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Design, Synthesis, and Biological Evaluation of Novel Polyamine Transport System Probes and their Application to Human Cancers

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DESIGN, SYNTHESIS, AND BIOLOGICAL EVALUATION OF NOVEL POLYAMINE TRANSPORT SYSTEM PROBES AND THEIR APPLICATION TO HUMAN CANCERS

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the College of Sciences at the University of Central Florida Orlando, Florida

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2012

Major Professor: Otto Phanstiel IV
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ABSTRACT

The mammalian polyamine transport system (PTS) has been of interest due to its roles in cancer and maintaining cellular homeostasis. Polyamines are essential growth factors which are tightly controlled via a balance of biosynthesis, metabolism, import, and export. This work focused on the development and biological testing of polyamine transport probes to help understand the molecular requirements of the PTS. This was mediated through the use of a CHO (PTS active) and CHO-MG* (PTS deficient) screen, where compounds demonstrating high toxicity in CHO and low toxicity in CHO-MG* were considered PTS selective.

The first chapter focused on the development of polyamine-based drugs which are both metabolically stable to polyamine oxidase (PAO) activity and are hyperselective for targeting the PTS. This approach was optimized by combining a di-substituted aryl design with terminal N-methylation of the appended polyamine chains to generate a new class of superior PTS agonists. The metabolic stability of these compounds was demonstrated in CHO and CHO-MG* in the presence and absence of a known PAO inhibitor, aminoguanidine (AG). Highly PTS selective compounds were then tested in the NCI-60 cell line screen to demonstrate the effectiveness of polyamine-based drugs in cancer therapy. During this screen, the MALME-3M (human melanoma) cell line was identified as being very sensitive to these PTS targeting drugs. Further studies using MALME-3M and its normal counterpart, MALME-3, showed excellent targeting of the cancer line over MALME-3. For example, the MeN44Nap44NMe compound showed 59-fold higher toxicity in MALME-3M over MALME-3.
The second chapter focused on the development of potential polyamine transport inhibitors (PTIs) for use in combination therapy with α-difluoromethylornithine (DFMO). This therapy is predicated upon reducing sustained polyamine depletion within cells by inhibiting both polyamine biosynthesis with DFMO and polyamine transport with the PTI ligand. Potential PTIs were identified by blocking the uptake of spermidine in DFMO-treated CHO and L3.6pl cells. Previous work has identified a tri-substituted polyamine-based design as an effective PTI. Low toxicity and a low $K_i$ value in a L1210 screen were good predictors for PTI efficacy. The structural requirements for a potent PTI were explored by modulating the toxicity through the introduction of amide bonds, and also by determining the number and orientation of the polyamine messages (appended to an aryl core) required for efficient inhibition of polyamine uptake. These experiments showed that a tri-substituted design and a triamine message (homospermidine) appended was optimal for PTI potency.

The final chapter focused on the development of Dihydromotuporamine C derivatives as non-toxic anti-metastatic agents. Dihydromotuporamine C demonstrated good anti-invasive properties with tumor cells. Derivatives were made in an effort to reduce the cytotoxicity of the parent and improve the anti-migration potency. The motuporamine derivatives all have a polyamine message (norspermidine or homospermidine) appended to make a macrocycle core, making them prime targets to evaluate as potential PTS ligands in the CHO and CHO-MG* screen. Each compound was also tested in the highly metastatic pancreatic cancer cell line L3.6pl to determine both its IC$_{50}$ value and maximum tolerated dose (MTD). The anti-migration assay was performed at the lowest MTD obtained (0.6 µM) in order to compare the series at the same non-toxic dose. The results suggested that as the $N'$-amine center was moved further from the
macrocyclic ring, an increased ability to inhibit cell migration and reduced toxicity was observed.

These collective findings provide new tools for cell biologists to modulate and target polyamine transport in mammalian cells. Future applications of these technologies include new cancer therapies which are cell-selective and inhibit the spread of tumors.
ACKNOWLEDGMENTS

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<tr>
<td>$^{13}$C</td>
<td>Carbon 13 isotope</td>
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<tr>
<td>$^1$H</td>
<td>Hydrogen 1 isotope</td>
</tr>
<tr>
<td>AcOH</td>
<td>Acetic Acid</td>
</tr>
<tr>
<td>AG</td>
<td>Aminoguanidine</td>
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<tr>
<td>Ar</td>
<td>Aromatic system</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>Cbz</td>
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<tr>
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</tr>
<tr>
<td>Cmpd</td>
<td>Compound</td>
</tr>
<tr>
<td>d</td>
<td>Doublet</td>
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<tr>
<td>D₂O</td>
<td>Deuterated water</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>DFMO</td>
<td>α-Difluoromethylornithine</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>FBS</td>
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<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HSpd</td>
<td>Homospermidine</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>L1210</td>
<td>Murine leukemia cells</td>
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L3.6pl  Metastatic human pancreatic cancer

LiAlH$_4$  Lithium aluminum hydride

m  Multiplet

$M$  Molarity (moles/L)

MALME-3  Fibroblast derived normal skin cells

MALME-3M  Fibroblast derived malignant melanoma

MeOH  Methanol

mg  Milligram

MHz  Megahertz

min  Minute

mL  Milliliter
<table>
<thead>
<tr>
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<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>mmol</td>
<td>Millimole</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrum</td>
</tr>
<tr>
<td>MsCl</td>
<td>Methanesulfonyl chloride</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>NaBH₄</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NH₄OH</td>
<td>Ammonium hydroxide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>ODC</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PAO</td>
<td>Polyamine oxidase</td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>PSV</td>
<td>Polyamine sequestering vesicle</td>
</tr>
<tr>
<td>PTI</td>
<td>Polyamine transport inhibitor</td>
</tr>
<tr>
<td>PTS</td>
<td>Polyamine transport system</td>
</tr>
<tr>
<td>Put</td>
<td>Putrescine</td>
</tr>
<tr>
<td>r.t.</td>
<td>Room temperature</td>
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<tr>
<td>Spd</td>
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<tr>
<td>Spm</td>
<td>Spermine</td>
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<tr>
<td>SSAT</td>
<td>Spermidine/spermine-(N^1)-acetyl transferase</td>
</tr>
<tr>
<td>t</td>
<td>Triplet</td>
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TEA  Triethylamine

THF  Tetrahydrofuran

TLC  Thin layer chromatography

TMS  Tetramethyilsilane

UV  Ultraviolet

δ  ppm
CHAPTER I: DEVELOPMENT OF HYPERSELECTIVE POLYAMINE TRANSPORT LIGANDS AND SELECTIVITY AGAINST SPECIFIC HUMAN CANCERS

I.1 Abstract

The absolute cellular requirement for polyamine growth factors makes maintenance of polyamine homeostasis critical for life. Polyamine homeostasis is accomplished via a balance of polyamine biosynthesis, degradation, and transport. Rapidly-dividing cancer cells have been shown to have high polyamine transport activity compared to normal cells, likely due to their high requirement for polyamine growth factors. Therefore, the polyamine transport system (PTS) is a therapeutically relevant target as it can provide selective drug delivery to cancer cells. This chapter describes the synthesis and biological evaluation of new multimeric polyamine derivatives as efficient PTS ligands. A series of arylmethyl-polyamine derivatives were synthesized and evaluated for their ability to target the polyamine transport system. The architectures of these probes addressed two caveats in PTS drug design: a) PTS selectivity and b) metabolic stability to amine oxidases.

I.2 Introduction

Polyamines (1-3) are low molecular weight aliphatic amines and are essential growth factors for cells. Polyamines play essential roles in transcription, translation, and chromatin remodeling. Tumor cells have been shown to contain elevated polyamine levels and have active polyamine transport systems to import exogenous polyamines. This special characteristic of cancer cells allows for cell selective drug delivery of polyamine-drug conjugates to particular cell types. The polyamine transport system (PTS) is an important target as many cancer cells
need to import polyamines in order to sustain their growth rate, especially in the presence of polyamine biosynthesis inhibitors like difluoromethylornithine (DFMO).\(^1,3\) DFMO resembles the natural substrate ornithine for ornithine decarboxylase (ODC), a key enzyme in putrescine (1) biosynthesis.\(^1,3\)

Although the PTS has been recognized as an important target for cell selective drug delivery, the PTS is still poorly understood. What is known is that the PTS is an energy-requiring and carrier mediated process.\(^4\) Recently, two models of mammalian polyamine transport have been proposed by Poulin\(^5\) and Belting\(^6\), respectively. Poulin suggested that polyamines enter the cell through an active plasma membrane transporter, followed by the sequestration into polyamine sequestering vesicles (PSVs).\(^5a\) In order for polyamines to internalize within these PSVs, a vesicular H\(^+\)/polyamine carrier is needed to facilitate the import and escape from the PSV.\(^5a\) It was also found that the PSVs colocalized with acidic vesicles of the late endocytic compartment and the trans Golgi.\(^5a\) Belting, on the other hand, provided a multi-step endocytic process where polyamines bind to heparan sulfate proteoglycans in caveolae.\(^6b\) Once the polyamines have bound to heparan sulfate, they are then endocytosed via a caveolin-dependent process and their heparan sulfate chains are subsequently cleaved and further processing by NO liberates the polyamines.\(^6b\)

Drug conjugates, which join a cytotoxic agent to a polyamine, have exhibited selective cytotoxicity to cancer cells compared to their normal cell counterparts.\(^1,3\) A method was developed to investigate if polyamine-drug conjugates were indeed targeting the polyamine transport system. Delcros \textit{et.al.} demonstrated that the Chinese hamster ovary (CHO) cell line was very effective in identifying drug conjugates which are PTS selective.\(^7\) This cell line was chosen
along with its transport deficient mutant (CHO-MG) in order to demonstrate which drug conjugates selectively target the PTS. The CHO-MG cell line developed by Flintoff et al. is polyamine transport deficient and was developed by treating the CHO cell line with ethyl methanesulfonate and subsequent dosing of the surviving cells with cytotoxic methylglyoxal-bis(guanylhydrazone) (MGBG, a known PTS ligand) to determine their resistance. The surviving cells that were found to be resistant to MGBG were also found to have a marked decrease (less than 1% as compared to the wild type) in the uptake of radiolabeled spermidine, demonstrating a defective PTS. When evaluating the polyamine drug conjugates, the respective IC\textsubscript{50} values are determined in CHO and CHO-MG, and the ratio (CHO-MG IC\textsubscript{50}/CHO IC\textsubscript{50}) is used to represent the PTS selectivity of the compound. Compounds with high CHO-MG/CHO IC\textsubscript{50} ratios are considered PTS selective.

![Figure I-1](image-url)

**Figure I-1.** Structures of the native polyamines (putrescine, Put 1, spermidine, Spd 2, and spermine, Spm 3) and the non-native polyamine (homospermidine, HSpd 4) and MGBG
Various types of $N$-alkylpolyamines have been synthesized and shown to provide promising anticancer activity. The PTS is sensitive to the polyamine structure used; where the number of nitrogens present and the distance between each plays an integral part. However, while the PTS is sensitive in its molecular recognition of the polyamine chain, it can still accommodate large $N^i$-substituents. 

Previous work in the Phanstiel lab has shown that the number of methylene spacer units, the size of the $N^i$ substituent, and the degree of the $N^i$ substitution all influence PTS mediated delivery. Initially, it was found that an anthryl-homospermidine conjugate 5a had a 150-fold higher cytotoxicity in CHO cells than in the mutant cell line, CHO-MG, which was PTS deficient. Utilization of the polyamine transporter was also supported by the ability of exogenous Spd to outcompete and effectively rescue the cells from the toxicity of the polyamine based drug. It was also observed that the proper polyamine sequence was necessary to demonstrate PTS selectivity with homospermidine being optimal. After demonstrating the high selectivity of 5a for targeting the PTS, the next question was whether the addition of another polyamine sequence to the drug platform would enhance the PTS selectivity; in other words would twice the number of polyamine messages increase the PTS selectivity? In order to answer this question, compounds 6a-8a were synthesized and evaluated in CHO and CHO-MG. Rewardingly these agents showed a dramatic increase in PTS selectivity (e.g., CHO-MG/CHO IC$_{50}$ ratios; 5a: 150, 6a: >2222). Clearly, di-substituted designs dramatically improved targeting. While this was a definite advance, there were other factors to consider in terms of optimizing drug design.
Several factors were shown to affect PTS selectivity and required further structural optimizations. For example, Polyamine oxidase (PAO) activity severely reduces the PTS selectivity of polyamine-based drugs via drug degradation. This degradation is inhibited when aminoguanidine (AG), a known inhibitor of PAO, is added to the growth medium. Polyamine oxidase is known to be present in fetal bovine serum as well as in human blood. Specifically, polyamine oxidase (PAO) is an important enzyme responsible for converting $N^\prime$-acetyl spermine (54) to spermidine (2) and also for converting $N^\prime$-acetyl spermidine (53) to putrescine (1).

Since polyamine oxidase targets primary amines, it also metabolizes polyamine-based drugs. In terms of drug design, the primary amine can be protected by $N$-methylation to avoid the metabolic and degradative effects of polyamine oxidase activity. $N$-alkylation of the polyamine termini has many effects on the biological properties of polyamines, including enhanced stability in the culture medium. In short, polyamine-oxidase-mediated degradation can be prevented via $N$-alkylation.

**Figure I-2. Polyamine Biosynthesis**
For example, compound 5a, which lacks a terminal N-methyl group, showed a profound loss in PTS selectivity in the absence of AG (CHO-MG/CHO IC₅₀ ratio with AG: 150; without AG: 4). The N-methylated derivative of 5a, compound 5b, was able to retain its PTS selectivity in the absence of AG (CHO-MG/CHO IC₅₀ ratio of 12.3 with AG, and 11.5 without AG).

The next step was then to combine the di-substituted design shown to be so effective in PTS targeting with the N-methylation technology to generate a superior PTS agonist. Therefore, compounds 6b-8b were generated with the expectation that they would show enhanced metabolic stability over their non-methylated counterparts (6a-8a) while maintaining high PTS selectivity in the absence of AG.

