-3-butenoic acid, Bradbury et al. 1990) and di- and tri-peptides (Erion et al. 1994; Zabriskie et al. 1992) have also shown to inhibit PAM activity. Like Disulffiram, PBA also causes irreversible PAM inhibition via a covalent bond (Bradbury et al. 1990). In vivo, PBA administered subcutaneously (sc) inhibited serum PAM activity for up to 3 hours, had a muted effect after 6 hours, but had no detectable effect after 24 hours, indicating that PBA has a short duration of action (Ogonowski et al. 1997). D-Phenylalanyl-L-phenylalanyl-D-vinylglicine is a competitive PHM inhibitor that results in the cleavage of the terminal D-vinylglicine (Zabriskie et al. 1992). It is agreed that substrate-like inhibitors need to be stereospecific at the penultimate position because D-isomers are much less potent then their natural L-counterparts (Ping et al. 1995; Feng et al. 2000). Non-peptide inhibitors include benzamides and glyoxylic acid derivatives (Katopodis and May, 1990) and thioglycolic acid analogs (Merkler et al. 2008). Excluding several of the peptide analogs, these inhibitors all have potencies in the millimolar to micromolar range and therefore are not potent enough to be considered “drug-like”.

Erion et al. (1994) synthesized the “first potent inhibitors of PAM” with potencies in the low nanomolar range. These compounds utilized a peptide backbone attached to imidazoles, thioimidizoles, or thiolates which interact with the copper inside the PAM active site. Though these compounds exhibited excellent potency when tested against
purified enzyme, the authors admit that the compounds have a kinetically slow binding process and a limited intracellular bioavailability. Another group (Meckler et al. 2008) found that thioglycolic acid analogs have significantly different binding affinities for PAM enzyme that was purified from mammalian cells than that from insect cells. It is possible that this specificity between species could result in development of a PAM inhibitor that has specific activity against one form of PAM while leaving other isomers of PAM uninhibited.

Based on previous studies, a series of potential PAM inhibitors have been synthesized in our laboratory. These inhibitors are tri-peptide compounds designed to resemble PAM substrates attached to an acyl group on their amino-terminus. Structures of the acyl groups used are found in Table 1.1. Amino acids such as Methionine, Histidine, and the modified amino acid S-Methyl-Cysteine have chelating properties and were utilized in the penultimate and antepenultimate positions. Some compounds contained modified amino acids, depicted in Table 1.2.
Table 1.1: Structures of acyl-groups used as attachments to the amino-terminus of tripeptide probes. A complete list of the acyl-tripeptides tested can be found in Table 2.2.

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<th>Acyl Structure</th>
<th>Abbreviation</th>
<th>Parent Acid</th>
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<tbody>
<tr>
<td><img src="image" alt="tBuaa" /></td>
<td>tBuaa</td>
<td>tert-Butylacetic Acid</td>
</tr>
<tr>
<td><img src="image" alt="Phaa" /></td>
<td>Phaa</td>
<td>Phenylacetic Acid</td>
</tr>
<tr>
<td><img src="image" alt="Inaa" /></td>
<td>Inaa</td>
<td>Indole-3-acetic Acid</td>
</tr>
<tr>
<td><img src="image" alt="Inca" /></td>
<td>Inca</td>
<td>Indole-2-carboxylic Acid</td>
</tr>
<tr>
<td><img src="image" alt="Pyaa" /></td>
<td>Pyaa</td>
<td>4-Pyrindylacetic Acid</td>
</tr>
<tr>
<td><img src="image" alt="Thpa" /></td>
<td>Thpa</td>
<td>3-(2-Thienyl)propionic Acid</td>
</tr>
<tr>
<td><img src="image" alt="Adca" /></td>
<td>Adca</td>
<td>Adamantanecarboxylic Acid</td>
</tr>
<tr>
<td><img src="image" alt="Thba" /></td>
<td>Thba</td>
<td>4-(2-Thienyl)butyric Acid</td>
</tr>
<tr>
<td><img src="image" alt="Thaa(2)" /></td>
<td>Thaa(2)</td>
<td>(2-Thienyl)acetic Acid</td>
</tr>
<tr>
<td><img src="image" alt="Thaa(3)" /></td>
<td>Thaa(3)</td>
<td>(3-Thienyl)acetic Acid</td>
</tr>
</tbody>
</table>
Thca  (2-Thienyl)carbonic Acid

Adaa  Adamantylacetic Acid
Table 1.2: Modified amino acid structures employed in synthesized acyl-tripeptide test agents.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Abbreviation</th>
<th>Amino Acid Modification</th>
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</thead>
<tbody>
<tr>
<td><img src="image" alt="TyrOBn structure" /></td>
<td>TyrOBn</td>
<td>O-Benzyl-Tyrosine</td>
</tr>
<tr>
<td><img src="image" alt="MetO structure" /></td>
<td>MetO</td>
<td>Methionine-Sulfoxide</td>
</tr>
<tr>
<td><img src="image" alt="PheNO2 structure" /></td>
<td>PheNO2</td>
<td>p-Nitro-Phenylalanine</td>
</tr>
<tr>
<td><img src="image" alt="CysMe structure" /></td>
<td>CysMe</td>
<td>S-Methyl-Cysteine</td>
</tr>
<tr>
<td><img src="image" alt="NpAla structure" /></td>
<td>NpAla</td>
<td>(2-Naphthyl)-Alanine</td>
</tr>
</tbody>
</table>
The compounds were tested against PAM enzyme to determine their inhibitory potency. The most potent compounds were the tested against DU145 CaP cells to determine their effect on cellular growth. CD3-246 was the most potent PAM inhibitor tested, and was therefore moved into further stages of tests. The structure of CD3-246 is illustrated in Figure 1-1.

Figure 1-1: Chemical structure of the lead acyl-tripeptide agent Thba-(D)Tyr-Met-Gly, designated as compound CD3-246. The asterisk denotes unnatural (D) stereochemistry at the Tyrosine asymmetric center; Methionine has the natural (L) stereochemistry, while the terminal Glycine does not possess an asymmetric center. The acronym Thba indicates 4-(2-Thienyl)-butyryl, as indicated in Table 1.1.

This thesis will describe the multiple stages of in vitro testing resulting in the selection of CD3-246 as the lead agent, subsequent in vivo evaluation of its pharmacokinetic and anticancer efficacy, and preliminary studies directed toward delineating its mechanism of action.
Chapter 2

PAM Enzyme Inhibition

To determine the effectiveness of each synthesized PAM inhibitor, described in Chapter 1, each compound was incubated at five concentrations with PAM enzyme secreted from DU145 prostate cancer cells. Compounds with a $K_i$ less than 2 $\mu$M were then tested against PAM from other prostate cancer sources and from muscle, small cell lung cancer, and medulloblastoma. Unless otherwise stated, all products in this thesis were purchased from Fisher Scientific (Hanover Park, IL).

2.1 Cell Maintenance

The PAM-expressing prostate cancer (CaP) cell lines DU145 and PC3 were received from the National Cancer Institute (NCI) Department of Cancer Treatment and Diagnosis Tumor Repository (Bethesda, MD). LNCaP, a prostate cancer cell line that does not express the PAM enzyme, was purchased from ATCC (Manassas, VA). Other PAM-expressing cell lines used in the following studies include TE671 (human medulloblastoma, received from Mariola Klis, University of Toledo, Toledo, OH), H889 (human small cell lung carcinoma, ATCC), and TT (human thyroid carcinoma, ATCC).
All cells were cultured in RPMI 1640 medium containing 2.0 mM L-glutamine (Sigma, St. Louis, MO) and 20 mM HEPES buffer (Sigma), supplemented with 5% fetal bovine serum (FBS), 5% NuSerum IV (NS), 100 IU penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (PSA) within a 37 °C, 100% humidity, 5% CO₂ Revco Ultima II tissue culture incubator. Each cell line was routinely passaged once per week and culture media was replaced twice per week. Cells were discarded when they reached passage twenty.

### 2.2 Secreted PAM Collection

To collect soluble, secreted PAM, cellular media collections were performed. Cells were passaged and allowed to attach for 3 days in maintenance media, at which time the maintenance media was removed and replaced with RPMI 1640 medium that contained 2.0 mM L-glutamine and 20 mM HEPES buffer, supplemented with 10 µg/ml transferrin, 10 µg/ml insulin, and 10 ng/ml selenium (TIS), and PSA. This media lacks added growth factors and has been used previously to create conditions where cellular growth becomes dependent on PAM-activated growth factors (Rocchi et al. 2001; Iwai et al. 1999). One ml aliquots of media were collected on days 14, 18, and 23 and the media was replaced. Ten percent (v/v) glycerol was added to each sample. The samples were stored at -80 °C and used in the PAM inhibition assay described in Section 2.4.
2.3 Cellular PAM Collection

In order to collect membrane-bound PAM from CaP and other tissue types, cellular extractions were performed. PAM was extracted from prostate cancer cell lines as described by Trendel et al. (2008). Four Nunc T225 tissue culture flasks, which contained CaP cells pre-cultured for 6-7 days in media supplemented with 1% dextran-coated charcoal steroid-depleted FBS (SD-FBS) for DU145 and PC3 cells or 2.5% SD-FBS for LNCaP cells, were chilled on ice. The media was collected and the flasks rinsed two times with 10 ml ice cold phosphate buffered saline (PBS, pH 7.4, made in-house). The cells were scraped into 15 ml of ice cold PBS and centrifuged at 100 × g for 5 min at 4 °C. The supernatant was discarded and the cell pellet was resuspended in 7 ml 20 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid, Sigma)/10 mM mannitol buffer (pH 7.4) containing the protease inhibitors PMSF (phenylmethylsulfonyl fluoride, 30 µg/ml), leupeptin (2 µg/ml), benzamidine (16 µg/ml) and 1 µM copper sulfate. This mixture was homogenized via 30 strokes in a dounce homogenizer. The suspension was centrifuged at 1000 × g for 5 min at 4 °C. The resulting pellet, "P1", containing the nuclear fraction of the cells, was suspended in 1.0 ml of buffer. 0.2 ml of the supernatant, "S1", which contained the cytosol and membrane fractions of the cells, was collected and the remaining S1 supernatant was centrifuged at 30,000 × g for 30 min at 4 °C. The supernatant, "S30", containing the cytosolic fraction of the cells, was collected in 1-ml aliquots. The pellet, "P30", was suspended in 3.5 ml TES/mannitol buffer with leupeptin, benzamidine, 1 µM copper sulfate, and 1% Triton X100 (Sigma). Suspended P30 sample (0.2 ml) was collected and the remaining P30 fraction was centrifuged at 100,000 × g for 60 min at 4 °C. The resulting supernatant, "S100",...
contained the membrane fraction of the cells and was collected in 0.5- to 1.0- ml aliquots. The insoluble cellular fraction, "P100", was suspended in 1.0 ml TES/mannitol buffer with protease inhibitors, 1 µM copper sulfate, and 1% Triton X100.