The CHO/CHO-MG screen closely resembles the differences between a cancer cell line and healthy cells. CHO cells can be envisioned as cells with a constitutively active PTS (similar to many cancer types), while CHO-MG models a healthy normal cell where polyamine transport activity is minimal. A success in this area would be a significant advance because the ability to target the PTS of cancers could provide new drugs which have lower toxicity in healthy tissues. Since the polyamine transport system is not fully understood in mammals, these compounds also represent novel molecular probes to study polyamine transport properties.

Chapter 1 investigates the combination of these two technologies (enhanced targeting via di-substituted drug designs and N-methylated polyamines) to provide metabolically stable drugs with excellent PTS targeting.
Figure I-3. Structures of compounds 5-8

I.3 Results and Discussion

Synthesis. The regional strategy was to generate a modular core platform which could have the terminal N-alkyl groups introduced in the last step. As shown in Scheme I-1, the synthesis of 6b-8b began with commercially available 4-amino-1-butanol, 9. By first Boc-
protecting 9 to make 10, the alcohol could be selectively mesylated to give 11. Next, mesylate 11 was reacted with another equivalent of aminoalcohol 9 via nucleophilic substitution to give the secondary amine 12. The subsequent Boc introduction step sequestered the free 2° nitrogen in 12 as a carbamate through the use of di-tert-butyl dicarbonate to provide Boc protected 13. Both amines were protected to provide for regiochemical control and provided a more stable compound amenable to long term storage.\\(^{3f}\)

**Scheme I-1.** Synthesis of 6b (1st Attempt)

\(^{a}\)Reagents: (a) 10%TEA/MeOH, di-tert-butyl dicarbonate; (b) MsCl, TEA/CH\(_2\)Cl\(_2\); (c) 4-amino-1-butanol, CH\(_3\)CN; (d) 4 M HCl, EtOH; (e) 25% MeOH/CH\(_2\)Cl\(_2\), anthracene-9,10-dicarboxaldehyde; (f) 50% MeOH/CH\(_2\)Cl\(_2\), NaBH\(_4\); (g) MsCl, TEA/CH\(_2\)Cl\(_2\); (h) 2.0 M methylamine in MeOH
The next stage of the synthesis was initiated by first reacting 13 with 4 M HCl to remove both Boc protecting groups to generate 14, the HCl salt. Next, 14 was reacted with anthracene-9,10-dicarboxaldehyde and NaBH₄ via reductive amination to provide diol 15. In this sequence, the Schiff base was first generated by reacting 14 with the anthracene-9,10-dicarboxaldehyde, and then NaBH₄ reduced the resultant imine intermediate to the corresponding secondary amine, 15. After completion of this step, all four free amines were globally protected again using di-tert-butyl dicarbonate to provide 16. Boc-protected diol 16 was next reacted with methanesulfonyl chloride to convert both alcohol groups to mesylates, i.e. bismesylate 17. The key step was to react 17 with methylamine to provide 18. The first attempt at this step was done using 17 dissolved in acetonitrile and adding it to a 2.0 M solution of methylamine in methanol. After these two reagents were refluxed overnight, the ¹H NMR showed a mixture of products. After performing column chromatography on the crude mixture, 18 could not be isolated in high purity or high yield (12%). In order to improve upon the yield and purity issues, a new strategy needed to be developed.

When attempting the reaction of 17 with methylamine again, a new approach was tried. This attempt did not use acetonitrile, but instead ran the reaction in the methanol provided by the methylamine solution. The other alteration to this method was to dissolve 17 in a minimal amount of dichloromethane and add this solution dropwise to a 150-fold excess of a methylamine/methanol solution over 20 minutes. With these changes in the procedure, 18 was provided in much higher yield (52%). After seemingly successfully making 18, the final step of removing the Boc protecting groups was done by again using 4 M HCl, ultimately providing the final product, 6b. Elemental analysis and ¹H NMR, however, showed that both 18 and 6b were
not in fact pure, and the purification of **18** proved to be far more difficult than originally thought as a mixture of the desired product and alcohol byproducts were observed.

After purification of **18** via the synthetic pathway seen in Scheme 1 had failed after multiple attempts, a new synthetic route was explored. As shown in Scheme 2, the methylated amine was generated first instead of in the penultimate step. The amino alcohol **9** was reacted with ethyl formate in ethanol, and the resulting formamide **19** was reduced to **20** using LiAlH_4 in THF.\(^{19}\) This methylated amine was then Boc protected using di-tert-butyl dicarbonate to generate **21** in 84% yield. The production of **21** was confirmed by \(^1\)H NMR.\(^{20}\) This protection was necessary as the next step generated the mesylate **22** using methanesulfonyl chloride. Mesylate **22** was reacted with putrescine in refluxing acetonitrile to afford **23** in 90% yield. This proved to be very valuable as now the \(N\)-methyl polyamine chain was generated prior to reductive amination to avoid the issues observed in Scheme I-1. Subsequent reductive amination of **23** with anthracene-9,10-dicarboxaldehyde and NaBH_4 provided the di-substituted system **24**. However, the tetra-amine was difficult to purify directly due to its multiple amines. Therefore, **24** was globally Boc protected to give **25**, which could then in principle be purified by column chromatography. Unfortunately, the purification of **25** proved to be unsuccessful. The difficulty ultimately proved to be that when globally Boc protecting **24**, some polyamine **23** was also present which also becomes Boc protected. These two compounds were then seen to have nearly identical \(R_f\) values, and could not be separated from one another.
Scheme I-2. Synthesis of 6b (2nd Attempt)

**Reagents:** (a) Ethyl formate, EtOH; (b) LiAlH₄, THF; (c) 10% TEA/MeOH, di-tert-butyl dicarbonate; (d) MsCl, TEA/CH₂Cl₂; (e) putrescine, K₂CO₃, CH₃CN; (f) 25% MeOH/CH₂Cl₂, anthracene-9,10-dicarboxaldehyde; (g) 50% MeOH/CH₂Cl₂, NaBH₄; (h) 4 M HCl, EtOH

After the synthetic routes shown in Schemes I-1 and I-2 failed to afford compound 6b in high purity, another synthetic route was explored, Scheme I-3. This synthesis began with the generation of mesylate 22 from alcohol 21 in 95% yield. Mesylate 22 was then reacted with mono-Cbz-protected putrescine (generated by a known procedure)²¹ in a nucleophilic substitution reaction to generate compound 26 in 50% yield. The secondary amine in compound
26 was then Boc protected to give compound 27 in 99% yield. The Cbz group on compound 28 was then attempted to be removed by hydrogenation conditions (Pd/C), but surprisingly little conversion was achieved. Although speculative, the proximity of the Boc groups may create a sterically crowded environment, making it more difficult than initially thought to remove the Cbz group. A second attempt at hydrogenation was also made through the addition of acetic acid, which helped in the conversion, but still only allowed for 50% conversion over a one week time period. While this synthetic route produced the desired polyamine, 28, it did so at the cost of very low conversion when removing the Cbz group. This prompted a change in the approach to making compound 28, as it was still the desired target needed for making the final compounds 6b-8b.

**Scheme 1-3.** Synthesis of 6b (3rd Attempt)

![Scheme 1-3](image)

**Reagents:** (a) MsCl, TEA/CH₂Cl₂; (b) mono-Cbz-putrescine, K₂CO₃, CH₃CN; (c) 10% TEA/MeOH, di-tert-butyl dicarbonate; (d) Pd/C, AcOH, H₂, MeOH; (e) 25% MeOH/CH₂Cl₂, anthracene-9,10-dicarboxaldehyde; (f) 50% MeOH/CH₂Cl₂; (g) 4 M HCl, EtOH
While multiple synthetic routes had been approached to generate compounds 6b-8b in high purity, none had proved successful. This led to the development of a procedure to alleviate the purification issues mentioned for Scheme I-2, and the conversion issues mentioned for Scheme I-3. As discussed earlier, the desired polyamine for the synthesis of 6b-8b was compound 28.

In prior work, selectively-protected triamines could be separated from their respective condensation products with arylaldehydes. This method takes advantage of the distinct polarity differences between primary and secondary amines. In order to use this approach, a method was needed to selectively introduce a Boc group onto a secondary amine in the presence of a primary amine. Previous work has shown that the chemoselectivity of imine formation with salicylaldehyde occurs with primary amines exclusively even in the presence of secondary amines. With the primary amine sequestered as an imine, the secondary amine can then be selectively Boc-protected. Subsequent imine hydrolysis then regenerated the desired primary amine, 28. Using this approach, polyamine 28 was synthesized with excellent regioselective control in 47% overall yield from 23.

As shown in Scheme I-4, the synthesis was approached by reacting polyamine 28 with the appropriate aryl dialdehyde via reductive amination. Fortunately, compounds 29-31 were successfully generated (55-74% yield) and purified. The methodology developed to generate 28 proved to be invaluable as the purification of 29-31 was straightforward. Finally, with compounds 29-31 generated, they were treated with 4 M HCl to obtain the respective final compounds 6b-8b in near quantitative yields.
Scheme I-4. Synthesis of 6b (4th Attempt)

Reagents: (a) salicylaldehyde, Na$_2$SO$_4$, 25% MeOH/CH$_2$Cl$_2$; (b) di-tert-butyl dicarbonate, MeOH; (c) 0.1 $M$ HCl, EtOH; (d) aq. Na$_2$CO$_3$, CH$_2$Cl$_2$; (e) aryl-dialdehyde, 25% MeOH/CH$_2$Cl$_2$; (f) NaBH$_4$, 50% MeOH/CH$_2$Cl$_2$; (g) 4 $M$ HCl, EtOH

Biological Evaluation. Once synthesized, the conjugates were initially screened for toxicity in CHO, and CHO-MG* cells. CHO cells were chosen along with a mutant PTS deficient line (CHO-MG*) in order to comment on polyamine transport selectivity.$^{3c-e,7}$ The CHO and CHO-MG* results are shown in Table 1.

The CHO-MG* line was derived from the original CHO-MG line obtained from Flintoff.$^8$ Due to a freezer failure, the CHO-MG line had to be reisolated from a thermally compromised lot. This reisolation was successful in providing a mutant line, CHO-MG*, with the same phenotype (deficient polyamine transport). However, this cell line proved to be slightly more sensitive to polyamine drugs making the CHO-MG/CHO ratio reduced. For example, CHO-MG/CHO IC$_{50}$ ratio for 5a was 150, whereas the CHO-MG*/CHO IC$_{50}$ ratio for 5a was 43. As the original stocks of CHO-MG were no longer available from their original source, CHO-MG* were used in the PTS screen.
**CHO and CHO-MG* Studies. IC₅₀ Determinations.** CHO cells were chosen along with a mutant cell line (CHO-MG*) to comment on how the synthetic conjugates gain access to the cells.³⁻⁷ As discussed earlier, the CHO-MG* cell line is polyamine-transport deficient and represents a model for alternative modes of entry (other than PTS) including passive diffusion or use of another transporter. The CHO cell line on the other hand, represents cells with high polyamine transport activity.⁸⁻¹¹ A comparison of toxicity in these two CHO cell lines provided a screen that would detect selective use of the polyamine transporter. High utilization of the PTS by the polyamine compounds would be very toxic to CHO cells. However, reduced toxicity would be expected in CHO-MG* cells.³⁻⁷ Ultimately, a CHO-MG*/CHO IC₅₀ ratio was determined for each compound; where a high ratio would be achieved for highly PTS selective compounds.
Table I-1. Biological Evaluation of Polyamine Derivatives (5-8) in CHO and CHO-MG* Cells in the Presence and Absence of AG<sup>a,b,c,d</sup>

<table>
<thead>
<tr>
<th>Compound</th>
<th>CHO-MG* IC&lt;sub&gt;50&lt;/sub&gt; (µM) w/ AG</th>
<th>CHO IC&lt;sub&gt;50&lt;/sub&gt; (µM) w/ AG</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ratio&lt;sup&gt;c&lt;/sup&gt; w/ AG</th>
<th>CHO-MG* IC&lt;sub&gt;50&lt;/sub&gt; (µM) w/o AG</th>
<th>CHO IC&lt;sub&gt;50&lt;/sub&gt; (µM) w/o AG</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ratio&lt;sup&gt;c&lt;/sup&gt; w/o AG</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a&lt;sup&gt;a&lt;/sup&gt; (Ant44)</td>
<td>13.7 (±1.3)</td>
<td>0.32 (±0.01)</td>
<td>42.8</td>
<td>2.2 (±0.1)</td>
<td>1.5 (±0.02)</td>
<td>1.4</td>
</tr>
<tr>
<td>5b&lt;sup&gt;a&lt;/sup&gt; (Ant44NMe)</td>
<td>10.72 (±1.2)</td>
<td>2.8 (±0.2)</td>
<td>3.8</td>
<td>11.3 (±2.2)</td>
<td>2.1 (±0.06)</td>
<td>5.4</td>
</tr>
<tr>
<td>6a&lt;sup&gt;b&lt;/sup&gt; (44Ant44)</td>
<td>&gt;100</td>
<td>0.028 (±0.001)</td>
<td>&gt;3571</td>
<td>8.4 (±0.7)</td>
<td>4.0 (±0.3)</td>
<td>2.1</td>
</tr>
<tr>
<td>6b&lt;sup&gt;d&lt;/sup&gt; (MeN44Ant44NMe)</td>
<td>&gt;100</td>
<td>0.083 (±0.004)</td>
<td>&gt;1204</td>
<td>&gt;100</td>
<td>0.084 (±0.002)</td>
<td>&gt;1190</td>
</tr>
<tr>
<td>7a&lt;sup&gt;d&lt;/sup&gt; (44Nap44)</td>
<td>&gt;100</td>
<td>0.022 (±0.002)</td>
<td>&gt;4545</td>
<td>52.1 (±7.5)</td>
<td>5.5 (±0.5)</td>
<td>9.5</td>
</tr>
<tr>
<td>7b&lt;sup&gt;b&lt;/sup&gt; (MeN44Nap44NMe)</td>
<td>&gt;100</td>
<td>0.044 (±0.002)</td>
<td>&gt;2272</td>
<td>&gt;100</td>
<td>0.039 (±0.001)</td>
<td>&gt;2564</td>
</tr>
<tr>
<td>8a&lt;sup&gt;b&lt;/sup&gt; (44Bn44)</td>
<td>19.6 (±0.8)</td>
<td>0.027 (±0.001)</td>
<td>727</td>
<td>56.5 (±3.5)</td>
<td>10.3 (±0.9)</td>
<td>5.5</td>
</tr>
<tr>
<td>8b&lt;sup&gt;b&lt;/sup&gt; (MeN44Bn44NMe)</td>
<td>51.5 (±2.6)</td>
<td>0.030 (±0.001)</td>
<td>1715</td>
<td>54.0 (±2.4)</td>
<td>0.041 (±0.002)</td>
<td>1316</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cells were incubated for 48 h at 37°C with the respective conjugate. <sup>b</sup>1 mM AG was incubated with cells for 24 h prior to drug addition. <sup>c</sup>The ratio denotes the (CHO-MG*/CHO) IC<sub>50</sub> ratio, a measure of PTS selectivity. <sup>d</sup>All experiments were done in triplicate.