The extraction process was performed in a 4 ºC cold room. Samples were collected on ice, 10% (v/v) glycerol was added, and stored at -80 ºC. A schematic representation of this collection process can be found in Figure 2-1. The samples were then assayed for PAM activity and protein content as described in Trendel et al. (2008).
4 T225 Flasks (cells cultured 6-7 days in media supplemented with steroid-depleted FBS)

Chilled flasks on ice, collected media

**Cell Media** (Extracellular PAM)
140 ml total, about 35 ml per flask

Rinsed flasks 2 times with 10 ml ice cold PBS
Scraped cells into 15 ml ice cold PBS, collected cells in centrifuge tubes on ice

**Centrifugation** (<1000g, 5 min, 4 °C)

**Supernatant** (Cell-free, discard)

Cell Pellet
Resuspend cells in 7 ml 20 mM TES/10 mM mannitol buffer pH 7.4 containing protective enzyme inhibitors (PMSF, protease inhibitors) and 1 µM CuSO₄

**Homogenization**
Ruptured cells via 30 strokes with Dounce homogenizer

**Centrifugation** (<1000g, 5 min, 4 °C)

**Pellet, P1** (Nuclear Fraction)
Resuspend & collected in 1 ml buffer

**Supernatant, S1** (Cytosol and Membrane Fractions)
6.5 ml total, transferred to ultracentrifuge tube and collected 0.2 ml sample

**Ultracentrifugation** (30,000g, 30 min, 4 °C)

**Supernatant, S30** (Cytosolic PAM)
6.2 ml total, collected in 1 ml aliquots

Pellet, P30
Resuspend in 3.5 ml TES/Mannitol buffer with protease inhibitors and 1 µM CuSO₄ (no PMSF), added Triton X100 to 1%, collect 0.2 ml sample

**Ultracentrifugation** (100,000g, 60 min, 4 °C)

**Supernatant, S100** (Membrane PAM)
3.4 ml total, collected in 0.5-1 ml aliquots

Pellet, P100 (Insoluble Fraction)
Resuspend in 1 ml TES/mannitol buffer with protease inhibitors and 1 µM CuSO₄
Added Triton X100 to 1%

Figure 2-1: Procedures for the extraction of cellular PAM enzymes. Extraction procedures were carried out in a refrigerated (4 °C) cold room. Samples were collected on ice, glycerol was added to 10% by volume, and samples were stored at -80 °C until testing. Procedure volumes were increased or decreased as needed for more or fewer flasks.
2.4 PAM Inhibition Assay

2.4.1 CaP PAM Inhibition Assay

Each agent was tested to determine the concentration that produced a 50% reduction in PAM activity (Inhibitory Concentration, IC$_{50}$). Tests were conducted as previously described (Trendel et al. 2008), except each enzyme was incubated in duplicate in the presence of five serial dilutions of inhibiting agent or inhibitor-free control. Briefly, 20 µl PAM enzyme from either cell extraction or media collection was incubated for 1-2 hours with 180 µl of incubation mixture that contained 150 mM NaMES, 10 mM L-ascorbic acid (Sigma), 1% ethanol, 0.1 mg/ml beef liver catalase (Sigma), 1% DMSO, and 1 or 10 µM copper sulfate, depending on sample type. During incubation, samples were shaken at 37 °C. 50 µM TNP-(D)Tyr-Val-Gly, a synthetic PAM substrate that has a detection of 344 nm (Katopodis and May, 1990; Feng et al. 2000), was also included in the incubation mixture. After incubation, the reaction was quenched by adding 20 µl 3 N perchloric acid (Aldrich). Samples were then centrifuged at 16,000 × g at 4 °C for 2 min. 180 µl of sample was transferred to an autosample vial (Waters Corp., Milford, MA) and stored at 4 °C until analysis by high-performance liquid chromatography (HPLC). PAM inhibition was quantified via HPLC detection of hydroxylated TNP-intermediate, TNP-(D)Tyr-Val-OH and TNP-(D)Tyr-Val-NH$_3$, its amidated TNP-product, compared to uninhibited sample.

Known PAM inhibitors were included to validate this assay, including the copper chelators disulfiram (Aldrich) and DDC (Aldrich), and the substrate-like inhibitor PBA (Aldrich). Dopamine was included as a negative control, as it is the product of dopamine
β-hydroxylase which has some homology with PAM (Prigge et al. 2000), but has very little inhibitory effect on PAM.

HPLC analysis was performed on a Waters Alliance® Model 2695 Separations Module with a membrane degasser, refrigerated autosampler set at 4 °C, heated chamber set to 35 °C, and a Waters Dual Wavelength Detector (Model 2487) monitoring absorbance at 344 nm. The TNP-labeled substrate, intermediate, and product were separated on a Supelco Discovery RP Amide C16 column (15 cm × 4.6 mm, 5 μm, Sigma-Aldrich) preceded by a pre-column filter from MAC MOD (Chadds Ford, PA). 50 μl injections were eluted with 1.5 ml/min mobile phase containing 55% fresh 0.01 M sodium acetate buffer (pH 3.8, Sigma-Aldrich, made in-house) and 45% HPLC grade acetonitrile. Total assay time was 4 min per sample, with retention times of 1.9 min for the hydroxylated intermediate, 2.3 min for substrate, and 3.6 min for amidated product. Standards containing 0 to 200 μM substrate, 0 to 50 μM intermediate, and 0 to 50 μM product were prepared in incubation mixture daily and analyzed at the beginning, middle, and end of each HPLC run. Calibration curves generated from the standard peak areas were used to quantify analyte levels in the inhibition samples. The sum of the intermediate and product represented total PAM conversion. A sample chromatograph is shown in Figure 2.2.
Figure 2-2: Representative chromatograph of incubation sample prepared by incubating 50 µM substrate for 2 hr with 10 µM CuSO₄ and DU145 PAM extract. The extract was collected as described in Figure 1.1. The test fraction depicted above represents a resuspended pellet P30.

The Hill equation gave excellent fits for all four standard inhibitors and was used to determine IC₅₀ values for each inhibitor. Because IC₅₀ values are dependent on the concentration of substrate in the samples, the Kᵢ (inhibitor constant) values provided a better comparison between inhibitors tested. Kᵢ values were estimated from these IC₅₀ values using simple competitive inhibitor model relationships. The relationship between Kᵢ and the IC₅₀ for a competitive inhibitor measured using a substrate with Michaelis-Menten constant Kₘ and at a substrate concentration of [S] is given by Segel (1993) as

\[ Kᵢ = \frac{Kₘ \times IC₅₀}{Kₘ + [S]} \]

so that Kᵢ values could be calculated from measured IC₅₀ values as long as the Kₘ value for the substrate was known. Copper chelators do not follow a competitive inhibitor model, so the calculated Kᵢ values for these compounds were included only for crude comparisons. Kₘ values, the concentration of substrate at which enzyme activity is half of its maximum activity, used for each PAM type (extracted or collected in media) in
these calculations were estimated by Lineweaver-Burke analysis of kinetic rates measured without inhibitor in duplicate with six serial dilution concentrations of the TNP-(D)Tyr-Val-Gly substrate (Segel, 1993). $K_m$ values for CaP PAM are shown in Table 2.1. All acyl-tripeptides were first screened in PAM collected from DU145 TIS media. Calculated $K_i$ values are listed in Table 2.2.

Table 2.1: Values of the TNP-(D)Tyr-Val-Gly Michaelis-Menten constant $K_m$ measured for each of the CaP PAM enzymes. Column n indicates the number of duplicate measurements for each substrate concentration.

<table>
<thead>
<tr>
<th>CaP PAM Enzyme</th>
<th>n</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145 TIS</td>
<td>6</td>
<td>6.8 ± 0.5</td>
</tr>
<tr>
<td>DU145 S100</td>
<td>4</td>
<td>10.9 ± 0.7</td>
</tr>
<tr>
<td>PC3 TIS</td>
<td>4</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>PC3 S100</td>
<td>4</td>
<td>10.3 ± 0.6</td>
</tr>
</tbody>
</table>
Table 2.2: Inhibitor constant $K_i$ values measured with DU145 TIS PAM for inhibitor standards and all acyl-tripeptide test agents. Column n indicates the number of samples tested. Acyl-tripeptide results are grouped by peptide families based on the most active member of each family, with agents for a given peptide family listed from most to least active. The unnatural stereoisomer of a naturally occurring amino acid is indicated by a (D). Acyl group and modified amino acid structures are depicted in Tables 1.1 and 1.2.

<table>
<thead>
<tr>
<th>CD3 ID#</th>
<th>Compound</th>
<th>n</th>
<th>$K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Disulfiram (Antabuse)$^\dagger$</td>
<td>3</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>Standard</td>
<td>Diethylthiocarbamate (DDC)$^\ddagger$</td>
<td>3</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Standard</td>
<td>4-Phenyl-3-Butenoic Acid (PBA)</td>
<td>4</td>
<td>0.68 ± 0.07</td>
</tr>
<tr>
<td>246</td>
<td>Thba-(D)Tyr-Met-Gly</td>
<td>18</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>255</td>
<td>Adaa-(D)Tyr-Met-Gly</td>
<td>6</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>405</td>
<td>Thba-(D)TyrOBn-Met-Gly</td>
<td>4</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>236</td>
<td>Inaa-(D)Tyr-Met-Gly</td>
<td>6</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td>247</td>
<td>Thaa(3)-(D)Tyr-Met-Gly</td>
<td>3</td>
<td>0.61 ± 0.11</td>
</tr>
<tr>
<td>245</td>
<td>Thaa(2)-(D)Tyr-Met-Gly</td>
<td>3</td>
<td>0.65 ± 0.08</td>
</tr>
<tr>
<td>237</td>
<td>Thpa-(D)Tyr-Met-Gly</td>
<td>3</td>
<td>0.67 ± 0.04</td>
</tr>
<tr>
<td>234</td>
<td>Inca-(D)Tyr-Met-Gly</td>
<td>3</td>
<td>0.76 ± 0.07</td>
</tr>
<tr>
<td>244</td>
<td>Thca(2)-(D)Tyr-Met-Gly</td>
<td>3</td>
<td>0.90 ± 0.12</td>
</tr>
<tr>
<td>299</td>
<td>Thba-(D)Tyr-MetO-Gly</td>
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<td>2.02 ± 0.10</td>
</tr>
<tr>
<td>297</td>
<td>(D)Tyr-Met-Gly</td>
<td>3</td>
<td>70.1 ± 4.8</td>
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<tr>
<td>269</td>
<td>Thba-(D)Tyr-(D)Met-Gly</td>
<td>4</td>
<td>311 ± 20</td>
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<tr>
<td>270</td>
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<tr>
<td>298</td>
<td>Thba-(D)Phe-Met-Gly</td>
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<td>0.34 ± 0.01</td>
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<tr>
<td>257</td>
<td>Thba-(D)PheNO$_2$-Met-Gly</td>
<td>6</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>189</td>
<td>Inaa-Phe-Met-Gly</td>
<td>3</td>
<td>1.65 ± 0.11</td>
</tr>
<tr>
<td>207</td>
<td>Phaa-Phe-Met-Gly</td>
<td>3</td>
<td>2.23 ± 0.18</td>
</tr>
<tr>
<td>188</td>
<td>tBuaa-Phe-Met-Gly</td>
<td>3</td>
<td>3.71 ± 0.37</td>
</tr>
<tr>
<td>256</td>
<td>Inaa-(D)Trp-Met-Gly</td>
<td>6</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>235</td>
<td>Inaa-Trp-Met-Gly</td>
<td>3</td>
<td>1.17 ± 0.18</td>
</tr>
<tr>
<td>238</td>
<td>Thba-(D)Phe-CysMe-Gly</td>
<td>3</td>
<td>0.76 ± 0.06</td>
</tr>
<tr>
<td>275</td>
<td>Thba-(D)Tyr-Met-(D)Ala</td>
<td>3</td>
<td>2.05 ± 0.29</td>
</tr>
<tr>
<td>276</td>
<td>Inaa-(D)Tyr-Met-(D)Ala</td>
<td>3</td>
<td>2.21 ± 0.32</td>
</tr>
<tr>
<td>296</td>
<td>Thba-Leu-Met-Gly</td>
<td>3</td>
<td>3.26 ± 0.24</td>
</tr>
<tr>
<td>226</td>
<td>Adca-(D)Tyr-His-Gly</td>
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<td>9.2 ± 1.0</td>
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<tr>
<td>214</td>
<td>Inaa-(D)Tyr-His-Gly</td>
<td>3</td>
<td>9.4 ± 0.7</td>
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<tr>
<td>209</td>
<td>Inaa-Tyr-His-Gly</td>
<td>3</td>
<td>10.6 ± 0.8</td>
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<tr>
<td>210</td>
<td>Phaa-(D)Tyr-His-Gly</td>
<td>3</td>
<td>157 ± 8</td>
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<tr>
<td>224</td>
<td>Phaa-Tyr-His-Gly</td>
<td>3</td>
<td>29.6 ± 10.8</td>
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<tr>
<td>208</td>
<td>tBuaa-Tyr-His-Gly</td>
<td>3</td>
<td>50.6 ± 1.4</td>
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<tr>
<td>191</td>
<td>Inaa-Phe-His-Gly</td>
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<td>9.8 ± 0.5</td>
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<td>219</td>
<td>Pyaa-Phe-His-Gly</td>
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<td>30.1 ± 8.9</td>
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<td>192</td>
<td>Phaa-Phe-His-Gly</td>
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<td>31.9 ± 4.0</td>
</tr>
<tr>
<td>190</td>
<td>tBuaa-Phe-His-Gly</td>
<td>3</td>
<td>35.0 ± 7.7</td>
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<td>223</td>
<td>Inaa-NpAla-His-Gly</td>
<td>3</td>
<td>19.6 ± 2.5</td>
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<td>212</td>
<td>Phaa-NpAla-His-Gly</td>
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<td>66.8 ± 11.4</td>
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<td>33.7 ± 3.7</td>
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<td>Inaa-Met-His-Gly</td>
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<td>Phaa-Met-His-Gly</td>
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<td>109 ± 15</td>
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<td>295</td>
<td>Thba-(D)Tyr-Met-NH$_2$</td>
<td>3</td>
<td>549 ± 249</td>
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</table>

$^\dagger$Chelating standards unlikely to be competitive inhibitors so estimated $K_i$ unreliable but provided for comparison.
Acyl-tripeptides with DU145 TIS media PAM $K_i$ values of less than 2 $\mu$M were also tested against PAM found in DU145 S100, PC3 TIS media, and PC3 P100. Disulfiram, DDC, and PBA were included as standard PAM-inhibiting agents and select acyl-tripeptides were included for SAR studies. Michaelis-Menten $K_m$ constants used to calculate $K_i$ from the measured $IC_{50}$ values are provided in Table 2.1 and calculated $K_i$ values are listed in Table 2.3.