As reported earlier,<sup>3g</sup> dramatic differences in CHO and CHO-MG* cytotoxicity (Table I-1) were observed for 5a, however, the difference was not as great as the original CHO-MG line used (e.g., CHO-MG/CHO IC<sub>50</sub> ratio of 5a: 148, CHO-MG*/CHO IC<sub>50</sub> ratio of 5a: 43). This slightly modified CHO-MG* cell line still showed less sensitivity to the polyamine conjugate drugs than CHO, however, not to the extent as the original cell line, CHO-MG. This led to the
determination of the IC\textsubscript{50} values of all drugs previously tested in this modified CHO-MG* cell line to facilitate comparisons.

This new CHO screen again showed a dramatic increase in PTS selectivity when a second polyamine message was attached to the cytotoxic (aryl) core (e.g. CHO-MG*/CHO IC\textsubscript{50} ratio of 6a: >3571, CHO-MG*/CHO IC\textsubscript{50} ratio of 5a: 43). This was expected as an increase in the number of appended polyamine messages should increase the drug conjugates’ ability to gain entry via the PTS. As observed earlier\textsuperscript{12}, however, adding a third polyamine message did not further increase in PTS selectivity, but instead showed a tremendous decrease in PTS selectivity for compound 32 (CHO-MG*/CHO IC\textsubscript{50} ratio of 1). While compound 32 was found to be an inferior PTS ligand, it would be found to be an excellent PTS inhibitor, which will be discussed later in Chapter 2.

![Structure of 32 (Trimer44)](image)

**Figure I-4. Structure of 32 (Trimer44)**

Despite these unprecedented PTS selectivities that were found for the di-substituted polyamine conjugates, they were found to have one distinct limitation. This limitation was found to be their sensitivity to serum amine oxidase. Aminoguanidine\textsuperscript{13} (AG) is a known inhibitor of serum amine oxidases and is routinely added (1 mM) during the cell culture experiments to avoid
polyamine drug degradation by the serum oxidase. Experiments performed in the absence of AG showed a severe decrease in PTS selectivity (e.g. CHO-MG*/CHO IC$_{50}$ ratio with AG of 6a: $>3571$, CHO-MG*/CHO IC$_{50}$ ratio without AG of 6a: 2.1). However, $N$-methyl derivative 5b showed that metabolic stability could be achieved via $N$-methylation, (CHO-MG*/CHO IC$_{50}$ ratio with AG=3.8, CHO-MG*/CHO IC$_{50}$ ratio without AG=5.4).

By design, compounds 6b-8b combined the best features of the di-substituted platforms with the $N$-methylation strategy. Rewardingly, compounds 6b-8b retained their high selectivity in targeting the PTS with and without the AG additive. For example, each of the $N$-methylated derivatives (5b, 6b, 7b, and 8b) in Table I-1 retained their PTS targeting property even in the absence of AG. Compounds 6b-8b proved that a polyamine based drug conjugate could be designed to be metabolically stable while at the same time being highly PTS selective.
Figure I-5. NCI 60 cell-line screen of 6a (44Ant44)\textsuperscript{a}

\textsuperscript{a}High cytotoxicity was observed in compounds with bars extending to the right
Figure I-6. NCI 60 cell-line screen of 6b (MeN44Ant44NMe)\textsuperscript{a}  

\textsuperscript{a} High cytotoxicity was observed in compounds with bars extending to the right.
MALME-3 and MALME-3M Studies. IC$_{50}$ Determinations. Once compounds 6a and 6b were evaluated in CHO and CHO-MG*, these compounds were sent to NCI to conduct its 60 cell-line screen to determine if these compounds could also be potent anti-cancer agents for human cancer cell lines. Due to the robotic aspects of the NCI screen, AG was not added during their screens of 6a and 6b. This screen showed that 6a was toxic to a wide range of different cancer cells and thus was chosen to advance to the hollow fiber tube assay. While this finding was interesting in its own right, it likely is an artifact of drug degradation. An even more interesting finding was the incredible selectivity 6b seemed to possess for the MALME-3M cell line. While 6b inhibited growth for some of the cell lines, it was only the MALME-3M cell line where it was seen to both inhibit growth and kill cells.

The sensitivity of the MALME-3M cell line to 6b was reconfirmed by NCI through a 5-dose assay where a dose dependent response was observed. Armed with this insight, the MALME-3M (melanoma) cell line and its non-cancerous counterpart MALME-3 were purchased from ATCC. These matched cell lines provided a method to assess PTS selectivity for the cancer line (MALME-3M) over the non-cancerous cell line (MALME-3). These results obtained for 5-8 are shown in Table I-2.
**Table I-2.** Biological Evaluation of Polyamine Derivatives (5-8) in MALME-3 and MALME-3M Cells in the Presence of AG<sub>a,b,c,d</sub>

<table>
<thead>
<tr>
<th>Compound</th>
<th>MALME-3 IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>MALME-3M IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ratio&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a (Ant44)</td>
<td>0.83 (±0.03)</td>
<td>0.27 (±0.01)</td>
<td>3.1</td>
</tr>
<tr>
<td>5b (Ant44NMe)</td>
<td>0.62 (±0.01)</td>
<td>0.45 (±0.01)</td>
<td>1.4</td>
</tr>
<tr>
<td>6a (44Ant44)</td>
<td>0.69 (±0.02)</td>
<td>0.017 (±0.001)</td>
<td>41</td>
</tr>
<tr>
<td>6b (MeN44Ant44NMe)</td>
<td>1.00 (±0.01)</td>
<td>0.062 (±0.002)</td>
<td>16</td>
</tr>
<tr>
<td>7a (44Nap44)</td>
<td>1.27 (±0.09)</td>
<td>0.018 (±0.001)</td>
<td>71</td>
</tr>
<tr>
<td>7b (MeN44Nap44NMe)</td>
<td>0.82 (±0.06)</td>
<td>0.014 (±0.001)</td>
<td>59</td>
</tr>
<tr>
<td>8a (44Bn44)</td>
<td>0.09 (±0.01)</td>
<td>0.005 (±0.0002)</td>
<td>18</td>
</tr>
<tr>
<td>8b (MeN44Bn44NMe)</td>
<td>0.02 (±0.001)</td>
<td>0.01 (±0.0001)</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cells were incubated for 96 h at 37°C with the respective conjugate. <sup>b</sup>1 mM AG was determined to be non-toxic and incubated with MALME-3M and MALME-3 cells for 24 h prior to drug addition. This facilitated comparisons between the drugs tested. <sup>c</sup>The ratio denotes the (MALME-3/MALME-3M) IC<sub>50</sub> ratio, a measure of selectivity. <sup>d</sup>All experiments were done in triplicate using RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin.

When compounds 5-8 were tested in MALME-3M and MALME-3, it was discovered that a 96 h viability assay was necessary due to the slow growth rates of MALME-3M (doubling time=48 h) and MALME-3 (doubling time=96 h) when using the media described for Table I-2. With this assay in place, compounds 5-8 all showed high cytotoxicity (IC<sub>50</sub>&lt;500 nM) in MALME-3M, with the di-substituted platforms (6-8) proving to be the most potent as seen in Table I-2. It was also seen, with the exception of compound 7b, that the non-methylated
derivatives (5a, 6a, and 8a) all showed enhanced toxicity over their methylated counterparts in the presence of AG. The aryl core of each compound also demonstrated a significant effect as the benzylic compounds (8a and 8b) proved to be the most toxic, while the toxicities for the anthryl and naphthyl derivatives were very similar. As expected, the mono-substituted compounds 5a and 5b were significantly less toxic in MALME-3M and exhibited low selectivity for the MALME-3M cell line.

Despite the enhanced toxicity of 8a and 8b in MALME-3M, they demonstrated the poorest selectivity for the MALME-3M cell line over the MALME-3 cell line in the di-substituted series. This result is consistent with findings observed in CHO/CHO-MG*, where the benzylic derivatives (8a and 8b) also exhibited the lowest PTS selectivity. This enhanced toxicity, yet modest of selectivity could be attributed to the benzylic core being more prone to metabolism, and being degraded into toxic metabolites. Prior work showed that the mono-substituted benzyl-homospermidine motif was converted to free homospermidine, whereas the naphthyl and anthryl derivatives were dramatically less prone to degradation.

In the present studies, the anthryl and naphthyl cores (6-7) demonstrated increased selectivity for MALME-3M over MALME-3. A surprising find, however, was that 6b showed a significantly lower selectivity than its non-methylated anthryl derivative, 6a, and also lower than its naphthyl derivative, 7b. This finding for compounds 6a, 7a, and 7b was very interesting, and could be attributed to enhanced uptake of polyamines through the PTS of cancer cells (i.e. MALME-3M) when compared to the lower level of uptake for non-cancerous cells (i.e.
MALME-3). The hypothesis that these toxicities were attributed to uptake through the PTS was tested in a series of spermidine (Spd) rescue experiments shown in Figures I-6 through I-11.

**Figure I-7.** Ability of Spd to rescue MALME-3M and MALME-3 cells treated with compound 6a (44Ant44)\(^{a,b,c,d}\)

\(^a\)Cells were incubated for 96 h at 37°C with compound 6a at 0.02 µM (MALME-3M) and 0.6 µM (MALME-3). \(^b\)1 mM AG was determined to be non-toxic and was incubated with cells for 24 h prior to drug addition. \(^c\)Control is no drug control. \(^d\)All experiments were done in triplicate using RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin.
Figure I-8. Ability of Spd to rescue MALME-3M and MALME-3 cells treated with compound 6b (MeN44Ant44NMe)\textsuperscript{a,b,c,d}

\textsuperscript{a}Cells were incubated for 96 h at 37°C with compound 6b at 0.06 µM (MALME-3M) and 1 µM (MALME-3). \textsuperscript{b}1 mM AG was determined to be non-toxic and was incubated with cells for 24 h prior to drug addition. \textsuperscript{c}Control is no drug control. \textsuperscript{d}All experiments were done in triplicate using RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin.
Figure I-9. Ability of Spd to rescue MALME-3M and MALME-3 cells treated with compound 7a (44Nap44)\textsuperscript{a,b,c,d}

\textsuperscript{a}Cells were incubated for 96 h at 37°C with compound 7a at 0.02 µM (MALME-3M) and 1 µM (MALME-3). \textsuperscript{b} 1 mM AG was determined to be non-toxic and was incubated with cells for 24 h prior to drug addition. \textsuperscript{c} Control is no drug control. \textsuperscript{d} All experiments were done in triplicate using RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin.
Figure I-10. Ability of Spd to rescue MALME-3M and MALME-3 cells treated with compound 7b (MeN44Nap44NMe)\textsuperscript{a,b,c,d}

\textsuperscript{a}Cells were incubated for 96 h at 37°C with compound 7b at 0.02 µM (MALME-3M) and 0.8 µM (MALME-3). \textsuperscript{b}1 mM AG was determined to be non-toxic and was incubated with cells for 24 h prior to drug addition. \textsuperscript{c}Control is no drug control. \textsuperscript{d}All experiments were done in triplicate using RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin.
Figure I-11. Ability of Spd to rescue MALME-3M and MALME-3 cells treated with 8a (44Bn44)a,b,c,d

Cells were incubated for 96 h at 37°C with compound 8a at 0.01 µM (MALME-3M) and 0.1 µM (MALME-3). b 1 mM AG was determined to be non-toxic and was incubated with cells for 24 h prior to drug addition. c Control is no drug control. d All experiments were done in triplicate using RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin.
Figure I-12. Ability of Spd to rescue MALME-3M and MALME-3 cells treated with 8b (MeN44Bn44NMe) \(^{a,b,c,d}\)

\(^a\)Cells were incubated for 96 h at 37°C with compound 8b at 0.01 µM (MALME-3M) and 0.02 µM (MALME-3). \(^b\)1 mM AG was determined to be non-toxic and was incubated with cells for 24 h prior to drug addition. \(^c\)Control is no drug control. \(^d\)All experiments were done in triplicate using RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin.

**Spd Rescue Experiments.** As stated above, in an effort to determine if the cytotoxicities seen for compounds 6-8 were polyamine transport related, MALME-3M and MALME-3 were dosed near the IC\(_{50}\) value of each drug. These cells were also treated with increasing amounts of Spd to see if it could outcompete compounds 6-8 for cellular entry and thus rescue the cells. Compounds 6-7 each showed that exogenous Spd was able to significantly rescue (~90% viability) MALME-3M and MALME-3 cells from the cytotoxicity of these compounds. This supports the premise that these compounds gain access to these cells primarily via the PTS. When exogenous Spd was added to 8a and 8b (Figures I-10 and I-11 respectively), a much lower
level of rescue was observed in MALME-3M (<80% viability). However, complete rescue was observed in MALME-3. Since the viability of MALME-3M cells could be partially rescued by Spd, compounds 8a and 8b may gain access via the PTS, along with non-PTS related pathway(s). These results correlate well with the finding that 6 and 7 (anthryl and naphthyl cores respectively) prefer MALME-3M and its upregulated PTS over MALME-3. In contrast, 8a and 8b did not demonstrate the same degree of selectivity.