Table 2.3: Inhibitor constant $K_i$ values measured with DU145 S100, PC3 TIS, and PC3 S100 PAM for acyl-tripeptides with DU145 TIS PAM $K_i$ values less than 2 $\mu$M, as well as PAM inhibitor standards and selected acyl-tripeptide SAR probe molecules. Results are arranged and grouped exactly as in Table 2.2. The unnatural stereoisomer of a naturally occurring amino acid is indicated by (D). Acyl group and modified amino acid structures are depicted in Tables 1.1 and 1.2.

<table>
<thead>
<tr>
<th>CD3 ID#</th>
<th>Compound</th>
<th>DU145 S100</th>
<th></th>
<th>PC3 TIS</th>
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<th>PC3 S100</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>n $K_i$ (µM)</td>
<td></td>
<td>n $K_i$ (µM)</td>
<td></td>
<td>n $K_i$ (µM)</td>
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<tr>
<td>Standard</td>
<td>Disulfiram$^\dagger$</td>
<td>3  2.68 ± 0.74</td>
<td>3  0.05 ± 0.01</td>
<td>3  0.89 ± 0.07</td>
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<td>Standard</td>
<td>DDC$^\ddagger$</td>
<td>3  2.51 ± 0.49</td>
<td>3  0.10 ± 0.02</td>
<td>3  4.08 ± 0.48</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>PBA$^\ddagger$</td>
<td>3  0.58 ± 0.05</td>
<td>3  0.28 ± 0.03</td>
<td>3  0.71 ± 0.08</td>
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<tr>
<td>246</td>
<td>Thba-(D)Tyr-Met-Gly</td>
<td>5  1.18 ± 0.09</td>
<td>7  0.27 ± 0.02</td>
<td>5  1.26 ± 0.13</td>
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<tr>
<td>255</td>
<td>Adaa-(D)Tyr-Met-Gly</td>
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<td>4  0.31 ± 0.03</td>
<td>3  2.38 ± 0.37</td>
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<td>Inaa-(D)Tyr-Met-Gly</td>
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<td>3  1.30 ± 0.06</td>
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<tr>
<td>247</td>
<td>Thaa(3)-(D)Tyr-Met-Gly</td>
<td>5  2.14 ± 0.13</td>
<td>3  0.36 ± 0.04</td>
<td>3  1.61 ± 0.26</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>245</td>
<td>Thaa(2)-(D)Tyr-Met-Gly</td>
<td>4  2.43 ± 0.24</td>
<td>3  0.40 ± 0.01</td>
<td>3  2.10 ± 0.09</td>
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<tr>
<td>237</td>
<td>Thpa-(D)Tyr-Met-Gly</td>
<td>3  1.86 ± 0.15</td>
<td>3  0.42 ± 0.01</td>
<td>3  1.80 ± 0.32</td>
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<td>3  2.68 ± 0.42</td>
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<td>3  0.61 ± 0.07</td>
<td>3  2.87 ± 0.48</td>
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<tr>
<td>298</td>
<td>Thba-(D)Phe-Met-Gly</td>
<td>5  1.14 ± 0.11</td>
<td>6  0.24 ± 0.01</td>
<td>4  1.21 ± 0.02</td>
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</tr>
<tr>
<td>257</td>
<td>Thba-(D)PheNO$_2$-Met-Gly</td>
<td>3  1.64 ± 0.11</td>
<td>4  0.29 ± 0.04</td>
<td>3  1.50 ± 0.20</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>189</td>
<td>Inaa-Phe-Met-Gly</td>
<td>3  5.31 ± 1.66</td>
<td>3  0.79 ± 0.06</td>
<td>3  3.69 ± 0.14</td>
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<td></td>
</tr>
<tr>
<td>256</td>
<td>Inaa-(D)Trp-Met-Gly</td>
<td>3  1.62 ± 0.05</td>
<td>3  0.30 ± 0.05</td>
<td>3  1.47 ± 0.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>235</td>
<td>Inaa-Trp-Met-Gly</td>
<td>3  3.65 ± 0.52</td>
<td>3  0.72 ± 0.05</td>
<td>3  4.19 ± 0.50</td>
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</tr>
<tr>
<td>238</td>
<td>Thba-(D)Phe-CysMe-Gly</td>
<td>3  2.14 ± 0.10</td>
<td>3  0.47 ± 0.06</td>
<td>3  2.46 ± 0.34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>275</td>
<td>Thba-(D)Tyr-Met-(D)Ala</td>
<td>3  4.44 ± 0.24</td>
<td>3  1.73 ± 0.31</td>
<td>3  6.07 ± 0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>214</td>
<td>Inaa-(D)Tyr-His-Gly</td>
<td>3  15.2 ± 5.7</td>
<td>3  3.63 ± 0.23</td>
<td>3  13.8 ± 2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>211</td>
<td>Inaa-(D)His-His-Gly</td>
<td>3  179 ± 21</td>
<td>3  17.8 ± 1.2</td>
<td>3  202 ± 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>222</td>
<td>Phaa-Met-His-Gly</td>
<td>3  243 ± 69</td>
<td>3  63 ± 13</td>
<td>3  181 ± 37</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^\dagger$Chelating standards unlikely to be competitive inhibitors, so estimated $K_i$ unreliable but provided for comparison.
2.4.2 Non-CaP PAM Inhibition Assay

The next stage of inhibition screening of the acyl-tripeptide test agents was against PAM enzymes derived from tissues other than prostate cancer. Three non-CaP cell lines were selected for collection of PAM enzymes. The muscle cancer cell line TE671 was identified as a viable PAM source based on screening the cell media. The small cell lung cancer (SCLC) cell line H889 was selected as a representative human neuorendocrine cell line known to produce high levels of PAM (Vos et al. 1996). The thyroid medullary carcinoma cell line TT was chosen as a representative human glandular-derived cell line known to produce active forms of several PAM-processed hormones, including calcitonin and gastrin-releasing peptide (Zabel and Grzeszkowiak, 1997).

Extracellular media PAM was collected from each of these cell lines by culturing cells in serum-free TIS media. Culture media was collected after 14 and 18 days of incubation as described in Section 2.2. Membrane particulate S100 cell extract PAM was collected from each cell line using the extract procedures described in Section 2.3. TIS and S100 PAM enzymes collected from each of these samples exhibited sufficient activity for biochemical PAM inhibition screening (≥ 3% conversion of TNP-(D)Tyr-Val-Gly substrate over a 2 hr or less incubation time). In an effort to save time and minimize unnecessary repetition of similar measurements, however, the S100 PAM extracts from the H889 and TT cells were not used in the agent screening assays. The Michaelis-Menten constants, $K_m$, for the TNP-(D)Tyr-Val-Gly substrate with each of the
Non-CaP PAM enzymes used for agent screening were measured as described in Section 2.4.1. The $K_m$ values are summarized in Table 2.4.

Table 2.4: Values of the TNP-(D)Tyr-Val-Gly Michaelis-Menten constant $K_m$ measured for each of the Non-CaP PAM enzymes used for agent screening. Column n indicates the number of duplicate measurements for each substrate concentration.

<table>
<thead>
<tr>
<th>CaP PAM Enzyme</th>
<th>n</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE671 TIS</td>
<td>4</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>TE671 S100</td>
<td>4</td>
<td>26.5 ± 6.8</td>
</tr>
<tr>
<td>H889 TIS</td>
<td>4</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>TT TIS</td>
<td>4</td>
<td>4.07 ± 0.04</td>
</tr>
</tbody>
</table>

Inhibitor screening with these non-CaP PAM enzymes was then performed on all acyl-tripeptides with $K_i < 2$ µM for all four CaP-derived PAM enzymes, along with selected acyl-tripeptide SAR probe molecules and the PAM inhibitor standards Disulfiram and PBA. Results of these screening tests are summarized in Table 2.5. The results for a given test agent with each of the TIS PAM enzymes were very similar. This suggests that the test agents exhibit little or no detectable selectivity for the PAM from any of the representative tissue cell lines tested.
Table 2.5: Inhibitor constant $K_i$ values measured with non-CaP PAM enzymes for acyl-tripeptides with $K_i < 2 \, \mu M$ for all CaP PAM enzymes, as well as two PAM inhibitor standards and selected acyl-tripeptide SAR probe molecules. Acyl-tripeptide results are arranged and grouped exactly as in Table 2.2. The unnatural stereoisomer of a naturally occurring amino acid is indicated by a (D). Acyl group and modified amino acid structures are depicted in Figures 1-1 and 1-2.

<table>
<thead>
<tr>
<th>ID#</th>
<th>Compound</th>
<th>TE671 TIS</th>
<th>TE671 S100</th>
<th>H889 TIS</th>
<th>TT TIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n $K_i$ (µM)</td>
<td>n $K_i$ (µM)</td>
<td>n $K_i$ (µM)</td>
<td>n $K_i$ (µM)</td>
</tr>
<tr>
<td>Stnd</td>
<td>Disulfiram§</td>
<td>3 0.05 ± 0.01</td>
<td>3 1.92 ± 0.27</td>
<td>3 0.05 ± 0.01</td>
<td>3 0.06 ± 0.01</td>
</tr>
<tr>
<td>Stnd</td>
<td>PBA</td>
<td>3 0.31 ± 0.02</td>
<td>3 1.26 ± 0.12</td>
<td>3 0.39 ± 0.04</td>
<td>3 0.51 ± 0.04</td>
</tr>
<tr>
<td>246</td>
<td>Thba-(D)Tyr-Met-Gly</td>
<td>5 0.34 ± 0.02</td>
<td>5 2.54 ± 0.18</td>
<td>5 0.27 ± 0.03</td>
<td>5 0.25 ± 0.02</td>
</tr>
<tr>
<td>255</td>
<td>Adaa-(D)Tyr-Met-Gly</td>
<td>3 0.44 ± 0.02</td>
<td>3 3.92 ± 0.09</td>
<td>3 0.36 ± 0.04</td>
<td>3 0.37 ± 0.01</td>
</tr>
<tr>
<td>236</td>
<td>Inaa-(D)Tyr-Met-Gly</td>
<td>3 0.42 ± 0.05</td>
<td>3 2.88 ± 0.18</td>
<td>3 0.35 ± 0.04</td>
<td>3 0.38 ± 0.01</td>
</tr>
<tr>
<td>298</td>
<td>Thba-(D)Phe-Met-Gly</td>
<td>4 0.32 ± 0.01</td>
<td>4 2.38 ± 0.12</td>
<td>4 0.25 ± 0.01</td>
<td>4 0.24 ± 0.01</td>
</tr>
<tr>
<td>257</td>
<td>Thba-(D)PheNO$_2$-Met-Gly</td>
<td>3 0.43 ± 0.05</td>
<td>3 3.41 ± 0.39</td>
<td>3 0.32 ± 0.03</td>
<td>3 0.33 ± 0.01</td>
</tr>
<tr>
<td>256</td>
<td>Inaa-(D)Trp-Met-Gly</td>
<td>3 0.45 ± 0.05</td>
<td>3 3.99 ± 0.72</td>
<td>3 0.39 ± 0.03</td>
<td>3 0.36 ± 0.01</td>
</tr>
<tr>
<td>238</td>
<td>Thba-(D)Phe-CysMe-Gly</td>
<td>3 0.85 ± 0.08</td>
<td>3 3.88 ± 0.46</td>
<td>3 0.60 ± 0.06</td>
<td>3 0.71 ± 0.05</td>
</tr>
<tr>
<td>275</td>
<td>Thba-(D)Tyr-Met-(D)Ala</td>
<td>3 2.85 ± 0.24</td>
<td>3 12.1 ± 1.2</td>
<td>3 2.20 ± 0.17</td>
<td>3 2.10 ± 0.29</td>
</tr>
<tr>
<td>214</td>
<td>Inaa-(D)Tyr-His-Gly</td>
<td>3 5.31 ± 0.76</td>
<td>3 31.4 ± 1.8</td>
<td>3 4.31 ± 0.54</td>
<td>3 4.11 ± 0.31</td>
</tr>
</tbody>
</table>

§Chelating standard unlikely to be competitive inhibitor, so estimated $K_i$ unreliable but provided for comparison.

A graphical comparison of the acyl-tripeptide activity against the CaP and non-CaP derived PAM enzymes is provided in Figure 2-3 for the agents included in all stages of these tests. Points that fall above the identity line represent agents that exhibited less activity for the specified PAM than for DU145 TIS PAM, while points below the identity line had more activity for the specified PAM than with DU145 TIS PAM. Compounds with the desired inhibitor specificity for CaP PAM versus non-CaP PAM are indicated by having all of the non-CaP solid symbols located well above (less active than) the corresponding open symbol measurements for CaP PAM enzymes. While the TE671 S100 measurements did show somewhat less activity than the corresponding CaP PAM values, there were no agents that showed less activity for any of the other three non-CaP
PAM enzymes versus the CaP PAM enzymes. Thus our laboratory has developed acyl-peptide agents with good inhibitory activity against CaP PAM enzymes, but no agents have been identified with substantial specificity for CaP versus non-CaP PAM. While this was not our original goal, *in vitro* and *in vivo* results reported in Chapters 3 and 4 offer encouraging evidence that the acyl-tripeptide inhibitors can provide effective treatment of androgen-insensitive CaP without the desired CaP-specific inhibition profile.

![Graph showing comparison of Ki values for acyl-tripeptide agents measured with CaP and non-CaP PAM enzymes.](image)

**Figure 2-3.** Comparison of \( K_i \) values for acyl-tripeptide agents measured with CaP and non-CaP PAM enzymes. Note that points above the identity line represent test agents having less activity toward the specified PAM than toward DU145 TIS PAM, while points below the identity line have more activity toward the specified PAM than toward DU145 TIS PAM. Results for DU145 TIS PAM fall on the identity line by definition.
Chapter 3

PAM-Dependent DU145 Growth Inhibition

The DU145 cellular growth inhibition assay was performed to measure the change in DU145 growth specifically due to inhibiting the PAM enzyme. This assay was modified from the NCI screening program (Skehan et al. 1990; Monks et al. 1991) and utilized media supplemented with 10% serum or TIS. Media supplemented with 10% serum contained all necessary growth factors and therefore was not dependent on PAM activity for cellular growth. Alternatively, cellular growth was dependent on PAM activation of endogenous growth factors in the media that contained TIS. Disulfiram and PBA were used as positive controls. Only the test agents with the lowest $K_i$ values from the enzyme inhibition assay, as well as select agents for structure-activity-relationship (SAR) comparisons were included in this study.

The PAM-expressing CaP cell line DU145 was pre-cultured for seven days in RPMI 1640 medium supplemented with 1% SD-FBS and PSA. Twenty-four thousand cells were then loaded in 1 ml RPMI media/1% SD-FBS onto a 24-well plate and incubated at 37 °C, 5% CO$_2$, and 100% humidity overnight. The media was removed and replaced with 1 ml of either RPMI 1640 supplemented with 5% FBS and 5% NS, or RPMI 1640 supplemented with TIS. Both media conditions also contained PSA and
0.25% DMSO. Cells were exposed to 3 concentrations of 10-fold dilutions of test agent. Control wells contained only the 0.25% DMSO.

Plates were incubated with test agent for 48 hours. The plates were fixed with 250 µl of 50% trichloroacetic acid, which caused the cell membrane to rupture and deposited cellular proteins to the bottom of the well. After fixing, plates were incubated at 4 ºC for 60-90 minutes, rinsed five times with ice-cold water, and then air dried. After drying, the proteins were stained with 250 µl 0.4% sulforhodamine B in 1% acetic acid at room temperature, rinsed five times with 1% acetic acid, and air dried again. The stained proteins were resuspended in 750 µl 10 mM Tris-Base (Tris(Hydroxymethyl)Aminomethane), pH 10.5. One plate was also fixed immediately following test agent addition to represent the cell number at the start of test agent exposure and then processed with the other plates. The optical density was taken at 565 nm on a SpectraMax M5 plate reader. The difference between the optical density at the beginning and end of the agent exposure period provided a measure of the cell growth. The ratio for a given agent level versus control wells provided a measure of the fraction of control cell growth. GI_{50} values (the test agent concentration that causes a 50% reduction in cell growth versus the control wells) were estimated for each agent by using the Hill Equation.

Results of the PAM-dependent DU145 growth inhibition assay are shown in Figures 3-1 to 3-3. The anticancer standard agents vinblastine and docetaxel, which are used clinically to treat androgen-independent CaP, are shown in Figure 3-1. These agents showed very little difference between media conditions, indicating that they are not dependent on PAM for their anticancer activity. The chelating PAM inhibitor standards
disulfiram and DDC, which are effective PAM inhibitors but act through a general chelating action rather than a PAM active site-specific chelating mechanism, both exhibited unusual and unexpected responses as illustrated in Figure 3-2. Growth inhibition versus concentration in the PAM-dependent TIS media followed a normal sigmoidal shape for these chelators. However, the fraction of control cell growth for serum media (growth is independent of PAM activity) appeared to unexpectedly level off or even increase with increasing agent concentration at intermediate agent levels before eventually dropping off to low levels of cellular growth at higher levels of agent concentration. One possible explanation for such ambiguous behavior may lie in the promiscuous inhibitory nature of nonspecific metal chelating agents. These unexplainable results were consistent with repeated testing and indicated these general chelating agents do not provide reliable PAM-related CaP growth inhibition. The PAM substrate-like inhibitor standard PBA exhibited no detectable difference between the two growth conditions in Figure 3-2, indicating that it also does not cause PAM-specific growth inhibition.

Figure 3-1: PAM-dependent CaP growth inhibition assay results for anticancer agent standards.
Figure 3-2: PAM-dependent CaP growth inhibition assay results for PAM inhibitor standards.

Alternatively, the results in Figure 3-3 for our acyl-tripeptide compounds tested in this assay showed that these substrate-like PAM-localized chelating agents exhibit no detectable activity (NDA) in the serum media where growth factors were provided without the need for PAM activation, but showed considerable growth inhibition at higher concentrations in the serum-free TIS media where PAM activity was needed to produce growth factors. This significant result is consistent across every acyl-tripeptide agent tested in this assay. Collectively, these assay results verified that acyl-tripeptides could be utilized as effective inhibitors of PAM-dependent CaP cell growth, while the PAM inhibitor standards PBA and DDC employed previously in NCI lung cancer
research (Iwai et al, 1999) did not provide reliable PAM-related CaP cell growth inhibition.
Figure 3-3: PAM-dependent DU145 growth inhibition assay results for acyl-tripeptide test agents. The consistent inhibition of only the TIS media condition is significant in that it clearly demonstrates these agents were selective inhibitors of PAM-dependent DU145 cellular growth.
A summary of the GI$_{50}$ values measured in this assay is provided in Table 3.1 for both media conditions for all compounds tested. The GI$_{50}$ value was defined as the agent concentration that reduces DU145 cell growth over the two day agent exposure period by 50% versus untreated control cells (DMSO vehicle only) grown in the same media. Anticancer agents vinblastine and docetaxel showed similar GI$_{50}$ values in both types of media, as expected for agents that inhibit cell growth through mechanisms unrelated to PAM activity. The chelators disulfiram and DDC actually exhibited a lower GI$_{50}$ (higher growth inhibition activity) in the serum media than in the TIS media, which is the opposite of that expected for PAM-related growth inhibition. This reversal was due to the odd shape of the growth inhibition curve for these chelating agents in serum media. The disulfiram growth inhibition curve for serum media was so unusual that three GI$_{50}$ values could actually be defined, one for the lowest concentration where the curve first dropped below 50% of control growth (value listed in Table 3-1), a second for the intermediate concentration where the curve rose back above 50% of control growth, and a third for the higher concentration where the curve dropped back down below 50% of control growth for the final time. Like the anticancer standards that had no PAM inhibitory mechanism, the GI$_{50}$ values for the PAM inhibitor standard PBA showed no significant difference between the two media conditions. Alternatively, as shown previously in Figure 3-3, the acyl-tripeptide agents exhibited no measurable effect on cell growth in the serum media where PAM inhibition was expected to have no effect on growth, and hence no GI$_{50}$ value could be defined for these agents in this growth condition. The acyl-tripeptides all showed some level of growth inhibition in the serum-
free TIS media, but only two of the agents (CD3-246 and CD3-257) reach 50% of control growth at the highest concentration tested.

Table 3.1: PAM-dependent DU145 cell growth inhibition results for all compounds tested. GI$_{50}$ values represent the agent concentration that reduced cell growth during a 48-hour exposure period by 50% versus untreated (DMSO vehicle only) control cells in the same media. Cells cultured in serum media received the full complement of growth factors delivered in the serum supplement and hence did not require PAM activation of growth factors for cell multiplication. Cells cultured in serum-free TIS media did not receive any serum-supplied growth factors and must rely on the production and PAM-activation of exogenous growth factors. Acyl group and modified amino acid structures are depicted in Tables 1.1 and 1.2.

<table>
<thead>
<tr>
<th>CD3 ID#</th>
<th>Compound</th>
<th>Serum Media</th>
<th>TIS Media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>$GI_{50}$ (µM)</td>
</tr>
<tr>
<td>Anticancer Stnd</td>
<td>Vinblastine</td>
<td>3</td>
<td>0.0023 ± 0.0002</td>
</tr>
<tr>
<td>Anticancer Stnd</td>
<td>Docetaxel</td>
<td>3</td>
<td>0.0040 ± 0.0003</td>
</tr>
<tr>
<td>PAM Inh Stnd</td>
<td>Disulfiram</td>
<td>5</td>
<td>0.58 ± 0.02</td>
</tr>
<tr>
<td>PAM Inh Stnd</td>
<td>DDC</td>
<td>5</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>PAM Inh Stnd</td>
<td>PBA</td>
<td>5</td>
<td>1911 ± 230</td>
</tr>
<tr>
<td>246</td>
<td>Thba-(D)Tyr-Met-Gly</td>
<td>5</td>
<td>NDA</td>
</tr>
<tr>
<td>255</td>
<td>Adaa-(D)Tyr-Met-Gly</td>
<td>5</td>
<td>NDA</td>
</tr>
<tr>
<td>236</td>
<td>Inaa-(D)Tyr-Met-Gly</td>
<td>4</td>
<td>NDA</td>
</tr>
<tr>
<td>298</td>
<td>Thba-(D)Phe-Met-Gly</td>
<td>5</td>
<td>NDA</td>
</tr>
<tr>
<td>257</td>
<td>Thba-(D)PheNO$_2$-Met-Gly</td>
<td>3</td>
<td>NDA</td>
</tr>
<tr>
<td>256</td>
<td>Inaa-(D)Trp-Met-Gly</td>
<td>3</td>
<td>NDA</td>
</tr>
<tr>
<td>275</td>
<td>Thba-(D)Tyr-Met-(D)Ala</td>
<td>4</td>
<td>NDA</td>
</tr>
</tbody>
</table>

NDA – No detectable activity, i.e., no measurable effect of test agent on cell growth at concentrations up to 1 mM.
The lead PAM-inhibitor agent employed for in vivo and auxiliary testing was selected through a review of all in vitro screening results. The acyl-tripeptide agents that consistently showed the lowest $K_i$ values (highest PAM inhibition activity) throughout all stages of biochemical screening with CaP and non-CaP PAM enzymes were Thba-(D)Tyr-Met-Gly (CD3-246) and Thba-(D)Phe-Met-Gly (CD3-298). These acyl-tripeptides were the top two agents for every source of PAM enzyme tested; CD3-246 was the most active for two sources of PAM and CD3-298 was the most active for the other six types of PAM. However, neither compound was significantly better than the other for any type of PAM tested. Other acyl-tripeptides that exhibited consistently high activity in the biochemical screening assays included compounds CD3-236, -255, and -257. For the CaP cell growth inhibition assays, CD3-246 and CD3-257 clearly exhibited the best PAM-dependent CaP cellular growth inhibition. Considering all of these findings together, CD3-246 was used for further stages of testing. The structure of this compound is illustrated in Figure 1-1.
Chapter 4

In Vivo Testing of CD3-246

After the PAM enzyme inhibition assays and DU145 cellular growth inhibition assays were complete, CD3-246 emerged as the lead test agent and was moved into in vivo and auxiliary testing to test its efficacy in a mammalian model. In vivo testing was broken into 3 stages: Pharmacokinetic and toxicity (PK/tox) analysis in BALB/c mice, PK/tox analysis in immune-compromised BALB/c (nu/nu) mice, and efficacy against implanted DU145 tumor xenographs. In order to perform PK analysis, an HPLC (High performance Liquid Chromatography) method was developed to quantitate Thba-(D)Tyr-Met-Gly (CD3-246) and its potential PAM product Thba-(D)Tyr-Met-NH₂ (CD3-295), as described in Section 4.1.