It is also interesting to note that while Spd was able to rescue both MALME-3M and MALME-3 from the toxic effects of 6-8, a large excess of Spd was necessary. As seen in Figures I-7 through I-12, between 10 and 100 µM Spd was needed to get significant rescue. Since compounds 6-8 exhibited ~50% viability at very low doses (0.01 µM to 0.06 µM for MALME-3M, and 0.02 µM to 1 µM for MALME-3), a very large molar excess of Spd was necessary to outcompete these compounds. These results can be partially attributed to significant differences in $K_i$ values (lower $K_i$=better binding affinity) observed in L1210 cells for compounds 6a ($K_i$=0.39 µM), 7a ($K_i$=0.17 µM), and 8a ($K_i$=0.52 µM) as compared to Spd ($K_i$=2.46 µM). These findings suggest that these di-substituted compounds have a much higher affinity for the PTS as compared to Spd, thus requiring a large molar excess of Spd to outcompete 6-8 for the PTS.

The Spd rescue results coupled with the high sensitivity of MALME-3M to compounds 5-8, leads to three different hypotheses. One hypothesis is that these compounds are toxic primarily due to their ability to exploit the upregulated PTS of cancer cells, allowing them to deliver their toxic cargo. The other hypothesis is that these alkylated polyamines led to an
increase in SSAT activity, which in turn caused an increase in native polyamine metabolism, followed by an increase in export, which ultimately leads to cell death. This second hypothesis is predicated on work done by Porter et.al., who showed that when MALME-3M was treated with alkylated polyamines, a dramatic increase in SSAT activity was observed, leading to decreased levels of native polyamine pools.\textsuperscript{9, 11a, b, 17b, 17d, 23} The third hypothesis is that a combination effect of decreased levels of native polyamines and increased levels of the drug causes the MALME-3M cells to be more sensitive to cell death, thus allowing the cytotoxic cargo to have a more profound effect. Future studies are needed to address these remaining questions.
<table>
<thead>
<tr>
<th>Compound</th>
<th>MALME-3M IC$_{50}$ (µM) w/ AG</th>
<th>MALME-3M IC$_{50}$ (µM) w/o AG</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a (Ant44)</td>
<td>0.48 (±0.03)</td>
<td>0.29 (±0.03)</td>
</tr>
<tr>
<td>5b (Ant44NMe)</td>
<td>0.60 (±0.06)</td>
<td>0.62 (±0.06)</td>
</tr>
<tr>
<td>6a (44Ant44)</td>
<td>0.088 (±0.004)</td>
<td>0.29 (±0.005)</td>
</tr>
<tr>
<td>6b (MeN44Ant44NMe)</td>
<td>0.125 (±0.004)</td>
<td>0.16 (±0.02)</td>
</tr>
<tr>
<td>7a (44Nap44)</td>
<td>0.043 (±0.001)</td>
<td>0.43 (±0.06)</td>
</tr>
<tr>
<td>7b (MeN44Nap44NMe)</td>
<td>0.104 (±0.002)</td>
<td>0.168 (±0.006)</td>
</tr>
<tr>
<td>8a (44Bn44)</td>
<td>0.0102 (±0.0002)</td>
<td>0.4 (±0.02)</td>
</tr>
<tr>
<td>8b (MeN44Bn44NMe)</td>
<td>0.007 (±0.0001)</td>
<td>0.007 (±0.0004)</td>
</tr>
</tbody>
</table>

$^a$Cells were incubated for 48 h at 37°C with the respective conjugate. $^b$1 mM AG was determined to be non-toxic and incubated with MALME-3M cells for 24 h prior to drug addition. $^c$All experiments were done in triplicate using RPMI 1640 supplemented with 10% Nu-Serum IV and 1% penicillin/streptomycin.

**IC$_{50}$ Determinations in the Presence and Absence of AG.** Prior to the IC$_{50}$ determinations for MALME-3M and MALME-3 in a 96 h assay, IC$_{50}$ values were determined for MALME-3M in a 48 h assay using 10% Nu-Serum IV. In order to verify the enhanced metabolic stability of methylated compounds 5b, 6b, 7b, and 8b over their non-methylated counter parts (5a, 6a, 7a, and 8a) in MALME-3M, their IC$_{50}$ values were determined with and without AG. While the mono-substituted compounds 5a and 5b showed little to no difference in IC$_{50}$ values in the absence of AG, a dramatic change was seen for the di-substituted compounds (6-8). While the
methylated compounds demonstrated the same IC$_{50}$ values in the presence and absence of AG, a profound increase in IC$_{50}$ value (4 to 70 fold) was observed for the non-methylated derivatives in the absence of AG. This trend was also observed in CHO (Table I-1), where an absence of AG led to increased IC$_{50}$ values for the non-methylated derivatives and not the methylated derivatives. This finding suggests that in future in vivo studies compounds 6b, 7b and 8b could be dosed without the addition of AG.

**I.4 Summary**

Studies in CHO and CHO-MG* illustrated that a combination of a di-substituted platform coupled with terminal N-methylation of the appended polyamine chains is a hyperselective PTS design with improved metabolic stability. These results have also demonstrated that the aryl core plays a key role with the anthryl or naphthyl cores being the most PTS selective design. These trends were also observed in the MALME-3M/MALME-3 screen, where enhanced metabolic stability was observed, and the anthryl and naphthyl systems gave the best selectivity. The ability to significantly rescue MALME-3M cells with Spd for compounds 6a, 6b, 7a, and 7b further points to their utilization of the PTS and demonstrates the PTS-mediated cell-selective drug delivery in a human melanoma line, MALME-3M over its normal counterpart MALME-3.

**I.5 Experimental**

**I.5.1 Materials.**

Silica gel (32-63 µm) and chemical reagents were purchased from commercial sources and used without further purification. All solvents were distilled prior to use. All reactions were carried out under an N$_2$ atmosphere. $^1$H and $^{13}$C spectra were recorded at 400 or 75 MHz,
respectively. TLC solvent systems were listed as volume percents, and NH₄OH referred to concentrated aqueous NH₄OH. All tested compounds provided satisfactory elemental analyses.

I.5.2 Biological Studies.

CHO and CHO-MG* cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Note: the media must contain L-proline (2µg/mL) for proper growth of the CHO-MG* cells. MALME-3M and MALME-3 cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin for 96 h experiments, and RPMI 1640 medium supplemented with 10% Nu-Serum IV and 1% penicillin/streptomycin for 96 h experiments. All cells were grown at 37°C under a humidified 5% CO₂ atmosphere. Aminoguanidine (1 mM) was added to the growth medium to prevent oxidation of the drugs by the enzyme (bovine serum amine oxidase) present in calf serum. Cells in early to mid-log phase were used.

IC₅₀ Determinations. Cell growth was assayed in sterile 96-well microtiter plates (Costar 3599, Corning, NY, USA). CHO and CHO-MG* cells were plated at 10,000 cells/mL. MALME-3M and MALME-3 cells were plated at 5,000 cells/mL. Drug solutions (10 µL per well) of appropriate concentration were added after an overnight incubation for each CHO cell line (90 µL). 48 h MALME-3M experiments were conducted using 10% Nu-Serum IV, and 96 h MALME-3M and MALME-3 experiments were conducted using 10% FBS. After exposure to the drug for 48 h (CHO, CHO-MG*, and MALME-3M) or 96 h (MALME-3M and MALME-3), cell growth was determined by measuring formazan formation from 3-(4,5-dimethylthiazol-2-
yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium, inner salt (MTS) using a SynergyMx Biotek microplate reader for absorbance (490 nM) measurements.\textsuperscript{24}

**I.5.3 Synthetic Procedures and Characterization**

\textbf{N\textsubscript{1},N\textsubscript{1}'-(Anthracene-9,10-diylbis(methylene))bis(N\textsubscript{4}-(4-(methylamino)butyl)butane-1,4-diamine), 6b.} Orange solid (93\%); \textsuperscript{1}H NMR (D\textsubscript{2}O) δ 8.33 (dd, 4H), 7.82 (dd, 4H), 5.18 (s, 4H), 3.38 (t, 4H), 3.16 (12H), 2.76 (s, 6H), 1.84 (m, 16H); \textsuperscript{13}C NMR (D\textsubscript{2}O) δ 185.5, 129.9, 127.7, 125.1, 124.0, 48.2, 47.5, 46.9, 46.8, 42.9, 32.7, 22.9, 22.8, 22.7, 22.6. HRMS (FAB) \textit{m/z} calc for C\textsubscript{34}H\textsubscript{56}N\textsubscript{6} (M + H)\textsuperscript{+} 549.4566; found 549.4639. Elemental Analysis: C\textsubscript{34}H\textsubscript{56}N\textsubscript{6}Cl\textsubscript{6}•1.25H\textsubscript{2}O theory C: 51.69, H: 8.23, N: 10.64; found C: 51.66, H: 8.05, N: 10.49.

\textbf{N\textsubscript{1},N\textsubscript{1}'-(Naphthalene-1,4-diylbis(methylene))bis(N\textsubscript{4}-(4-(methylamino)butyl)butane-1,4-diamine), 7b.} Yellow solid (98\%); \textsuperscript{1}H NMR (D\textsubscript{2}O) δ 8.25 (dd, 2H), 7.83 (dd, 2H), 7.75 (s, 2H), 4.85 (s, 4H), 3.34 (t, 4H), 3.15 (t, 12H), 2.75 (s, 3H), 1.83 (m, 16H); \textsuperscript{13}C NMR (D\textsubscript{2}O) δ 134.3, 132.2, 131.5, 130.9, 126.8, 51.2, 51.0, 50.2, 49.9, 35.7, 25.9, 25.8, 25.6. HRMS (FAB) \textit{m/z} calc for C\textsubscript{30}H\textsubscript{54}N\textsubscript{6} (M + H)\textsuperscript{+} 499.4410; found 499.4479. Elemental Analysis: C\textsubscript{30}H\textsubscript{54}N\textsubscript{6}Cl\textsubscript{6}•2H\textsubscript{2}O theory C: 47.82, H: 8.56, N: 11.15; found C: 47.85, H: 8.35, N: 10.82.

\textbf{N\textsubscript{1},N\textsubscript{1}'-(1,4-Phenylenebis(methylene))bis(N\textsubscript{4}-(4-(methylamino)butyl)butane-1,4-diamine), 8b.} While solid (99\%); \textsuperscript{1}H NMR (D\textsubscript{2}O) δ 7.61 (s, 4H), 4.28 (s, 4H), 3.21-3.12 (t, 16H), 1.78 (m, 16H); \textsuperscript{13}C NMR (D\textsubscript{2}O) δ 135.0, 133.4, 53.5, 51.1, 49.7, 49.4, 35.6, 25.7, 25.6, 25.5. HRMS (FAB) \textit{m/z} calc for C\textsubscript{26}H\textsubscript{52}N\textsubscript{6} (M + H)\textsuperscript{+} 449.4253; found 449.4326. Elemental Analysis: C\textsubscript{26}H\textsubscript{58}N\textsubscript{6}Cl\textsubscript{6} theory C: 46.79, H: 8.76, N: 12.59; found C: 46.69, H: 8.82, N: 12.33.
(4-hydroxybutyl)carbamic acid tert-butyl ester, 10.\textsuperscript{3f} A solution of 4-amino-1-butanol 9 (5 g, 56.1 mmol) in TEA/MeOH (1:7 v/v, 150 mL) was stirred at 0°C for 10 min. A solution of di-tert-butyl dicarbonate (18.4 g, 84.1 mmol) in MeOH (40 mL) was added dropwise over 10 min. The mixture was stirred for 1 h under N\textsubscript{2} atmosphere. The temperature was allowed to gradually rise to room temperature, and the solution was then stirred overnight. The solution was evaporated under reduced pressure, and the residue was dissolved in CH\textsubscript{2}Cl\textsubscript{2} and washed three times with deionized water. The organic layer was separated, dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated to give 10 as a colorless oil 10 (9.78 g, 92%) that was used in the next step without further purification. R\textsubscript{f}=0.4 (40% acetone/hexane); \textsuperscript{1}H NMR (CDCl\textsubscript{3}) δ 4.96 (br s, 1H, NH), 3.62 (m, 2H, OCH\textsubscript{2}), 3.11 (m, 2H, NCH\textsubscript{2}), 2.49 (m, 1H, OH), 1.55 (m, 4H, CH\textsubscript{2}), 1.41 (s, 9H, CH\textsubscript{3}). Matched literature values.\textsuperscript{25}

Methanesulfonic Acid 4-tert-Butoxycarbonylaminobutyl Ester, 11.\textsuperscript{25} To a solution of the alcohol 10 (9.78 g, 51.4 mmol) and TEA (35.8 mL, 257 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (130 mL) at 0°C, methanesulfonyl chloride (29.5 g, 257 mmol) was added dropwise over 30 min under a N\textsubscript{2} atmosphere. The reaction mixture was stirred at 0°C for 1 h and was slowly warmed to room temperature and stirred overnight under N\textsubscript{2}. The reaction mixture was then cooled to 0°C, and a 4 M NaOH solution (50 mL) was added slowly with vigorous stirring. The organic phase was separated and washed with water (2 x 70 mL). The organic phase was separated, dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}, filtered and concentrated to give the product 11 (12.7 g, 92%) as a clear oil that was used in the next step without further purification. R\textsubscript{f}=0.5 (40% acetone/hexane); \textsuperscript{1}H NMR (CDCl\textsubscript{3}) δ 4.85 (br s, 1H, NH), 4.27 (t, 2H, OCH\textsubscript{2}), 3.16 (m, 2H, NCH\textsubscript{2}), 3.01 (s, 3H, CH\textsubscript{3}), 1.78 (quin, 2H, CH\textsubscript{2}), 1.59 (quin, 2H, CH\textsubscript{2}), 1.43 (s, 9H, CH\textsubscript{3}). Matched literature values.\textsuperscript{25}
[4-(4-Hydroxybutylamino) butyl] carbamic Acid tert-Butyl Ester, 12. The mesylate 11 (12.65 g, 47.2 mmol) and 4-amino-1-butanol (12.62 g, 142 mmol) were dissolved in acetonitrile (30 mL). The mixture was then stirred at 75°C reflux under a N₂ atmosphere overnight. After the confirmation of the disappearance of the mesylate by TLC, the solution was concentrated under reduced pressure. The residue was dissolved in CH₂Cl₂ (30 mL) and washed three times with aqueous sodium carbonate. The organic layer was separated, dried with anhydrous Na₂SO₄, filtered, and concentrated under vacuum to give 12 as a yellow oil (10.3 g; 84%). R₂f=0.33 (1:7:92, NH₄OH/MeOH/CH₂Cl₂; ¹H NMR (CDCl₃) δ 5.05 (br s, 1H, NHCO), 3.54 (t, 2H, OCH₂), 3.07 (m, 2H, BocNCH₂), 2.61 (m, 4H, NCH₂), 1.64 (quin, 4H, CH₂), 1.55 (quin, 4H, CH₂), 1.42 (s, 9H, CH₃). Matched literature values.

(4-tert-Butoxycarbonylaminobutyl)-(4-hydroxybutyl)-carbamic Acid tert-Butyl Ester, 13. A solution of 12 (10.3 g, 39.6 mmol) in triethylamine/MeOH (1:7 v/v, 160 mL) was stirred at 0°C for 10 min. A solution of di-tert-butyl dicarbonate (13.0 g, 56.4 mmol) in MeOH (40 mL) was added dropwise over 10 min. The mixture was stirred for 1 h at 0°C. The temperature was then allowed to gradually rise to room temperature, and the solution was stirred overnight under a N₂ atmosphere. The solution was then evaporated under reduced pressure, and the residue was dissolved in CH₂Cl₂ and washed with deionized water several times. The organic layer was separated, dried over anhydrous Na₂SO₄, filtered, and concentrated to give 13 as an oil. Column chromatography gave 13 as a colorless oil, (8.75 g, 53% over two steps). R₂f=0.35 (0.5:3:96.5 NH₄OH/MeOH/CH₂Cl₂); ¹H NMR (CDCl₃) δ 7.24 (br s, 1H, NH), 3.62 (t, 2H, OCH₂), 3.20 (t, 6H, NCH₂), 2.0 (br s, 1H, OH), 1.6 (m, 8H, CH₂), 1.42 (s, 18H, CH₃). Matched literature values.
4-(4-Amino-butylamino)-butan-1-ol, 14. A solution of Boc-protected 13 (630 mg, 1.75 mmol) was dissolved in absolute ethanol (20 mL) and stirred at 0°C for 10 min. A 4 N HCl solution (35 mL, 140 mmol) was added to the reaction mixture dropwise and stirred at 0°C for 30 min and then at room temperature overnight. The solution was concentrated in vacuo to give 14 as a white solid, (400 mg; 98% yield); $^1$H NMR (D$_2$O) $\delta$ 3.62 (t, 2H, NCH$_2$), 3.18 (m, 6H, CH$_2$), 1.75 (m, 6H, CH$_2$), 1.65 (m, 1H, CH$_2$).

4-{4-[(10-{[4-(4-Hydroxy-butylamino)-butylamino]-methyl}-anthracen-9-ylmethyl)-amino]-butylamino}-butan-1-ol, 15. To a stirred solution of 14 (370 mg, 1.6 mmol) in 25% MeOH/CH$_2$Cl$_2$ (20 mL) and TEA (0.57 mL, 4.0 mmol) was added 9,10-anthracenedicarboxaldehyde (170 mg, 0.72 mmol) under N$_2$. The mixture was stirred at room temperature overnight until the imine formation was complete (by $^1$H NMR). The solvent was removed in vacuo, and the solid residue was dissolved in 50% MeOH/CH$_2$Cl$_2$ (20 mL), and the solution was cooled to 0°C. NaBH$_4$ (164 mg, 4.3 mmol) was added in small portions to the solution, and the mixture was stirred at room temperature overnight. The solvent was removed in vacuo, and the solid residue was dissolved in CH$_2$Cl$_2$ (50 mL) and washed with 10% aqueous Na$_2$CO$_3$ solution (3 x 30 mL). The CH$_2$Cl$_2$ layer was dried over anhydrous Na$_2$SO$_4$, filtered, concentrated to give an oily residue. The oil was purified by flash column chromatography to yield 15 as a pale-yellow oil; (310 mg, 83%) $R_f$=0.25 (3:20:77 NH$_4$OH/MeOH/CH$_2$Cl$_2$; $^1$H NMR (CDCl$_3$) $\delta$ 8.39 (dd, 4H, CH), 7.45 (dd, 4H, CH), 4.65 (s, 4H, CH$_2$), 4.48 (t, 4H, CH$_2$), 2.90 (m, 8H, CH$_2$), 1.60 (m, 16 H, CH$_2$); $^{13}$C NMR (CDCl$_3$) $\delta$ 132.2, 130.2, 125.8, 125.0, 62.6, 50.5, 49.7, 49.5, 32.7, 28.9, 28.0, 27.7. HRMS (FAB) $m/z$ calc for C$_{32}$H$_{50}$N$_4$O$_2$ (H + M)$^+$ 522.3934, found
523.4007. Elemental Analysis: C$_{32}$H$_{50}$N$_4$O$_2$·1 H$_2$O theory C: 71.1, H: 9.70, N: 10.36; found C: 71.55, H: 9.62, N: 10.29.

{10-[(tert-Butoxycarbonyl)-[4-(tert-butoxycarbonyl-(4-hydroxy-butyl)-amino]-butyl]-amino]-methyl]-anthracen-9-ylmethyl}-[4-(tert-butoxycarbonyl-(4-hydroxy-butyl)-amino]-butyl]-carbamic acid tert-butyl ester, 16. A solution of 15 (310 mg, 0.60 mmol) in triethylamine/MeOH (1:7 v/v, 80 mL) was stirred at 0°C for 10 min. A solution of di-tert-butyl dicarbonate (651 mg, 3.0 mmol) in MeOH (20 mL) was added dropwise over 10 min. The mixture was stirred for 1 h under a N$_2$ atmosphere. The temperature was allowed to gradually rise to room temperature, and the solution was stirred overnight. The solution was evaporated under reduced pressure, and the residue was dissolved in CH$_2$Cl$_2$ and washed with deionized water. The organic layer was separated, dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated to give 16 as a colorless oil (501 mg, 91% yield) which was used in the next step without further purification; $^1$H NMR (CDCl$_3$) δ 8.45 (dd, 4H, CH), 7.59 (dd, 4H, CH), 5.59 (s, 4H, CH$_2$), 3.60 (s, 4H, CH$_2$), 2.8 (d, 12H, CH$_2$), 1.4 (m, 36H, CH$_3$); $^{13}$C NMR (CDCl$_3$) δ 155.9, 131.2, 130.0, 125.9, 125.1, 79.8, 79.5, 61.9, 46.5, 44.2, 41.5, 29.8, 28.3, 25.9, 25.8, 25.5, 24.6. HRMS (FAB) $m/z$ calc for C$_{52}$H$_{82}$N$_4$O$_{10}$ (H + M)$^+$ 922.6031, found 922.6104. Elemental Analysis:

C$_{52}$H$_{82}$N$_4$O$_{10}$·1 H$_2$O theory C: 66.36, H: 9.00, N: 5.95; found C: 66.33, H: 9.01, N: 5.83.

Methanesulfonic acid 4-{(tert-butoxycarbonyl)-[4-(tert-butoxycarbonyl-[10-[(tert-butoxycarbonyl-[4-(tert-butoxycarbonyl-(4-methanesulfonyloxy-butyl)-amino]-butyl]-amino)-methyl]-anthracen-9-ylmethyl]-amino]-butyl]-amino}-butyl ester, 17. To a solution of the alcohol 16 (485 mg, 0.53 mmol) and triethylamine (0.6 mL, 4.2 mmol) in CH$_2$Cl$_2$ (20 mL)
at 0°C, methanesulfonyl chloride (483 mg, 4.2 mmol) was added dropwise over 30 min under a N\textsubscript{2} atmosphere. The reaction mixture was stirred at 0°C for 1 h and was slowly warmed to room temperature and stirred overnight under N\textsubscript{2}. The reaction mixture was then cooled to 0°C, and a 1 M NaOH solution (20 mL) was added slowly with vigorous stirring. The organic phase was separated and washed with water (2x15 mL). The organic phase was separated, dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}, filtered and concentrated to give the product \textbf{17} as a colorless oil (500 mg, 88\%) which was used in the next step without further purification, \textit{R}\textsubscript{f}=0.24 (2:98 MeOH/CH\textsubscript{2}Cl\textsubscript{2}); \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \(\delta\) 8.45 (dd, 4H, CH), 7.60 (dd, 4H, CH), 5.58 (s, 4H, CH\textsubscript{2}), 4.23 (s, 6H, CH\textsubscript{3}), 3.10 (d, 12 H, CH\textsubscript{2}), 1.45 (m, 36 H, CH\textsubscript{3}).

\begin{verbatim}
[4-(tert-Butoxycarbonyl]-[10-[(tert-butoxycarbonyl]-[4-[tert-butoxycarbonyl-(4-
methylamino-butyl)-amino]-butyl]-amino)-methyl]-anthracen-9-ylmethyl]-amino]-butyl]-
(4-methylamino-butyl)-carbamic acid tert-butyl ester, \textbf{18}. A solution of 2.0 \textit{M} methylamine in MeOH (6 mL, 12 mmol) and K\textsubscript{2}CO\textsubscript{3} was brought to reflux while stirring. A solution of \textbf{17} (80 mg, 0.074 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (5 mL) was added dropwise over 15 min, and the reaction was stirred overnight at reflux under N\textsubscript{2}. The K\textsubscript{2}CO\textsubscript{3} was filtered off and the filtrate was concentrated under vacuum, and redissolved in CH\textsubscript{2}Cl\textsubscript{2} (50 mL) to precipitate unwanted inorganics, filtered again and concentrated, to give a crude residue. Column chromatography (1:11:88 NH\textsubscript{4}OH/MeOH/CH\textsubscript{2}Cl\textsubscript{2}) yielded 37 mg of \textbf{18} (53\%), \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \(\delta\) 8.42 (dd, 4H, CH), 7.59 (dd, 4H, CH), 5.59 (s, 4H, CH\textsubscript{2}), 2.80 (m, 12 H, CH\textsubscript{2}), 2.42 (s, 3H, CH\textsubscript{3}), 1.45 (m, 36H, CH\textsubscript{3}). Failed elemental analysis
\end{verbatim}
**N-(4-hydroxybutyl)formamide (19).** To a stirred solution of 9 (4.31 g, 48.4 mmol) in EtOH (50 mL) was added ethylformate (5.86 mL, 75.5 mmol) and the mixture was stirred at reflux for 18 hrs under N₂. The solution was evaporated under reduced pressure, and the crude product, 19, was used in the next step without further purification (4.8 g crude mass). ¹H NMR (CDCl₃) δ 8.02 (s, 1H), 3.57 (t, 2H), 3.24 (t, 2H), 1.56 (m, 4H).

**4-Methylamino-butanol-1-ol, 20.**¹⁹ The crude reaction mixture was dissolved in THF (25 mL) and added to a suspension of LiAlH₄ (5.50 g, 145 mmol) in THF (50 mL) dropwise under a drying tube while stirring. The reaction mixture was brought to reflux and monitored by TLC (20% EtOH/80% CHCl₃) and ¹H NMR (CDCl₃). After 2 hours, the starting material was consumed, and H₂O (4.16 mL) was added to the cooled reaction mixture, followed by 4 M NaOH (4.16 mL) and H₂O (12.5 mL) while stirring vigorously. The precipitate was then removed by filtration, and the filtrate concentrated in vacuo. The residue was redissolved in CHCl₃, dried over Na₂SO₄, filtered and concentrated under reduced pressure to give 20 as a colorless oil (2.76 g, 56%); ¹H NMR (CDCl₃) δ 3.74 (br, 2H), 3.57 (t, 2H), 2.62 (t, 2H), 2.43 (s, 3H), 1.50-1.75 (m, 4H). Matched literature values.¹⁹

**(4-Hydroxy-butyl)-methyl-carbamic acid tert-butyl ester, 21.**²⁰ A solution of 20 (2.76 g, 26.8 mmol) in TEA/MeOH (1:7 v/v, 100 mL) was stirred at 0°C for 10 min. A solution of di-tert-butyl dicarbonate (8.76 g, 40.2 mmol) in MeOH (20 mL) was added dropwise over 10 min. The mixture was stirred for 1 h under N₂ atmosphere. The temperature was allowed to gradually rise to room temperature, and the solution was stirred overnight. The solution was evaporated under reduced pressure, and the residue was dissolved in CH₂Cl₂ and washed with deionized
water. The organic layer was separated, dried over anhydrous Na₂SO₄, filtered, and concentrated to give a colorless oil 21 that was used in the next step without further purification (4.54 g, 84%); ¹H NMR (CDCl₃) δ 3.66 (t, 2H), 3.23 (t, 2H), 2.83 (s, 3H), 1.78 (br s, 1H), 1.51-1.60 (m, 4H), 1.44 (s, 9H). Matched literature values.²⁰

Methanesulfonic acid 4-(tert-butoxycarbonyl-methyl-amino)-butyl ester, 22. To a solution of the alcohol 21 (4.54 g, 22.24 mmol) and TEA (15.5 mL, 111 mmol) in CH₂Cl₂ (60 mL) at 0°C, methanesulfonyl chloride (12.73 g, 111 mmol) was added dropwise over 30 min under a N₂ atmosphere. The reaction mixture was stirred at 0°C for 1 hr and was slowly warmed to room temperature and stirred overnight under N₂. The reaction mixture was then cooled to 0°C, and a 1 M NaOH solution (500 mL) was added slowly with vigorous stirring. The organic phase was separated and washed with deionized water. The organic phase was again separated, dried over anhydrous Na₂SO₄, filtered and concentrated to give the product 22 as a colorless oil that was used in the next step without further purification (6.03 g, 96%); ¹H NMR (CDCl₃) δ 4.25 (t, 2H), 3.28 (t, 2H), 3.02 (s, 3H), 2.84 (s, 3H), 1.78 (m, 2H), 1.66 (m, 2H), 1.44 (s, 9H).