4.1 HPLC Detection of CD3-246 and CD3-295

An HPLC method was validated to quantify CD3-246 and also its potential PAM product, Thba-(D)Tyr-Met- NH₂ (CD3-295). The Waters Alliance® liquid chromatograph described in Section 2.4.1 was used with the detector wavelength set at 220 nm. A Synergi® Polar-RP™ analytical column (250 x 4.6 mm, 4µ) was used from Phenomenex® (Torrance, CA) preceded by a pre-column filter from MAC-MOD.
Analytical, Inc (Chadds Ford, PA). All chromatographic runs were carried out in an isocratic mode with a flow rate of 1 ml/min for 10 min. The mobile phase consisted of 60% 10 mM Ammonium Acetate (pH 4.0) and 40% acetonitrile. The injection volume was 100 µl, and the column and samples were kept at 35±5 °C and 4±5 °C, respectively. Under this condition, the retention time for CD3-246 was 4.1 min and 6.5 min for CD3-295. Standard solutions ranging from 1 μM – 100 μM for CD3-246 and CD3-295 were used to generate a linear calibration curve for peak areas versus concentrations of each compound. A representative HPLC chromatograph showing the separation of CD3-246 and CD3-295 is shown in Figure 4-1.

![Representative HPLC chromatogram for a sample containing CD3-246 and CD3-295.](image)

Figure 4-1: Representative HPLC chromatogram for a sample containing CD3-246 and CD3-295.

For analysis of *in vivo* plasma samples, whole blood was collected from mice by cardiac puncture in Microtainer Brand EDTA (ethylenediaminetetraacetic acid) tubes. Blood samples were centrifuged at 1600 × g for 15 min and up to 250 µL of plasma was collected and stored at -80 °C until assayed. After thawing, samples were prepared for analysis by adding 1000 µL of 4 ºC acetonitrile per 250 µL plasma, vortexing, incubating at room temperature for 2 min, then centrifuging at 16,000 × g for 2 min. Up to 1125 µL of supernatant was collected, vacuum centrifuged at 30 ºC until a two-phase pellet formed, and 250 µL of 4 ºC toluene was added per 1125 µL supernatant. Each sample
was vortexed, briefly centrifuged, and then vacuum centrifuged until dry. The dry residue was resuspended in 150 µL HPLC mobile phase, vortexed, incubated at room temperature for 2 min, then centrifuged at 16,000 × g for 2 min. Supernatant was transferred to an autosampler vial and analyzed by HPLC. Blank plasma standards spiked with 0, 1.0, 10.0, 100 µM CD3-246 were processed simultaneously and used to generate a calibration curve for the plasma samples during each HPLC run. The potential PAM-converted metabolite CD3-295 was not detected in plasma from animals treated with CD3-246, and therefore calibration curves for the metabolite were not generated.

4.2 CD3-246 Auxiliary Testing

Several auxiliary tests were performed with the lead agent CD3-246 in order to better understand its properties and prepare for in vivo testing. Biochemical-level kinetic experiments were carried out to determine whether the lead agent acts as a competitive, noncompetitive, or uncompetitive inhibitor of PAM enzymes. The lead agent was also incubated with PAM enzyme, carboxypeptidase enzyme, and serum to determine whether the compound was converted to inactive forms in the body.

4.2.1 Determining the Inhibition Kinetics

The standard technique for determining the nature of enzyme inhibition requires measuring the enzymatic reaction rate while simultaneously varying the concentrations of both test substrate and inhibitor. Once the measurements have been made with varying substrate and inhibitor levels, the type of inhibition can be determined using a Dixon plot and/or a Lineweaver-Burk plot analysis (Segel, 1993). For a Dixon plot analysis, the reciprocal of the reaction velocity (1/v) is plotted versus the inhibitor concentration [I] for each substrate concentration [S] tested, and the data for each substrate concentration
should fall on a straight line. For a competitive inhibitor, all of the substrate lines should intersect at a single point where \([I] = -K_i\) and \(1/v = 1/v_{\text{max}}\), while for a noncompetitive inhibitor they should intersect on the x-axis where \([I] = -K_i\). For an uncompetitive inhibitor the lines should be parallel and never intersect. Alternatively, for the Lineweaver-Burk plot analysis, the reciprocal of the reaction velocity \((1/v)\) is plotted versus the reciprocal of the substrate concentration \(1/[S]\) for each inhibitor concentration \([I]\) tested, and the data for each inhibitor concentration should fall on a straight line. For a competitive inhibitor, all of the inhibitor lines should intersect at a single point on the y-axis where \(1/v = 1/v_{\text{max}}\), while for a noncompetitive inhibitor they should intersect on the x-axis where \(1/[S] = -1/K_m\). For an uncompetitive inhibitor the lines should again be parallel and never intersect.

For the inhibitor mechanism tests with CD3-246 and DU145 TIS PAM, substrate conversion rate measurements were made with 6.25, 12.5, 25, and 50 µM TNP-(D)Tyr-Val-Gly substrate levels and inhibitor levels of 0.00, 0.20, 0.63, 1.99, 3.98, and 6.30 µM CD3-246. A total of four measurements were averaged for each substrate/inhibitor condition. The results were analyzed by a classical computer algorithm developed by Cleland (1979), which performed a simultaneous nonlinear regression fit to an entire set of rate measurements (4 substrate levels \(\times\) 6 inhibitor levels = 24 conditions for this case) with competitive, noncompetitive, and uncompetitive models, and determined which provides the best model for the data. The competitive inhibition model was found to provide the best fit to the CD3-246 data, with the fitted lines illustrated along with the measured data points via a Dixon plot in Panel A of Figure 4-2 and for a Lineweaver-Burk plot in Panel B of Figure 4-2. Two rate measurements that were below the TNP-
(D)Tyr-Val-Gly HPLC assay limit of quantitation (LOQ = 0.1 µM substrate conversion) are indicated on the Dixon and Lineweaver-Burk plots and were not used in the data analysis. These plots showed the optimized competitive model parameters provide an excellent fit to all measured rate values except the two rate measurements below the assay LOQ. Comparison of the parameter values derived through this fitting procedure (K_m = 6.3 ± 0.4 µM, K_i = 0.35 ± 0.02 µM) versus the independently measured parameter values reported in Table 2-1 (K_m = 6.8 ± 0.5) and Table 2-2 (K_i = 0.31 ± 0.01 µM) showed that the two independent methods exhibited excellent agreement. Based on these results, it became apparent that the lead agent served as a competitive inhibitor of DU145 TIS PAM, as expected for a substrate-like inhibitor that binds to the active site (Segel, 1993) while also not possessing additional reactive functional groups. Since all other acyl-tripeptide agents in this program are substrate-like inhibitors that bind at the active site, it was reasonable to assume that all of these agents were competitive inhibitors of PAM enzymes as well.
Figure 4-2: Measured TNP-(D)Tyr-Val-Gly substrate conversion rates and nonlinear regression fitted competitive inhibitor model results for CD3-246 with DU145 TIS PAM illustrated as (A) a Dixon plot and (B) a Lineweaver-Burk plot. See text for details.
4.2.2 Incubation with Carboxypeptidase A Enzyme

Carboxypeptidase enzymes cleave amino acids off of the carboxy-terminus of peptides. CD3-246 was incubated with Carboxypeptidase A to determine if carboxypeptidase enzymes in the body would be a source of compound elimination.

Carboxypeptidase A was purchased from Sigma-Aldrich. Before testing, the purchased enzyme was dissolved in 0.5 ml of 10% NaCl and centrifuged. The resulting supernatant was used as an enzyme stock solution. The final concentration of the enzyme stock solution was determined by absorbance at 278 nm. Incubations of 100 µM CD3-246 were performed with 10 nM, 1 µM, and 20 µM Carboxypeptidase A in pH 7.4 Tris buffer at 37 ºC for up to 4 hours. Each condition was tested in triplicate. Simultaneous incubations under the same conditions without Carboxypeptidase A exhibited no signs of agent breakdown (results not shown). Results in Figure 4-3 showed that 10 nM Carboxypeptidase A caused a small decrease in CD3-246 peak area over 4 hr without any detectable breakdown products. Figure 4-4 shows that 1 µM Carboxypeptidase A caused a substantial decrease in the CD3-246 peak area over 4 hr and the appearance of the primary peptidase breakdown product Thba-(D)Tyr-Met (terminal Gly removed by Carboxypeptidase A, metabolite standard prepared synthetically for peak identification) is evident. Figure 4-5 clearly demonstrates that 20 µM Carboxypeptidase A converted CD3-246 to the primary peptidase product Thba-(D)Tyr-Met nearly to completion in 30 min, and subsequent conversion to the secondary peptidase product Thba-(D)Tyr (Gly and Met sequentially removed by Carboxypeptidase A, metabolite standard also prepared synthetically for peak identification) was essentially complete after 1 hr. These results
indicated that Carboxypeptidase enzymes may cause substantial in vivo breakdown of the lead agent in tissues with high levels of the enzyme.

Figure 4-3: Chromatograms illustrating the incubation of 100 µM CD3-246 with 10 nM Carboxypeptidase A in pH 7.4 Tris buffer at 37 ºC for up to 4 hours. The peak area of CD3-246 decreased slightly with time but no break-down products were detected.

Figure 4-4: Chromatograms illustrating the incubation of 100 µM CD3-246 with 1 µM Carboxypeptidase A in pH 7.4 Tris buffer at 37 ºC for up to 4 hours. The peak area of CD3-246 decreased with time and the appearance of the primary peptidase break-down product Thba-(D)Tyr-Met (terminal Gly removed by Carboxypeptidase A) was evident.
Figure 4-5: Chromatograms illustrating the incubation of 100 µM CD3-246 with 20 µM Carboxypeptidase A in pH 7.4 Tris buffer at 37 ºC for up to 2 hours. Conversion of CD3-246 to the 1° peptidase product Thba-(D)Tyr-Met (terminal Gly removed by Carboxypeptidase A) was nearly complete after 30 min. Subsequent conversion to the 2° peptidase product Thba-(D)Tyr (Gly and Met removed sequentially by Carboxypeptidase A) was essentially complete after 1 hr.

4.2.3 Stability in Mouse Serum

Mouse serum samples (Pel-Freeze Biologicals, Rogers, AR) containing 60 µM or 600 µM CD3-246 were incubated at 37 ºC for up to 4 h, with each concentration tested in triplicate incubations at each time point. Results for both concentrations showed no trace of CD3-246 breakdown, with chromatograms for the 600 µM tests illustrated in Figure 4-6.
Figure 4-6: Chromatograms illustrating the stability of 600 µM CD3-246 in mouse serum incubated at 37 ºC for up to 4 hours. The chromatograms showed no sign of CD3-246 disappearance or break-down product formation.

4.2.4 Incubation with CaP Enzymes

To determine whether PAM enzymes convert the lead agent CD3-246 to an amidated metabolite, solutions containing 400 µM CD3-246 were prepared in fresh PAM-free TIS media and in DU145 TIS media containing high levels of PAM enzyme and then incubated at 37 ºC for up to 2 h. The results in Figure 4-7 showed that there was no difference in the CD3-246 peak area or evidence of any breakdown products (PAM-converted metabolite CD3-295 or any other form) in the chromatograms for fresh PAM-free TIS media solution that was not incubated, PAM-free TIS media solution incubated for 2 hr, or a DU145 TIS PAM media solution incubated for 2 hr. Hence the lead agent did not undergo detectable rates of conversion with the PAM enzymes over at least a 2 hr period of time, and significant conversion by PAM enzymes to inactive metabolites in the body appeared unlikely.
Figure 4-7: Chromatograms illustrating the stability of the lead agent CD3-246 after (A) 400 µM CD3-246 in fresh PAM-free TIS media without incubation; (B) 400 µM CD3-246 incubated for 2 hours at 37 ºC in fresh PAM-free TIS media; and (C) 400 µM CD3-246 incubated at 37 ºC for 2 hours in DU145 TIS media containing high levels of PAM. The chromatograms showed no sign of lead agent disappearance, PAM-converted amidated-product CD3-295, or any other breakdown products.