[4-(4-Amino-butylamino)-butyl]-methyl-carbamic acid tert-butyl ester, 23.
Putrescine (9.45 g, 107 mmol) was dissolved in acetonitrile (200 mL) with K₂CO₃ (14.79 g, 107 mmol) and stirred under N₂. Mesylate 22 (6.03 g, 21.4 mmol) dissolved in acetonitrile (60 mL) was added dropwise over 30 min while stirring under N₂. After 30 min, the reaction mixture was brought to reflux and stirred overnight. The reaction mixture was then cooled, solid K₂CO₃ was filtered off and the filtrate concentrated in vacuo. The residue was redissolved in CH₂Cl₂ (200 mL) and washed six times with sat. aqueous Na₂CO₃ to remove the unreacted putrescine. The
organic layer was then dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated to give the product 23 as a clear oil that was used in the next step without further purification, yield: 5.31 g (90%); $^1$H NMR (CDCl$_3$) $\delta$ 3.21 (t, 2H), 2.84 (s, 3H), 2.71 (t, 2H), 2.62 (t, 4H), 1.40-1.60 (m, 17H).

$[4-\text{-(4-}\{(10-\{(4-\text{(tert-Butoxycarbonyl-methyl-amino)-butylamino)}\text{-butylamino)}\text{-methyl)-anthracen-9-ylmethy}}lamino)\text{-butyl]-methyl-carbamic acid tert-butyl ester}, \text{24.} \text{To a stirred solution of 23 (100 mg, 0.37 mmol) in 25% MeOH/CH}_2\text{Cl}_2 \text{(11.5 mL) and TEA (0.25 mL, 0.831 mmol) under N}_2 \text{was added a solution of anthracene-9,10-dicarboxaldehyde (39.1 mg, 0.166 mmol) in 25% MeOH/CH}_2\text{Cl}_2 \text{(8.5 mL). The mixture was stirred at room temperature overnight until the imine formation was complete (by $^1$H NMR). The solvent was removed in vacuo and the solid residue was dissolved in 50% MeOH/CH}_2\text{Cl}_2 \text{(15 mL), and the solution was cooled to 0°C. NaBH}_4 \text{(83.0 mg, 2.19 mmol) was added in small portions to the solution, and the mixture was stirred at room temperature overnight. The solvent was removed in vacuo, and the solid residue was dissolved in CH}_2\text{Cl}_2 \text{(25 mL) and washed with 10 wt% aqueous Na}_2\text{CO}_3 \text{solution (3x15 mL). The organic layer was dried over anhydrous Na}_2\text{SO}_4, \text{filtered and concentrated to give an oily residue. A pale red oil 24 was obtained and taken onto the next step without further purification (110 mg crude mass); $^1$H NMR (CDCl$_3$) $\delta$ 8.39 (dd, 4H, CH), 7.58 (dd, 4H, CH), 4.72 (dd, 4H, CH$_2$), 3.19 (t, 4H, CH$_2$), 2.92 (s, 6H, CH$_3$), 2.84 (t, 8H, CH$_2$), 2.61 (m, 8H, CH$_2$), 1.85 (br s, 4H, NH), 1.41-1.65 (m, 52H).} \]
ylmethyl)-tert-Butoxycarbonyl-amino]-butyl]-tert-Butoxycarbonyl-amino)-butyl]-methyl-carbamic acid tert-butyl ester, 25. A solution of 24 (110 mg crude mass from previous step) in TEA/MeOH (1:7 v/v, 50 mL) was stirred at 0°C for 15 min. A solution of di-tert-butyl dicarbonate (184 mg, 0.844 mmol) in MeOH (10 mL) was added dropwise over 10 min. The mixture was stirred for 1 h under a N₂ atmosphere. The temperature was allowed to gradually rise to room temperature, and the solution was stirred overnight. The solution was evaporated under reduced pressure, and the residue was dissolved in CH₂Cl₂ and washed with deionized water. The organic layer was separated, dried over anhydrous Na₂SO₄, filtered, and concentrated to give a clear oil. The oil was purified by flash column chromatography R<sub>f</sub> = 0.21 (20% acetone/80% hexane) to afford 25 (80 mg, 42% over 2 steps); <sup>1</sup>H NMR (CDCl₃) δ 8.44 (dd, 4H, CH), 7.57 (dd, 4H, CH₂), 5.58 (dd, 4H, CH₂), 3.19 (s, 6H, CH₃), 2.91-3.11 (t, 6H, CH₂), 2.80 (m, 12H, CH₂), 1.85 (bs, 2H, NH), 1.10-1.75 (m, 68H). HRMS (FAB) m/z calc for C<sub>64</sub>H<sub>104</sub>N<sub>6</sub>O<sub>12</sub> (H + M)<sup>+</sup> 1149.5436, found 1148.7604. Elemental Analysis: C<sub>64</sub>H<sub>104</sub>N<sub>6</sub>O<sub>12</sub>·2 H₂O theory C: 64.84, H: 9.18, N: 7.09; found C: 64.89, H: 9.13, N: 6.81.

[4-(4-Benzylxycarbonylamino-butylamino)-butyl]-methyl-carbamic acid tert-butyl ester, 26. (4-Amino-butyl)-carbamic acid benzyl ester (2.21 g, 9.93 mmol) was dissolved in acetonitrile (100 mL) with K₂CO₃ (6.40 g, 46.3 mmol) and stirred under N₂. Mesylate 22 (2.35 g, 8.33 mmol) dissolved in acetonitrile (25 mL) was added dropwise over 30 min while stirring under N₂. After 30 min of stirring, the reaction mixture was brought to reflux and run overnight. The reaction mixture was then cooled, and solid K₂CO₃ was filtered off and the filtrate was concentrated in vacuo. The residue was redissolved in CH₂Cl₂ (100 mL) and washed with sat. Na₂CO₃ (aq) (3x60 mL). The organic layer was then separated, dried over anhydrous Na₂SO₄,
filtered, and concentrated to give the product 26 as a colorless oil. The oil was purified by flash column chromatography $R_f=0.29$ (0.2% NH$_4$OH/15% iPrOH/84.8% CH$_2$Cl$_2$) to afford 26 (760 mg, 20%); $^1$H NMR (CDCl$_3$) $\delta$ 7.28 (m, 5H), 5.09 (s, 2H), 3.19 (s, 4H), 2.80 (s, 3H), 2.59 (t, 4H), 1.4-1.6 (m, 17H).

(4-Benzoxycarbonylamino-butyl)-[4-(tert-butoxycarbonyl-methyl-amino)-butyl]-carbamic acid tert-butyl ester, 27. A solution of 26 (760 mg, 1.87 mmol) in TEA/MeOH (1:7 v/v, 100 mL) was stirred at 0°C for 15 min. A solution of di-tert-butyl dicarbonate (407 mg, 1.87 mmol) in MeOH (20 mL) was added dropwise over 10 min. The mixture was stirred for 1 h under N$_2$ atmosphere at room temperature. The solution was evaporated under reduced pressure, and the residue was dissolved in CH$_2$Cl$_2$ (50 mL) and washed with deionized water (3x30 mL). The organic layer was separated, dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated to give a colorless oil 27 (940 mg, 99%). The oil was taken to the next step without further purification. $^1$H NMR (CDCl$_3$) $\delta$ 7.28 (m, 5H), 5.10 (s, 2H), 3.18 (s, 8H), 2.81 (s, 3H), 1.4-1.6 (m, 26 H).

tert-Butyl (4-((4-aminobutyl)(tert-butoxycarbonyl)amino)butyl)(methyl)carbamate, 28. To a stirred solution of 23 (5.54 g, 20.3 mmol) and anhydrous Na$_2$SO$_4$ (23 g, 160 mmol) in 25% MeOH/CH$_2$Cl$_2$ (500 mL) at room temperature was added salicylaldehyde (2.47 g, 20.3 mmol) dropwise over 5 min, and the reaction was allowed to stir for 1 hour. After $^1$H NMR showed complete conversion to the imine, the reaction was cooled to 0°C and di-tert-butyl dicarbonate (4.42 g, 20.3 mmol) was added as a solid. The reaction was then stirred for 45 min at room temperature. The volatiles were then removed under reduced pressure and the residue was redissolved in absolute EtOH (400 mL) and cooled to 0°C. A 1 M HCl (30 mL) solution was
added dropwise and then the reaction was warmed to room temperature and allowed to stir for 2 hours. After hydrolysis was complete, the volatiles were removed under reduced pressure, and the residue was washed 3 times with deionized water to remove excess salicylaldehyde. The residue was then redissolved in CH$_2$Cl$_2$ and washed 3 times with sat. aq. Na$_2$CO$_3$ to generate the free base. The free base was then purified by column chromatography $R_f$=0.28 (1% NH$_4$OH/10% MeOH/89% CH$_2$Cl$_2$) to give 28 as a pale yellow oil (3.55 g, 47%). $^1$H NMR (CDCl$_3$) $\delta$ 3.18 (t, 6H), 2.82 (s, 3H), 2.71 (t, 2H), 1.25-1.65 (m, 26H); $^{13}$C NMR (CDCl$_3$) $\delta$ 155.8, 155.5, 79.3, 79.2, 48.6, 48.0, 49.6, 41.0, 40.7, 34.1, 28.6, 28.4, 28.0, 25.8, 25.2, 24.8.

**Di-tert-butyl (((anthracene-9,10-diyldi(methylene))bis(azanediyl))bis(butane-4,1-diyl))bis((4-((tert-butoxycarbonyl)(methyl)amino)butyl)carbamate), 29.** To a stirred solution of amine 28 (600 mg, 1.61 mmol) in 25% MeOH/CH$_2$Cl$_2$ (25 mL) was added a solution of anthracene-9,10-dicarboxaldehyde (172 mg, 0.73 mmol) in 25% MeOH/CH$_2$Cl$_2$ (20 mL). The reaction was then stirred at room temperature under N$_2$ overnight. After complete imine formation was determined by $^1$H NMR, the solvents were then removed in vacuo and the residue was redissolved in 50% MeOH/CH$_2$Cl$_2$ (25 mL). The solution was then cooled to 0°C followed by addition of NaBH$_4$ (166 mg, 4.38 mmol) in small portion and the mixture was stirred at room temperature for 2 hours. After complete reduction, the solvents were removed in vacuo and the residue was redissolved in CH$_2$Cl$_2$ and washed three times with aqueous Na$_2$CO$_3$, then dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo to give 29 as a yellow oil (505 mg, 73%). $R_f$=0.28 (6% MeOH/0.5% NH$_4$OH/93.5% CH$_2$Cl$_2$); $^1$H NMR (CDCl$_3$) $\delta$ 8.37 (dd, 4H), 7.53 (dd, 4H), 4.70 (s, 4H), 3.19 (t, 12H), 2.89 (t, 4H), 2.81 (s, 6H), 1.57-1.44 (m, 52H); $^{13}$C NMR (CDCl$_3$) $\delta$ 155.8, 155.6, 132.1, 130.1, 125.7, 124.9, 79.1, 50.4, 46.9, 46.0, 34.1, 28.5, 27.5. HRMS (FAB)
m/z calc for C_{54}H_{88}N_{6}O_{8} (M + H)^+ 949.3119; found 949.6736. Elemental Analysis:
C_{54}H_{88}N_{6}O_{8}·0.5 \text{H}_2\text{O} theory C: 67.68, H: 9.36, N: 8.77; found C: 67.78, H: 9.09, N: 8.57.

**Di-tert-butyl (((((naphthalene-1,4-diylbis(methylene))bis(azanediyl))bis(butane-4,1-diyl))bis((tert-butoxycarbonyl)azanediyl))bis(butane-4,1-diyl))bis(methylcarbamate), 30.**

Yellow oil (55%), \(R_f=0.29\) (10% MeOH/0.5% NH_4OH/89.5% CH_2Cl_2); \(^1^H\) NMR (CDCl_3) \(\delta\) 8.21 (dd, 2H), 7.52 (dd, 2H), 7.71 (s, 2H), 4.22 (s, 4H), 3.19 (t, 12H), 2.81 (s, 3H), 2.75 (t, 4H), 1.31-1.62 (m, 52H); \(^1^3^C\) NMR (CDCl_3) \(\delta\) 155.8, 155.6, 135.5, 132.2, 125.8, 125.6, 124.4, 79.2, 79.1, 51.8, 49.8, 46.9, 34.1, 28.5, 27.5. m/z calc for C_{50}H_{86}N_{6}O_{8} (M + H)^+ 899.6507; found 899.6601. Elemental Analysis: C_{50}H_{86}N_{6}O_{8} theory C: 66.78, H: 9.64, N: 9.35; found C: 66.55, H: 9.61, N: 9.13.