4.3 Design of in Vivo Dosing Schedule for CD3-246

A preliminary in vivo dosing schedule was selected using a molecular similarity analysis. The idea of a molecular similarity analysis is to identify previously studied compounds with a similar structure to the test agent of interest, and then use the reported properties of the similar compounds to predict the test agent properties (Klebe et al. 1994). The molecular similarity analysis was performed using the Scifinder Scholar database operated by the American Chemical Society (Washington, D.C.) as well as the Cambridgesoft (Cambridge, MA) Merck Index database. Each database was initially searched by entering the structure of CD3-246 into the built-in Tanimoto structure similarity ranking algorithm (Willett et al. 1998), after which the search results were refined to include only compounds with reported PK/tox results.

A total of ten compounds were identified with > 60% similarity and useful reported properties, summarized in Table 4.1. The reported values for these similar compounds were then utilized to generate the range of expected mouse parameter values.
for CD3-246, along with the worst case (values yielding the lowest plasma concentrations), best case (values yielding the highest plasma concentrations), median, and mean parameter values for CD3-246, summarized in Table 4.2. Simulated CD3-246 plasma concentrations generated for a 100 mg/kg, oral (po) or intraperitoneal (ip) dose of CD3-246 using a one compartment first-order absorption model with the worst case, median, mean, and best case parameter values have been illustrated in Figure 4-8. The maximum plasma concentration (\(C_{p,max}\)), and the time period after dosing when the plasma concentration would be above the CD3-246 HPLC assay limit of quantitation (LOQ = 1 µM) was predicted in the same manner.
Table 4.1: Toxicological and pharmacokinetic properties of compounds identified by the Tanimoto ranking (Willett et al. 1998) similarity search for CD3-246 (see Figure 1.3 for structure) described in the text. For parameters that varied by route of administration (LD$_{50}$, F, $t_{\text{max}}$, $f_u$), only data collected for oral (po) or intraperitoneal (ip) dosing was listed and used in the analysis. PK rate-related parameters $t_{1/2}$ and $t_{\text{max}}$ have been reported here as mouse values, with interspecies scaling based on the methods of Ward et al. (2005) employed to convert from human (h) or rat (r) values to mouse (m) values. All other parameters have been assumed equivalent between the different species.

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>Drug Name</th>
<th>Tanimoto Similarity (%)</th>
<th>LD$_{50}$ (mg/kg)</th>
<th>F (%)</th>
<th>$t_{\text{max}}$ (hr)</th>
<th>$t_{1/2}$ (hr)</th>
<th>V (ml/g)</th>
<th>$f_u$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Alacepril" /></td>
<td>Alacepril</td>
<td>69</td>
<td>3000 m ip</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td><img src="image" alt="Batimastat" /></td>
<td>Batimastat</td>
<td>84</td>
<td></td>
<td></td>
<td>$&gt;48$ h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Enalapril" /></td>
<td>Enalapril</td>
<td>64</td>
<td>60 h,r po</td>
<td>0.9</td>
<td>2 h</td>
<td>1.7 h</td>
<td>18 h po</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Enalaprilat" /></td>
<td>Enalaprilat</td>
<td>63</td>
<td>40 h po</td>
<td>4</td>
<td>30 h</td>
<td></td>
<td>43 h po</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Lisinopril" /></td>
<td>Lisinopril</td>
<td>63</td>
<td>25 h po</td>
<td>7</td>
<td>12 h</td>
<td>2.4 h</td>
<td>25 h po</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Spirapril" /></td>
<td>Spirapril</td>
<td>73</td>
<td>$&gt;450$ r po</td>
<td>1</td>
<td>2 h</td>
<td>0.4 h</td>
<td>$&gt;0$ h po</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Stepronin" /></td>
<td>Stepronin</td>
<td>63</td>
<td>$&gt;2500$ m po</td>
<td>0.4 h</td>
<td></td>
<td></td>
<td>1 h po</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Sufentanil" /></td>
<td>Sufentanil</td>
<td>77</td>
<td></td>
<td>2 h</td>
<td>0.9 h iv</td>
<td>2 h iv</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2: The range of toxicological (Tox) and pharmacokinetic (PK) parameters reported for molecules identified during the structure similarity search for CD3-246 described in the text. All parameter values have been scaled to the mouse and defined as described in Table 4.1. The worst case (values yielding the lowest plasma concentrations) and best case (values yielding the highest plasma concentrations) parameter values for the similarity compounds, as well as the mean and median of the similarity compound parameters, have been summarized as well. The maximum plasma concentration ($C_{p,max}$), time period when the plasma concentration is above the CD3-246 HPLC assay limit of quantitation (LOQ = 1 µM), and 24 hr urine sample concentration ($C_{u,24hr}$) have been predicted for a 100 mg/kg po/ip dose of CD3-246 using a one compartment first-order absorption model with worst case, median, mean, and best case parameter values.

<table>
<thead>
<tr>
<th>Tox/PK Parameters</th>
<th>$LD_{50}$ (mg/kg)</th>
<th>F (%)</th>
<th>$t_{max}$ (hr)</th>
<th>$t_{1/2}$ (hr)</th>
<th>V (ml/g)</th>
<th>$f_u$ (%)</th>
<th>Time Period above LOQ</th>
<th>$C_{p,max}$ (µM)</th>
<th>$C_{u,24hr}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>450-3000 po/ip</td>
<td>25-90</td>
<td>0.1-1.6 po</td>
<td>0.5-11</td>
<td>0.4-2.4</td>
<td>0-43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Worst Case</td>
<td>450</td>
<td>25</td>
<td>1.6</td>
<td>0.5</td>
<td>2.4</td>
<td>0</td>
<td>1hr-4hr</td>
<td>1.7</td>
<td>0</td>
</tr>
<tr>
<td>Median</td>
<td>1500</td>
<td>50</td>
<td>0.35</td>
<td>2.1</td>
<td>1.1</td>
<td>2</td>
<td>5min-12hr</td>
<td>81</td>
<td>64</td>
</tr>
<tr>
<td>Mean</td>
<td>1613</td>
<td>53</td>
<td>0.63</td>
<td>3.3</td>
<td>1.2</td>
<td>11</td>
<td>5min-18hr</td>
<td>74</td>
<td>64</td>
</tr>
<tr>
<td>Best Case</td>
<td>3000</td>
<td>90</td>
<td>0.1</td>
<td>&gt;11</td>
<td>0.4</td>
<td>43</td>
<td>5min-48hr</td>
<td>425</td>
<td>1060</td>
</tr>
</tbody>
</table>
Figure 4-8: Simulated plasma concentration values for a 100 mg/kg po/ip dose of CD3-246 using the worst case, median, mean, and best case PK parameters listed in Table 4.2 for compounds identified as similar in structure to CD3-246 (see text). The limit of quantitation (LOQ = 1 µM) for the CD3-246 HPLC assay is also indicated on the graph.

The tox/PK parameters and predicted plasma concentrations from this similarity analysis were then used to aid the selection of the dose, route of administration, and dosing schedule for the CD3-246 in vivo tests in mice. In an effort to avoid serious toxicological side effects, we used a dose that was at least ten-times lower than the expected LD$_{50}$ (lethal dose 50, the dose of agent that causes a 50% lethality rate). The similarity analysis suggested a dose in the range 45-300 mg/kg, while the mean/median LD$_{50}$ suggested a dose ≤ 150 mg/kg. We opted to employ a dose of 100 mg/kg to be on
the safe side of the mean/median values. This dose also caused the concentration of CD3-246 in the injection solution to approach the solubility limit for the suggested mouse injection volume of 10 ml/kg employed in these tests, so that a higher dose could not readily be used.

CD3-246 showed poor water solubility in preliminary studies (data not shown). This led to concerns about precipitation in an intravenous (iv) injection, which could cause circulatory problems. Intraperitoneal (ip) drug delivery was chosen because any precipitated agent would remain in the peritoneal cavity where it would slowly dissolve and be absorbed, whereas undissolved agent resulting from oral (po) delivery would be eliminated in feces before it had time to redissolve.

4.4 Injection Solution Preparation

A stock solution of 25 mg/kg CD3-246 in DMSO was prepared and aliquoted into separate vials for each day of animal injections, with the aliquots stored at -80°C. Each injection day, one 25 mg/kg CD3-246 DMSO solution aliquot was thawed and mixed with 2 parts DMSO aliquot and 3 parts pH 7.4 phosphate buffered saline (PBS) to produce a fresh 10 mg/ml CD3-246 solution in 60% PBS/40% DMSO. A fresh solution of 60% PBS/40% DMSO without CD3-246 was prepared each day for control animal injections, and this control solution was also used to dilute the 10 mg/ml CD3-246 solution for lower CD3-246 doses in the efficacy stage of tests described in Section 4.5. Remnants of the fresh 1 mg/ml CD3-246 solutions used for 10 mg/kg injections in the final stage of in vivo tests were diluted 1:19 and evaluated by triplicate HPLC injections versus fresh independently prepared 50 µM CD3-246 analytical standards at the beginning, middle, and end of the final in vivo tests. Measured deviations between the
injection solutions and the analytical standards were +0.8%, -2.7%, and -1.0% at the beginning, middle, and end, respectively. These results indicated that the CD3-246 injection solutions had an acceptably low level of variation (<±3%) on different injection days.

**4.5 PK/Tox Analysis of CD3-246 in BALB/c and BALB/c (nu/nu) Mice**

Only female mice were used in the following studies to eliminate androgens from the animal models. Mice were routinely monitored for changes in body weight, abnormal behavior, and changes in grooming. Animals were allowed food and water ad libitum. All animals were housed in the University of Toledo main campus Department of Laboratory Animal Resources. Experiments were performed under the guidance of the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animals, 1996) with approval from the University of Toledo Institutional Animal Care and Use Committee.

PK studies were first performed in twenty BALB/c mice over sixteen days, three injections per week (Monday, Wednesday, Friday), up to eight injections per animal. Fourteen animals received 100 mg/kg CD3-246, the highest dose to be used in the efficacy study, and six animals received vehicle control (40% DMSO/60% PBS). After the first injection, two animals that received test agent and one control animal were sacrificed at 30 and 60 min post-injection.

The remaining animals were housed two animals per cage in Nalgene metabolic cages with feeding apparatuses and urine collection tubes fit for mice to allow for urine collection. Urine was collected for 36 hours at 12-hr intervals in a Techniplast
refrigerated collection rack (3M12B9 series). Urine was also collected for 36 hours following the fourth and seventh injections. Animals were housed in normal cages when urine was not being collected.

Following the eighth (final) injection, the remaining ten animals that received test agent were sacrificed at 15, 30, 45, 60, and 90 min, two animals per time point. The four remaining control animals were sacrificed 30 and 60 min post-injection, two animals per time point. Prior to sacrifice, animals were sedated with 100 mg/kg Ketaset (Fort Dodge Animal Health, Overland, KS) and 10 mg/kg Xyla-Ject (Phoenix Pharmaceuticals, Belmont, CA) and whole blood was collected via cardiac puncture in EDTA-treated vials. Plasma was collected from the blood samples and stored at -80 °C until analyzed as described in Section 4.1. Animals were euthanized by exsanguination followed by cervical dislocation.

No differences were seen in body weights between control animals and those given 100 mg/kg CD3-246, nor were differences in health or behavior observed in any of the animals. The percent of initial body weight for each group is plotted versus the treatment period in Figure 4-9. Repeated measures ANOVA analysis indicated no significant differences (p < 0.05) between the percent of initial body weight of the groups on any of the test dates. Weight drops for both groups after day seven and day fourteen correlate with housing periods in the metabolic cages. The animals may have been stressed by this change or had more difficulty getting to the food in the metabolic cages.
Figure 4-9: Percent of initial body weight for control and CD3-246 treated animals in the first stage of CD3-246 in vivo testing in normal BALB/c mice. Repeated measures ANOVA analysis indicated no significant differences (p < 0.05) between the two groups on any of the test dates.

Plasma concentrations of CD3-246 were readily detectable for all time points after the final treatment injection, as illustrated in Figure 4-10. The measured plasma concentrations were fit to a one-compartment first-order absorption PK model by nonlinear regression analysis weighted by 1/sd. Using only two measurements for each time point did not provide a reliable measure of the PK parameters (as indicated by the outlier point at 45 min which was given a very low weighting in the fitting analysis due to its large standard deviation), but the crude estimates from this preliminary study offer some insight into the pharmacokinetics of CD3-246 in mice. The PK parameters from this crude analysis have been summarized in Table 4.3 along with the parameters predicted by the CD3-246 molecular similarity analysis described in Section 4.3. The maximum measured CD3-246 concentration (at 45 min) is also compared to the similarity analysis prediction in Table 4.3. Note that the volume of distribution (V) and
the bioavailability (F) could not be independently measured without employing an intravenous injection of the agent, but the combined parameter V/F could be estimated from the fitted model parameters. The fitted value of $t_{\text{max}}$ showed excellent agreement with the median value from the similarity analysis, while the fitted V/F matched the median and mean similarity predictions. The measured maximum plasma concentration was closest to the best case similarity value, but this 60 min measurement appears to be an outlier that is much higher than the value given by the PK model fit of the measured data. The fitted value for the drug half-life, $t_{1/2}$, of 0.30 hr (18 min) provided the only major deviation from the similarity analysis, with a value less (worse) than the worst case prediction. This indicated that the PK duration of the agent in the treated mice is actually much shorter than predicted, and shed doubt on the potential for three injections of 100 mg/kg CD3-246 per week to impact tumor growth.
Figure 4-10: CD3-246 plasma concentrations measured in treated animals after the eighth injection of the first in vivo study in normal BALB/c mice. The curve represents a weighted nonlinear regression fit to a one-compartment PK model with first-order absorption.

Table 4.3: Comparison of the estimated pharmacokinetic parameters and maximum measured plasma concentration for the first stage of CD3-246 in vivo study in normal mice, versus the corresponding values predicted by the CD3-246 molecular similarity analysis (see Table 4.2).

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>F (%)</th>
<th>t\textsubscript{max} (hr)</th>
<th>t\textsubscript{1/2} (hr)</th>
<th>V (ml/g)</th>
<th>V/F (ml/g)</th>
<th>C\textsubscript{p,max} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First In vivo Study in Normal Mice</td>
<td></td>
<td>0.33</td>
<td>0.30</td>
<td>2.2</td>
<td>326 ± 83</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Molecular Similarity Analysis</th>
<th>Worst Case</th>
<th>Median</th>
<th>Mean</th>
<th>Best Case</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F (%)</td>
<td>t\textsubscript{max} (hr)</td>
<td>t\textsubscript{1/2} (hr)</td>
<td>V (ml/g)</td>
</tr>
<tr>
<td>Worst Case</td>
<td>25</td>
<td>1.6</td>
<td>0.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Median</td>
<td>50</td>
<td>0.35</td>
<td>2.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Mean</td>
<td>53</td>
<td>0.63</td>
<td>3.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Best Case</td>
<td>90</td>
<td>0.1</td>
<td>&gt;11</td>
<td>0.4</td>
</tr>
</tbody>
</table>

PK studies were then performed in immune-compromised BALB/c (nu/nu) mice exactly as described above except these animals were housed five animals per cage and
no urine was collected. Because these animals are immune-compromised, they were fed autoclaved food, given irradiated water, and housed in sterile-filtered HEPA cages. Again, no differences were seen in body weights between control animals and those given 100 mg/ml CD3-246, nor were there detectable differences in health or behavior in any of the animals. The percent of initial body weight for each group is plotted versus the treatment period in Figure 4-11 with no significant differences (p < 0.05) between the percent of initial body weight of the groups on any of the test dates indicated by ANOVA analysis. The measured concentrations of CD3-246 in plasma after the final (eighth) injection from the second stage of PK testing is illustrated in Figure 4-12. As in the first set of testing, the measured plasma concentrations were fit to a one-compartment first-order absorption PK model by nonlinear regression analysis weighted by 1/sd. Using only two measurements for each time point did not provide a reliable measure of the pharmacokinetic parameters (as indicated once again by an outlier point at 45 min which was given a low weighting in the fitting analysis due to its large standard deviation), but the crude estimates from this study could offer some insight into the pharmacokinetics of CD3-246 in immune-compromised nude mice.
Figure 4-11: Percent of initial body weight for control and CD3-246 treated animals in the second stage of CD3-246 in vivo testing in immune-compromised BALB/c (nu/nu) mice. Repeated measures ANOVA analysis indicated no significant differences (p < 0.05) between the two groups on any of the test dates.
Figure 4-12: CD3-246 plasma concentrations measured in treated animals after the eighth injection during the second *in vivo* study in immune-compromised BALB/c (nu/nu) mice. The curve represents a weighted nonlinear regression fit to a one-compartment PK model with first-order absorption.

The PK parameters from this crude analysis have been summarized in Table 4.4, along with the parameters measured previously for normal mice and predicted by the CD3-246 molecular similarity analysis described previously in this section. As for the first study, the values of $t_{\text{max}}$ and $V/F$ for the second study showed reasonable agreement with the median and/or mean similarity predicted values. The measured maximum plasma concentration for the second study was substantially lower than that in the first study and was much closer to the mean/median similarity value. The fitted value for $t_{1/2}$ of 0.35 hr (21 min) was similar to that in the first study and again provided the only major deviation from the similarity analysis, with a value less than the worst case prediction. Again, this indicates that CD3-246 has a short duration of treatment in mice,
and questioned the likelihood that three injections of 100 mg/kg CD3-246 per week could affect tumor growth.

Table 4.4: Comparison of the estimated pharmacokinetic parameters and maximum measured plasma concentration for the first two stages of CD3-246 in vivo study in mice, versus the corresponding values predicted by the CD3-246 molecular similarity analysis (see Table 4.2).

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>F (%)</th>
<th>t_{\text{max}} (hr)</th>
<th>t_{1/2} (hr)</th>
<th>V (ml/g)</th>
<th>V/F (ml/g)</th>
<th>C_{p,max} (µM)</th>
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<td>First In vivo Study in Normal Mice</td>
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</table>

Molecular Similarity Analysis

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Worst Case</td>
<td>25</td>
<td>1.6</td>
<td>0.5</td>
<td>2.4</td>
<td>9.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Median</td>
<td>50</td>
<td>0.35</td>
<td>2.1</td>
<td>1.1</td>
<td>2.2</td>
<td>81</td>
</tr>
<tr>
<td>Mean</td>
<td>53</td>
<td>0.63</td>
<td>3.3</td>
<td>1.2</td>
<td>2.2</td>
<td>74</td>
</tr>
<tr>
<td>Best Case</td>
<td>90</td>
<td>0.1</td>
<td>&gt;11</td>
<td>0.4</td>
<td>0.4</td>
<td>425</td>
</tr>
</tbody>
</table>

4.6 Efficacy of CD3-246 in Implanted DU145 Xenografts in Immune-Compromised BALB/c (nu/nu) Mice

A third in vivo study was performed to test the efficacy of CD3-246 on androgen-independent DU145 xenografts implanted in female BALB/c nu/nu mice. DU145 CaP cells were pre-cultured for seven days in RPMI 1640 media supplemented with 1% SD-FBS and PSA. $1 \times 10^6$ DU145 cells were implanted subcutaneously in the right hind flank of each mouse in 100 µl of a 1:1 mixture of BD Matrigel™ Basement Membrane Matrix and RPMI 1640 with no added serums. A total of sixty animals were needed for
this study. To account for the fact that not all animals that receive cancer cells develop
tumors, eighty-seven mice were injected with DU145 cells. After two weeks, seventy-nine
animals had visible tumors and 8 animals showed no signs of tumors. Nine animals
had irregular shaped tumors and were not used in the study.

Mice with regularly shaped tumors were randomly put into three test groups,
where one group received vehicle-only (negative control, 15 animals), another received
20 mg/kg Docetaxel (positive control, 10 animals), and the third received CD3-246 (100,
32, or 10 mg/kg, 15, 10, and 10 animals, respectively). The positive control treatment
regimen was selected based on a literature review of successful docetaxel treatments of
CaP tumor xenografts in mice, where treatments ranged from 6-20 mg/kg docetaxel given
1-3 times per week by subcutaneous, intraperitoneal, or intravenous injection (Oudard et
al. 2003; Van Laar et al. 2003; Huang et al. 2002; Sirotnak et al. 2000). A docetaxel
treatment was selected at the high end of this range, 20 mg/kg three times per week,
which ensured a significant tumor growth inhibition response for the positive control
treatment. Animals were treated 3 times per week (Monday, Wednesday, Friday) by a 10
ml/kg ip injections. Injection solutions for the CD3-246 groups and control group were
prepared fresh before each injection as described in Section 4.4. Docetaxel injection
solutions were prepared fresh before each injection in a similar manner, with frozen
aliquots of 5 mg/ml docetaxel in DMSO thawed and diluted to 2 mg/ml with sterile PBS
on the day of injection. Body weights, tumor measurements, and general animal health
were also evaluated three times per week on the same days as the injections. Tumor
measurements were performed with Fisher Brand digital calipers by measuring the
longest dimension of the tumor (L, mm), and then the tumor width perpendicular to the
longest dimension (w, mm). The weight of the tumors (in mg) was then estimated by the standard equation $\frac{1}{2}Lw^2$ (Corbett et al. 1997), and the percent of initial tumor weight was calculated for each animal at each time point by dividing by the starting tumor weight for that animal on the first day of injections. Animals remained in the study for a total of five weeks of injections (15 total injections), or until they suffered health problems requiring early removal and euthanasia. Immediately after carbon dioxide/cervical dislocation euthanasia, tumors were removed, weighed, rinsed with PBS, flash frozen in liquid nitrogen, and stored at -80°C for future PAM expression analysis.

Mice treated with docetaxel were the only animals in this study that exhibited health problems associated with their treatments. The docetaxel treated mice began showing obvious signs of health problems by Day 7 of the study after only three injections, when all animals in the docetaxel treatment group exhibited distended abdomens and a bluish-grey skin tone. Three of the docetaxel treated animals died after only four injections on Day 9, and the decision was made to euthanize the remaining animals in this treatment group as all exhibited extreme abdominal inflammation and dark grey skin color. The selected docetaxel treatment regimen (20 mg/kg, 3 treatments per week) was obviously too high for the animals used in this study.

No animals in any of the other treatment groups exhibited any signs of ill-health over the entire five week treatment period, and all were euthanized as planned at the end of the five weeks. The percent initial body weight measurements for the different treatment groups in this study have been summarized in Figure 4-12. These results show that docetaxel treated mice sustained rapid and significant weight loss right from the start of the study until they were removed on Day 9. A repeated measures ANOVA analysis
could not be used to analyze these results because of the missing data points caused by the early removal of the docetaxel animals. A Student’s t-test analysis was employed as a measure of statistical significance (p < 0.05). Treatment group values that were statistically different from other groups have been indicated by the colorized number/letter codes in Figure 4-13. The color indicates the treatment group with the lower value and the number/letter code indicates the treatment group with the higher value. This analysis verified that the docetaxel group body weights became significantly different from many of the other groups early in the study, and was significantly different than all of the other groups by Day 9 when the animals were removed from the study. Most of the other group comparisons showed small differences between the groups, although the highest CD3-246 group was significantly below the lower CD3-246 treatment doses on a few test dates, and the control animals were significantly below one or more of the CD3-246 treatment groups on many occasions. This would appear to provide a fairly clear indication that the CD3-246 treatments did not have any significant negative effects on the animals’ body weight or general health.
Figure 4-13: Percent of initial body weight for all animal treatment groups in the in vivo tumor growth inhibition study performed in immune-compromised nude BALB/c mice with androgen-independent CaP tumor xenografts. The colorized number/letter codes indicate values that are significantly different (p < 0.05) by a Student’s t-test analysis. The color of the code indicates the treatment group with the lower value and the number/letter code indicates the treatment group with the higher value. A repeated measures ANOVA analysis could not be used due to the early removal of the docetaxel treated animals.