**Di-tert-butyl (((1,4-phenylenebis(methylene))bis(azanediyl))bis(butane-4,1-diyl))bis((4-((tert-butoxycarbonyl)(methyl)amino)butyl)carbamate), 31.** Yellow oil (74%), \(R_f=0.25\) (7% MeOH/0.5% NH_4OH/92.5% CH_2Cl_2); \(^1^H\) NMR (CDCl_3) \(\delta\) 7.27 (s, 4H), 3.76 (s, 4H), 3.20 (t, 12H), 2.83 (s, 6H), 2.64 (t, 4H), 1.53-1.45 (m, 52H); \(^1^3^C\) NMR (CDCl_3) \(\delta\) 155.7, 155.5, 138.9, 128.1, 79.0, 53.6, 49.0, 46.9, 34.0, 28.4, 27.3. HRMS (FAB) m/z calc for C_{46}H_{84}N_{6}O_{8} (M + H)^+ 849.6351; found 849.6423. Elemental Analysis: C_{46}H_{84}N_{6}O_{8} theory C: 65.06, H: 9.97, N: 9.90; found C: 65.02, H: 9.87, N: 9.69.
CHAPTER II: POLYAMINE TRANSPORT INHIBITORS: DESIGN, SYNTHESIS AND COMBINATION THERAPIES WITH DIFLUOROMETHYLORLNITHINE (DFMO)

II.1 Abstract

The development of polyamine transport inhibitors (PTIs) in combination with difluoromethylornithine (DFMO) presents another strategy to exploit the PTS in cancer. A success in this area would provide a novel combination therapy which would result in sustained intracellular polyamine depletion and apoptosis. Prior work demonstrated that a tri-substituted platform was an efficient design for blocking the import of polyamines. This chapter sought to identify the key structural aspects of PTIs needed to effectively inhibit polyamine import in mammalian cells (CHO and L3.6pl) in the presence of the polyamine biosynthesis inhibitor DFMO.

II.2 Introduction

In aggressive cancers there is a marked increase in polyamine biosynthesis along with the increase in polyamine uptake. One of the key enzymes involved in polyamine biosynthesis is the enzyme ornithine decarboxylase (ODC). For cancer cells, ODC is upregulated in an effort to increase the intracellular pools of polyamines via the biosynthetic pathway and is seen as a proto-oncogene. ODC is the key enzyme necessary for the conversion of ornithine to Put, which in turn can be converted to the higher polyamines, Spd and Spm. In this regard, ODC is the gateway to polyamine biosynthesis and cell growth. Early efforts to control cell growth led to the development of a well-tolerated suicide inhibitor of ODC, α-difluoromethylornithine (DFMO). Despite success in inhibiting ODC activity, polyamine transport is often upregulated.
by cells in order to compensate for the depleted intracellular polyamine pools. Thus, DFMO as a sole therapeutic was not as effective as initially thought.  

This shortcoming of DFMO has opened the door for the development of polyamine transport inhibitors (PTIs) in an attempt to block the compensatory response of upregulated polyamine import. The combination of DFMO and an effective PTI could be very effective in the overall depletion of intracellular polyamine levels. This has led to the synthesis and bioevaluation of potential polyamine transport inhibitors and is the subject of Chapter 2.

In order to optimize PTI designs, it was important to understand how the polyamine transporter functions. As described earlier, there have been two models proposed by Poulin\textsuperscript{5a} and Belting\textsuperscript{6}, respectively for mammalian polyamine transport. Knowledge of the polyanionic cell surface target (heparan sulfate) aided the development of poly-cationic PTI agents. In essence, PTIs which effectively bind to heparan sulfate could also competitively block the import of exogenous native polyamines. This area has recently been reviewed in detail.\textsuperscript{30}
Potential PTIs were evaluated in two cell lines to comment on their effectiveness in blocking native polyamine import. CHO and L3.6pl were chosen for this study. The CHO cell line was selected for the reasons discussed in Chapter 1, and is known to have an active PTS. The L3.6pl cell line is derived from a human metastatic pancreatic cancer cell line, and was chosen for several reasons. The first reason is that pancreatic cancer is of particular interest due to the low five-year survival rate of patients with metastatic pancreatic cancer (6%), indicating a need for new therapies. Secondly, it has been identified that an activated $K$-RAS gene (identified in approximately 90% of pancreatic cancers) leads to an increase in polyamine uptake in human cells, and one particular cell line that possesses this $K$-RAS mutation is L3.6pl. Lastly, it has also been found that L3.6pl shows good sensitivity to DFMO and our PTS selective compound 5a. These factors point to L3.6pl as a good candidate for testing the ability of a potential PTI to block the import of native polyamines.
Initially, polyamine-based drug conjugates were developed as potential PTS selective ligands. A 1,3,5-tri-substituted benzene motif (compound 32), however, demonstrated low $K_i$ values in L1210 murine leukemia cells while also exhibiting relatively high IC$_{50}$ values (IC$_{50} > 100 \mu$M) in CHO and CHO-MG cells, and a lack of PTS selectivity.$^{12}$ The $K_i$ value is important for determining the relative affinity of these polyamine-based compounds for polyamine recognition sites on the cell surface. The lower the $K_i$ value, the higher the affinity the compound for its target. Therefore, presumably the lower the $K_i$ value the better the compound can bind to the cell surface and block uptake of other polyamine-conjugates. The $K_i$ values were determined in L1210 cells by competitive uptake with [$^{14}$C]spermidine.$^{12}$ This finding for tri-substituted 32 indicated that this compound could have potential as a polyamine transport inhibitor.

Polyamine-based PTIs have been developed, and several lessons have been learned.$^{30}$ Poulin et al. developed a series of di-substituted branched polyamine-based PTIs with relatively low $K_i$ values and demonstrated their ability to inhibit the uptake of putrescine in the T-47D breast cancer cell line.$^{33}$ However, these compounds failed to demonstrate effectiveness in inhibiting uptake of the higher polyamines spermidine and spermine. It was expected that compounds with lower $K_i$ values (potentially a third polyamine message) could more effectively inhibit native polyamine import. Burns et al. demonstrated that polyamine-based PTIs could be very effective through the development of amino acid-spermine conjugates.$^{34}$ Their initial lead compound, Lys-Spm (35) proved to be relatively non-toxic while effectively inhibiting spermidine uptake both in vitro and in vivo.$^{34-35}$ Due to its published properties as a PTI, compound 35 was prepared and used as a positive control in both the CHO and L3.6pl experiments.
Figure II-2. Structures of compounds 8a and 32-35

A combination therapy where DFMO provides blockade of polyamine biosynthesis coupled with PTS inhibitors results in sustained intracellular depletion of polyamines and eventually cell death via apoptosis. This approach represents a novel therapeutic strategy for cancers with a heavy reliance on the PTS. The finding with 32 then led to the development of other tri-substituted amine systems like 33a and 33b. Since all three compounds (32, 33a, 33b) possessed three polyamine messages, the question was then asked whether a third polyamine arm was needed for PTS inhibition. Interestingly, the bioevaluation of the 1,4-di-substituted xyllyl motif 8a demonstrated high PTS selectivity and a low $K_i$. At first glance, this PTS ligand may
make a potent PTI as it competes effectively for the cell surface receptors. However, its high toxicity was an undesired property for the DFMO-PTI combination therapy, which may require chronic administration. Therefore, PTIs with low toxicity were more desirable. The 1,4-di-substituted system was a toxic PTS ligand and the related 1,3,5-tri-substituted system, 32, was a relatively non-toxic PTI. Could the 1,3-orientation be a method to modulate drug toxicity? This question led to the synthesis and bioevaluation of the meta-substituted (1,3-) benzene ring compounds (34a and 34b). A tandem strategy where DFMO provides blockade of polyamine biosynthesis coupled with PTS inhibitors results in intracellular depletion of polyamines and eventually cell death. Investigations of these systems definitively showed that the tri-substituted design is required for polyamine transport inhibition and low compound toxicity.

II.3 Results and Discussion

**Synthesis.** The synthesis of 1,3,5-tri-substituted derivative 32 was previously described by Kaur et al.$^{12}$, and the synthesis of Lysine-spermine conjugate 35 was previously described by Burns et al.$^{34a}$.

The syntheses of the two novel potential polyamine transport inhibitors (PTIs) 33a and 33b began with commercially available 1,3,5-benzenetricarboxylic acid chloride 36. Ironically, the triamide platform was chosen initially as an alternate synthetic pathway to generate large quantities of compound 32 but also provided new platforms to evaluate as PTIs. The synthesis began with the coupling of 36 with Boc-protected polyamine 37a or 37b. Each amine and 36 were mixed together for six hours under mildly basic conditions to give the respective triamides 38a and 38b in moderate yield (56% and 75% respectively). Triamides 38a and 38b were then
deprotected in the presence of 4 M HCl in EtOH to give the respective compounds 33a and 33b in high yield.

Triamide 38a was also subjected to LiAlH₄ in an attempt to reduce the amide groups to the desired amine functionality with the expectation to then deprotect the Boc groups to yield tri-substituted system 32. The LiAlH₄ reduction, however, was problematic and thus a different synthetic route needed to be developed to 32. This was previously accomplished by Kaur et al. by using the tri-aldehyde motif 1,3,5-benzene tricarboxaldehyde, and reductive amination.¹²

**Scheme II-1.**³⁶ Synthesis of 33a and 33b

![Scheme II-1](image)

³⁶**Reagents:** (a) K₂CO₃, Aliquat, CH₂Cl₂/H₂O; (b) 4 M HCl, EtOH

Di-substituted derivatives 34a and 34b were generated to determine if the third arm present in the parent compound 32 was necessary for PTS inhibition. This two-arm platform would also prove to be less challenging synthetically than the parent 32 as dialdehyde 39 is commercially available, whereas the trialdehyde necessary for the construction of 32 is not.¹² As shown in Scheme II-2, the synthesis began with the reductive amination of aldehyde 39 with the protected polyamine 37a or 37b.³⁶ Extra caution was taken to dry methanol and NaBH₄ so as to
minimize the generation of alcohol byproducts, which were difficult to separate from the desired product. This became evident in the purification of 40a, as the mono-alcohol side product and the excess amine used initially had very similar Rf values to the desired product.

The purification problem was alleviated through the use of a unique TLC solvent system (hexanes/NH4OH/freshly distilled THF) which ultimately led to the isolation of compound 40a. Deprotection of 40a gave the final HCl salt 34a. This provided 34a in 24% yield over 3 steps. The related compound 40b was isolated in 55% yield using the more conventional CH2Cl2/MeOH/NH4OH TLC solvent system. Finally, 40b was deprotected to give the di-substituted extended amine system 34b in 98% yield. In sum, 34b was generated in 54% yield over these steps.

**Scheme II-2.** Synthesis of 34a and 34b

\[ \text{39} \xrightarrow{a, b} \text{40a: R=Boc, R'=H} \xrightarrow{c} \text{34a: R=H, R'=H; 6 HCl} \]

\[ \text{40b: R=Boc, R'= (CH}_2)_4\text{NHBoc} \xrightarrow{c} \text{34b: R=H, R'= (CH}_2)_4\text{NH}_2; 8 \text{ HCl} \]

**Reagents:** (a) 25% MeOH/CH2Cl2, Boc-protected amine 37a or 37b; (b) 50% MeOH/CH2Cl2, NaBH4; (c) 4 M HCl, EtOH

**Biological Evaluation.** Once synthesized, the conjugates were screened for their respective toxicity in L3.6pl, CHO, and CHO-MG* cells. L3.6pl cells were selected as a model system of human pancreatic cancer and was obtained from Dr. Cheryl Baker at the MD Anderson Cancer Center in Orlando, FL. CHO cells were chosen along with a mutant PTS.
deficient line (CHO-MG*) in order to comment on polyamine transport selectivity. Note: See Chapter 1 regarding CHO-MG* cell description. The CHO/CHO-MG* results are shown in Table II-1.

**CHO and CHO-MG* Studies. IC\textsubscript{50} and MTD Determinations.** CHO cells were chosen along with a mutant cell line (CHO-MG*) to comment on how the synthetic conjugates gain access to the cells. As discussed earlier, the CHO-MG* cell line is polyamine-transport deficient and represents a model for alternative modes of entry (other than PTS) including passive diffusion or use of another transporter. The CHO cell line on the other hand, represents cells with high polyamine transport activity. A comparison of toxicity in these two CHO cell lines allowed for a screen that would detect selective use of the polyamine transporter. This is seen where high utilization of the PTS would be very toxic to CHO cells. In contrast, reduced toxicity would be expected in CHO-MG* cells. Ultimately, a CHO-MG*/CHO IC\textsubscript{50} ratio was determined. A high ratio is expected for highly PTS selective compounds. The results for this experiment are listed in Table II-1.
Table II-1. Biological Evaluation of Polyamine Derivatives (32-35) in CHO and CHO-MG* Cells<sup>a,b,c,d,e</sup>

<table>
<thead>
<tr>
<th>Compound</th>
<th>MTD (µM) in CHO</th>
<th>CHO-MG* IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>CHO IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ratio&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 (Trimer44)</td>
<td>80</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
<td>33a (Triamide44)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
<td>33b (Triamide444)</td>
<td>1</td>
<td>69.1 (±4.0)</td>
<td>16.0 (±0.7)</td>
<td>4.3</td>
</tr>
<tr>
<td>34a (mBn44)</td>
<td>0.01&lt;sup&gt;f&lt;/sup&gt;</td>
<td>30.0 (±0.8)</td>
<td>0.028 (±0.002)</td>
<td>1072</td>
</tr>
<tr>
<td>34b (mBn444)</td>
<td>0.01&lt;sup&gt;g&lt;/sup&gt;</td>
<td>13.4 (±0.8)</td>
<td>0.04 (±0.007)</td>
<td>335</td>
</tr>
<tr>
<td>35 (Lys-Spm)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup>CHO and CHO-MG* cells were incubated with 1 mM AG for 24 h prior to drug addition.  
<sup>b</sup>The ratio denotes the (CHO-MG*/CHO) IC<sub>50</sub> ratio, a measure of PTS selectivity.  
<sup>c</sup>Cells were incubated for 48 h at 37°C with the respective conjugate.  
<sup>d</sup>All experiments were done in triplicate.  
<sup>e</sup>ND: not determined due to the low toxicity in both CHO cell lines.  
<sup>f</sup>At 0.01 µM, 34a displayed ~90% viability.  
<sup>g</sup>At 0.01 µM, 34b displayed ~90% viability.