The percent initial tumor weight values for the different treatment groups exhibited some interesting and exciting results in Figure 4-13. As with the body weight analysis, a repeated measures ANOVA analysis could not be used because of the missing data points caused by the early removal of the docetaxel animals. Student’s t-test analysis was employed as a measure of statistical significance (p < 0.05). Treatment
group values that were statistically different from other groups have been indicated by the colorized number/letter codes in Figure 4-14. The color indicates the treatment group with the lower value and the number/letter code indicates the treatment group with the higher value. These results clearly showed that the docetaxel treatment started shrinking the tumors from the very beginning of the study, with significant differences versus all other treatments from Day 4 until the docetaxel treated mice were removed on Day 9.

The selected docetaxel treatment regimen was obviously too high for the animals used in this study, but the tumor growth results clearly demonstrated that this treatment was highly successful in not only reducing the tumor growth, but actually caused the tumors to significantly shrink. Thus, while the docetaxel treatment was detrimental to the animals’ health, it was successful as a positive control in demonstrating that the tumor growth could be slowed by an effective anticancer drug.

Also shown in Figure 4-14, the CD3-246 treatment group results were exciting because they exhibit a consistent dose-dependent reduction in tumor growth. The tumor growth for the lowest 10 mg/kg CD3-246 treatment dose was essentially identical to control animal results, indicating that this dose had no detectable effect on tumor growth. The intermediate 32 mg/kg CD3-246 dose actually appeared to provide the best dose for tumor growth inhibition, with a consistent and ever-widening gap between the control animal values and the 32 mg/kg CD3-246 animals across the five week duration of the study, and statistically significant differences versus control and/or 10 mg/kg CD3-246 treatment groups at numerous study time points. The highest 100 mg/kg CD3-246 treatment dose also exhibited a consistent and ever-widening difference versus the
control and 10 mg/kg CD3-246 results, but while these results are consistent they are not statistically significant.

The consistent effectiveness of the 32 mg/kg CD3-246 dose, and to a lesser extent the 100 mg/kg CD3-246 dose, in inhibiting androgen-sensitive CaP tumor xenograft growth (also shown in Figure 4-14) was all the more amazing given the pharmacokinetics of CD3-246 and the less than ideal dosage regimen that was used in this study. As measured in the previous two in vivo studies, the elimination half-life of CD3-246 was on the order of 20 min in mice. This short half life meant that CD3-246 plasma concentrations were at elevated levels only for several hours immediately after each injection. Giving the mice injections only three times per week should have only provided very brief exposure to the PAM inhibitor at widely spaced time intervals. It was fully expected that this type of exposure would at best only show very modest effects on the rate of tumor growth. On the contrary, however, the difference between the 32-100 mg/kg CD3-246 treatments and control animals were consistent and ever-widening throughout the duration of the study. There could be several explanations for these exciting results. First, as stated earlier, CD3-246 has a very low solubility in aqueous environments. It is thus possible that a significant portion of the CD3-246 precipitates out immediately following the ip injection. This could actually have worked to our advantage, as the precipitated agent could then have been slowly dissolved and absorbed, providing a slow rate continuous release of the agent into the blood stream. Alternatively, the highly lipophilic agent could have collected in lipophilic tissues and, again, been slowly but continuously released into the blood stream. As a final alternative, tissues with high PAM enzyme levels, such as the tumor, may have provided
an enzyme-bound storage depot for CD3-246. Since CD3-246 had a high affinity for PAM enzymes but did not appear to be converted to an amidated metabolite by PAM, a high level of the agent could be expected to build-up and remain in the vicinity of tissues with high PAM enzyme expression. Alternatively, CD3-246 could have been converted to some other active metabolite that had a longer half-life than CD3-246, which could have resulted in reduced tumor growth. Regardless of the explanation for the CD3-246 tumor inhibition effects, these preliminary findings clearly indicated that CaP PAM enzymes are a viable target for the treatment of androgen-independent CaP.
Figure 4-14: Percent of initial tumor weight for all animal treatment groups in the *in vivo* tumor growth inhibition study performed in immune-compromised nude BALB/c mice with androgen-independent CaP tumor xenografts. The colorized number/letter codes indicate values that were significantly different (p < 0.05) by a Student’s t-test analysis. The color of the code indicates the treatment group with the lower value and the number/letter code indicating the treatment group with the higher value. A repeated measures ANOVA analysis could not be used due to the early removal of the docetaxel treated animals.
Chapter 5

Mechanism Studies: DU145 Growth Inhibition Assay with Exogenous iAM and AM

\(iAM\) is the glycine-extended intermediate form of adrenomedullin (AM) that is inactive until it is amidated by PAM. The product of this amidation is AM, an endogenous growth factor. Previous studies have shown that adding 200 nM exogenous AM to DU145 cells causes a 20% increase in growth after four days of exposure (Rocchi et al. 2001). In preliminary studies, we have found an 18% increase in growth in DU145 cells after four days exposure to 800 nM exogenous AM (Phoenix Pharmaceuticals, Burlingame, CA). Likewise, we found a 17% increase in growth in DU145 cells after four days exposure to 800 nM exogenous \(iAM\) (Phoenix Pharmaceuticals, data not shown). To determine if CD3-246 specifically targets PAM in order to inhibit cell growth, DU145 cells were treated with CD3-246 in conjunction with AM and iAM.

It was hypothesized that DU145 cells treated with exogenous AM should increase cellular growth, independent of treatment with CD3-246. It was also predicted that DU145 cells treated with exogenous \(iAM\) should increase cellular growth in the absence of CD3-246 but decrease when treated with both \(iAM\) and CD3-246 simultaneously.

To further verify that CD3-246 caused inhibition of PAM enzyme, DU145 cells were incubated with three 2-fold dilutions of CD3-246 in RPMI 1640 media
supplemented with TIS and PSA in the presence or absence of 800 nM AM or iAM. As stated in Chapter 3, this media has been used by other groups to simulate conditions where the cells exhibited PAM-dependent growth. Tests were performed and processed as described in Chapter 3, except 30,000 cells were plated per well, only CD3-246 was tested, and cells were exposed to test agent for four days. The difference between the optical density at the beginning and end of the agent exposure period provided a measure of the cell growth. The ratio of cell growth at each condition versus untreated cellular growth provided a measure of the fraction of control growth.

Results of the PAM-dependent DU145 growth inhibition assay are illustrated in Figure 5-1. These results indicated that both AM and iAM stimulated DU145 growth at similar levels. There was also a similar level of increased DU145 cellular growth at the lowest concentration CD3-246 tested for AM- and iAM-treated cells. At 1 mM CD3-246, the growth of DU145 cells treated with both AM and iAM began to decrease, but both conditions remained significantly higher than cells that did not receive exogenous growth factors. When treated with 2 mM CD3-246, there was no statistical difference between any of the growth factor conditions. These results do not confirm or refute the hypothesis that inhibition of PAM is a major determinant for the growth inhibition caused by CD3-246.
Figure 5-1: PAM-dependent DU145 growth inhibition assay results for the test agent CD3-246 with exogenous AM or iAM shown as the ratio of control cell growth (n ≥ 5).

There are several possibilities that may explain our inconclusive results. One such possibility is that the DU145 CaP model employed in our assay was inadequate and/or that a number of additional replicates may be required to discover such differences above the ‘noisy’ growth environment of these cells. Another possibility is that AM may not have been the ideal PAM substrate to model PAM-dependence. Also, we only tested one concentration of AM and iAM. It is possible that adding higher levels of these growth factors may have stimulated growth more, particularly in the cells that received the highest concentration of CD3-246. Third, there are a number of growth factors other than AM endogenous to the DU145 CaP cells and these may have been inhibited by CD3-246 independent of treatment with AM and iAM. Lastly, it is possible that CD3-
246 acts specifically against PAM at lower concentrations, but becomes a general cytotoxic agent at high concentrations. Thus, additional studies still need to be performed to verify the mechanism of CD3-246 growth inhibition.
Chapter 6

Discussion

Our laboratory has identified the compound CD3-246, a novel PAM inhibitor that shows promise in reducing the growth of advanced, hormone-independent prostate cancer cells both in vitro and in vivo. In vitro CD3-246 significantly reduced DU145 CaP growth in a PAM-dependent manner in the high nanomolar range (0.31 ± 0.001 µM). Although this range is not as effective as the thiolate conjugated di-peptides reported by Erion et al (1994), which have potencies in the low nanomolar range, those inhibitors were pre-incubated with PAM for twenty min before adding a synthetic PAM substrate.

To demonstrate specificity to CaP PAM, DU145 cells were treated with standard anticancer agents, standard PAM inhibitors, or CD3-246 in TIS media, which lacks growth factors and relies upon PAM to activate endogenous growth factors, or in media containing necessary growth factors. Vinblastine and docetaxel, as well as disulfiram, DDC, and PBA, inhibited DU145 cell growth in both media types suggesting the mechanism of action is not PAM-dependent. However, DU145 cells grown in both media types and treated with CD3-246 exhibited a decrease of over 50% cell growth in TIS media (PAM-dependent) versus those in media containing necessary growth factors.
This suggests that CD3-246 inhibits cellular growth via a mechanism involving PAM autocrine growth loops.

To additionally confirm that the mechanism of growth inhibition is associated with PAM, DU145 cells in TIS media were treated with exogenous AM and iAM in the presence of CD3-246. Since AM is active independently of PAM activity and iAM relies on PAM to be amidated to become active, DU145 cells in the presence of iAM and CD3-246 should not have significant increases in growth. As expected, DU145 cells treated with AM and iAM had increased growth in the absence of CD3-246 and at the lowest concentration of agent tested. However, both AM- and iAM-treated cells showed decreased cell growth with increasing levels of CD3-246. The differences in CD3-246 inhibition of AM and iAM stimulated growth were not statistically significant and the exact mechanism of CD3-246 activity could not be ascertained from this assay.

CD3-246 has proven effective against implanted CaP xenographs. Untreated (control) DU145 xenographs grew three-fold over a five-week period. However, the animals that received 100 mg/kg and 32 mg/kg three times per week by ip injection exhibited only a two-fold increase in tumor size. The growth reduction seen in implanted DU145 xenografts was surprising due to the extremely short half-life (around 20 min) of CD3-246 in the test animals. CD3-246 also had low aqueous solubility that may have actually been in our favor, as the compound could have precipitated in the intraperitoneal cavity immediately upon injection and slowly dissolved over time, creating a low-dose and prolonged continuous exposure to the test agent. Alternatively, an active metabolite having a longer half-life may have formed from the quick break-down of CD3-246 in vivo.
Although animals treated with CD3-246 did not exhibit noticeable side effects, it has been demonstrated that reduced expression of PAM in PAM haploinsufficient (+/-) mice exhibit some physiological changes. PAM +/- mice have significantly lower body temperatures when exposed to cold temperatures due to vasoconstriction (Bousquet-Moore et al. 2010). Vasoconstriction also led to an inability of the PAM +/- mice to induce a shiver response to the cold temperature (Bousquet-Moore et al. 2009). Problems with vasoconstriction (Bousquet-Moore et al. 2009) were most likely due to the inactivation of vasopressin (Murphy et al. 1994) and neuropeptide Y (Jolicoeur et al. 1991; Jolicoeur et al. 1994) both of which are amidated by PAM.

PAM +/- mice have also shown some psychological differences from their wild type littermates. PAM +/- mice showed increased anxiety-like behaviors, were deficient in contextual and cued fear conditioning, required higher shock intensities to elicit a startle response (Gaier et al. 2010), as well as displaying increased seizure sensitivity (Bousquet-Moore et al. 2010). PAM activated factors may be responsible for these behaviors as Substance P is involved in pain regulation (Barak et al. 1999) and neuropeptide Y is related to anxiety-like behaviors (Heilig et al. 1989; Wahlestedt et al. 1993). Interestingly, wild type mice fed a diet deficient in copper display similar effects as PAM +/- mice. Feeding PAM +/- mice extra dietary copper can reverse some of these effects (Bousquet-Moore et al. 2010). PAM +/- is lethal in utero. Because CD3-246 is not targeted to prostate cancer cells, effects associated with inhibiting PAM systemically must be taken into consideration.

Taken together, in vitro and in vivo results indicate that CD3-246 may offer a potential treatment for hormone refractory CaP. While CD3-246 has promise, it is not
ideal. Therefore, future goals are to increase potency in the low nanomolar range, gain specificity to prostate cancer cells, and increase the half-life by generating analogs of CD3-246. As a first step to determine if continuous exposure to CD3-246 can further reduce tumor growth, our laboratory plans to utilize an Alzet® implantable pump that utilizes osmosis to deliver a continuous dose of agent for up to 6 weeks. This type of system should allow for controlled release of CD3-246 and overcome the problems associated with a short half-life in laboratory animals.
References