The first step in evaluating the above compounds as polyamine transport inhibitors was to use the CHO and CHO-MG* screen to determine if the compound was a PTS selective agent. While PTS selective compounds would be competitive inhibitors of polyamine transport, the goal here was to identify non-toxic PTIs which could be given long-term to patients. In this regard, toxic PTS-selective agonists were undesired. The maximum tolerated dose (MTD) of each agent (Table II-1) was determined so that when utilizing the compound as a PTI it could be stated that the toxicity was attributed to blockade of polyamine import and not to the intrinsic...
toxicity of the PTI compound. In this regard, non-toxic PTIs provided a higher therapeutic window as their doses could be significantly elevated to out-compete native polyamines for cell entry. As discussed earlier, 1,3,5-tri-substituted analogue 32 exhibited a very high IC$_{50}$ value in both CHO and CHO-MG* (>100 µM for both), demonstrating it to be fairly non-toxic. This was in stark contrast to the 1,4-di-substituted platform used for 8a which was very PTS selective (CHO-MG*/CHO IC$_{50}$ ratio: 727). This proved to be a very interesting finding as both 32 and 8a possess near identical $K_i$ values in L1210 cells (0.52 ± 0.11 and 0.49 ± 0.02 respectively). This indicated that while these two compounds bind to the cell surface equally as well, a feature is present which imparts significant toxicity with 8a and less so with 32.

To better understand these differences, new derivatives were made to investigate whether the third polyamine message of 32 was needed or if the orientation of these messages is a driver of this phenomenon. These two questions were answered with the synthesis and evaluation of 34a and 34b as they were designed to most closely mimic 32. These compounds are meta-substituted instead of being para-substituted seen in 8a. With 34a and 34b in hand, they were evaluated in the CHO and CHO-MG* screen.

The results for 34a and 34b in CHO and CHO-MG* proved to be very interesting and unexpected. Both compounds were shown to be highly PTS selective as seen in Table II-1. Compound 34a was seen to be the most PTS selective (CHO-MG*/CHO IC$_{50}$ ratio: 1072), and clearly demonstrated that the third polyamine arm of 32 eliminated toxicity. For example, in the CHO cell line, 32 is >3500-fold less toxic than 34a. Compound 34b was shown to be less PTS selective (CHO-MG*/CHO IC$_{50}$ ratio: 335) than its counterpart 34a, demonstrating that the
longer polyamine message lowered the PTS selectivity of the design. A similar finding was noted in the comparison of mono-substituted triamines and tetraamines. Clearly, tri-substituted designs were preferred due to their low toxicity. This lesson would also be used in the development of 33a and 33b as possible polyamine transport inhibitors.

Although 33a was a potential synthetic entry to 32, it also provided a unique opportunity to evaluate a triamide platform as a PTI agent. The amide motif sequestered one of the amine groups and altered the positive point charge at this position. In this regard, the triamides represent a tri-substituted design where the appended polyamine chains are further away from the aryl core. Triamide 33a effectively displays three putrescine messages whereas triamide 33b displays three homospermidine messages. Triamide 33b had relatively poor PTS selectivity in the CHO assay and 34a was not determined due to its low toxicity (Table II-1).

The high transport activity of CHO was also exploited to determine if compounds 32-35 were potent polyamine transport inhibitors. This assay was performed by first inhibiting ODC and polyamine biosynthesis through the addition of DFMO and reducing cell viability to ~50%. As stated earlier DFMO is a suicide inhibitor of ODC which in turn leads to an up-regulation of polyamine transport. Once cell viability was reduced to ~50%, Spd, a native polyamine was added to reproducibly rescue the cells’ viability back to 100%. A potent PTI would be expected to inhibit Spd entry and the cells treated with DFMO + Spd + PTI would be expected to resemble the DFMO-only control and gave 50% viability. With this assay in place, the effectiveness of each potential polyamine transport inhibitor could be tested. DFMO (1 mM) was added to cells
followed by addition of the putative PTI, followed by addition of 1 µM Spd. The results for this experiment are shown in Figures II-3, II-4, and II-5.

**DFMO Studies.** The relative effectiveness of a PTI to block polyamine import was tested through its ability to block Spd (1 µM) uptake in DFMO treated cells. The rescue event is best illustrated through the use of Spd as it is found to effectively rescue L1210 and CHO cells from polyamine based drugs and DFMO. For example, the $K_i$ values of the native polyamines (Put, Spd, Spm) were 208.2, 2.46, and 1.34 µM respectively in L1210 cells. While all three of the native polyamines could be used as a rescue agent, putrescine transport was easily blocked due to its high $K_m$ value and spermine at high doses was toxic to cells. In this regard, spermidine was more challenging to inhibit and was less toxic to cells and thus preferred.

When determining the relative effectiveness of compounds 32-35 in their ability to block Spd import, 33b, 34a, and 34b were severely limited by their MTD. The MTD as described earlier is important to be sure the toxicity was due to PTS inhibition and not intrinsic toxicity of the PTI agent. In CHO, 33b could only be tested up to 1 µM, and 34a and 34b could only be tested up to 0.01 µM. When tested at their MTD, none of these three compounds could prevent the rescue effect of Spd (1 µM). Fortunately, the remaining compounds 32, 33a and 35 all fully blocked the import of Spd (1 µM) at different concentrations. All three of these compounds showed a dose dependent response in their abilities to block Spd import. For example, as shown in Figure II-3, increasing levels of 32 provided decreased viability in the presence of DFMO (1 mM) and a typical rescuing dose of Spd (1 µM).
In a head to head comparison of \(32\), \(33a\), and \(35\) in CHO, compound \(32\) proved to be the superior inhibitor of Spd import. In CHO, this became most evident at 1 \(\mu\)M PTI, where cell viability is 50\% for \(32\) and cell viability is at 100\% for both \(33a\) and \(35\) at the same concentration. This clearly demonstrates that \(32\) is better at blocking Spd import and is the most potent of the series. Compounds \(33a\) and \(35\) are also effective inhibitors of Spd but required much higher concentrations (60 \(\mu\)M and 6 \(\mu\)M respectively) to be effective.

![Bar graph showing cell viability against different concentrations of compound 32.](image)

**Figure II-3.** Ability of \(32\) (Trimer44) to prevent Spd rescue of DFMO treated CHO cells\(^a,b,c\)

\(^a\)Cells were incubated for 48 h at 37\(^\circ\)C with the respective conjugate, DFMO (1 mM) and Spd (1 \(\mu\)M).

\(^b\)1 mM AG was incubated with cells for 24 h prior to drug addition.

\(^c\)Columns 4-9 represent experiments with DFMO (1 mM), Spd (1 \(\mu\)M) and increasing concentrations of \(32\).
**Figure II-4.** Ability of 33a (Triamide44) to prevent Spd rescue of DFMO treated CHO cells\(^{a,b}\)

\(^a\)Cells were incubated for 48 h at 37°C with the respective conjugate, DFMO (1 mM) and Spd (1 µM). \(^b\)1 mM AG was incubated with cells for 24 h prior to drug addition. \(^c\)Columns 4-9 represent experiments with DFMO (1 mM), Spd (1 µM) and increasing concentrations of 33a.

**Figure II-5.** Ability of 35 (Lys-Spm) to prevent Spd rescue of DFMO treated CHO cells\(^{a,b}\)

\(^a\)Cells were incubated for 48 h at 37°C with the respective conjugate, DFMO (1 mM) and Spd (1 µM). \(^b\)1 mM AG was incubated with cells for 24 h prior to drug addition. \(^c\)Columns 4-9 represent experiments with DFMO (1 mM), Spd (1 µM) and increasing concentrations of 35.
**L3.6pl Studies. IC\textsubscript{50} and MTD Determinations.** Compounds 32-35 were also tested in the metastatic human pancreatic cancer cell line, L3.6pl. This cell line was also chosen due to its \(K\)-RAS mutation which has been linked to increased polyamine uptake.\textsuperscript{32h,37} This increase in polyamine uptake lends itself to being targeted by polyamine transport inhibitors. The combination of DFMO and an effective PTI would allow for decreased polyamine levels in these cells ultimately leading to cell death. When compounds 32-35 were initially tested in L3.6pl, their IC\textsubscript{50} values followed the same trend as seen in CHO. The two main lessons learned in L3.6pl were the same as in CHO. First, increased polyamine length (33a vs. 33b and 34a vs. 34b) led to increased toxicity. Second, given the same orientation of the benzene ring, the two polyamine messages of 34a increased toxicity compared to the three polyamine chains of 32. These findings in CHO/CHO-MG\textsuperscript{*} and L3.6pl identified as potential polyamine transport inhibitors to be used in combination with DFMO.
Table II-2. Biological Evaluation of Polyamine Derivatives (32-35) in L3.6pl cells\textsuperscript{a,b,c}

<table>
<thead>
<tr>
<th>Compound</th>
<th>MTD (µM) in L3.6pl</th>
<th>L3.6pl IC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 (Trimer44)</td>
<td>10</td>
<td>66.5 (±2.4)</td>
</tr>
<tr>
<td>33\textsuperscript{a} (Triamide44)</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>33\textsuperscript{b} (Triamide444)</td>
<td>1</td>
<td>15.7 (±0.8)</td>
</tr>
<tr>
<td>34\textsuperscript{a} (mBn44)</td>
<td>0.1\textsuperscript{d}</td>
<td>52.2 (±2.0)</td>
</tr>
<tr>
<td>34\textsuperscript{b} (mBn444)</td>
<td>0.01\textsuperscript{e}</td>
<td>7.5 (±0.4)</td>
</tr>
<tr>
<td>35 (Lys-Spm)</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

\textsuperscript{a}L3.6pl cells were incubated with 250 µM AG for 24 h prior to drug addition. \textsuperscript{b}Cells were incubated for 48 h at 37°C with the respective conjugate. \textsuperscript{c}All experiments were done in triplicate. \textsuperscript{d}At 0.1 µM, 34\textsuperscript{a} displayed 108% viability. \textsuperscript{e}At 0.01 µM, 34\textsuperscript{b} displayed 97% viability.

**DFMO Studies.** In L3.6pl, 33\textsuperscript{b} could only be tested up to 1 µM, 34\textsuperscript{a} exhibited a MTD of 0.1 µM, and 34\textsuperscript{b} proved to be very toxic with a MTD at a dose limiting 0.01 µM. Again, when these compounds (33\textsuperscript{b}, 34\textsuperscript{a}, and 34\textsuperscript{b}) were tested at their MTD in L3.6pl, they were unsuccessful in inhibiting the uptake of Spd (1 µM). These results further illustrate the need for PTIs with low toxicity and high MTD values.

In L3.6pl, 32 and 35 were effective inhibitors of Spd import at 10 µM, where 33\textsuperscript{a} was not as effective at the same concentration. As shown in Figures II-6, II-7 and II-8, 32 was the superior PTI as it gave 52% viability at 4 µM, whereas 35 gave 75% viability at the same 4 µM dose. Compound 33\textsuperscript{a} proved to be far less effective as it was not able to approach the 50% viability mark when dosed even up to 100 µM.
Figure II-6. Ability of 32 (Trimer44) to prevent Spd rescue of DFMO treated L3.6pl cells.\textsuperscript{a,b,c}

\textsuperscript{a}Cells were incubated for 48 h at 37\textdegree C with the respective conjugate (32), DFMO (5 mM) and Spd (1 \textmu M). \textsuperscript{b}250 \textmu M AG was incubated with cells for 24 h prior to drug addition. \textsuperscript{c}Columns 4-9 represent experiments with DFMO (5 mM), Spd (1 \textmu M) and increasing concentrations of 32.

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Figure II-7. Ability of 33a (Triamide44) to prevent Spd rescue of DFMO treated L3.6pl cells.\textsuperscript{a,b,c}

\textsuperscript{a}Cells were incubated for 48 h at 37\textdegree C with the respective conjugate, DFMO (5 mM) and Spd (1 \textmu M). \textsuperscript{b}250 \textmu M AG was incubated with cells for 24 h prior to drug addition. \textsuperscript{c}Columns 4-9 represent experiments with DFMO (5 mM), Spd (1 \textmu M) and increasing concentrations of 33a.
Figure II-8. Ability of 35 (Lys-Spm) to prevent Spd rescue of DFMO treated L3.6pl cells\textsuperscript{a,b,c}.

\textsuperscript{a}Cells were incubated for 48 h at 37°C with the respective conjugate, DFMO (5 mM) and Spd (1 µM). \textsuperscript{b}250 µM AG was incubated with cells for 24 h prior to drug addition. \textsuperscript{c}Columns 4-9 represent experiments with DFMO (5 mM), Spd (1 µM) and increasing concentrations of 35.

II.4 Summary

These Spd import inhibition experiments indicate that a platform with three homospermidine messages provided the most potent PTI of the series evaluated. As stated earlier, the di-substituted (meta-substituted) analogues 34a and 34b were toxic PTS selective ligands which limited their application as PTIs. Their ability to be competitive inhibitors of polyamine transport could not be demonstrated in the DFMO + Spd assay due to their low MTD values. In short, these compounds proved to be too toxic in both CHO and L3.6pl to test high enough doses to outcompete Spd (1 µM). Therefore, the third polyamine arm was shown to be a key part of the PTI design in terms of its ability to block the import of native polyamines to retain a low toxicity profile.
Another key in the development of potential PTIs, is the influence of the polyamine message length on the toxicity of the compound. Initially it was thought that since the longer native polyamine chains (i.e. spermine) exhibit lower $K_i$ values, they should be superior PTIs. However, 33a-b and 34a-b demonstrated that this was not the case due to the toxicity of tetraamines. Both of the conjugates with the longer polyamine message exhibited a greater level of toxicity which translated to a very small therapeutic window for each compound. While it is possible that both of these compounds could in fact block the import of Spd more effectively than their shorter polyamine counterparts, their low MTD did not allow for this evaluation at doses which outcompeted Spd (1 µM). In summary, several factors were identified for potent PTIs: a) triamine arms provided decreased toxicity and a presumed higher therapeutic window; b) the distance of the triamine from the aryl core also played a role as 33b represents three homospermidine messages surrounding an aryl core similar to 32. However, these polyamine messages are further away from each other and reside at five bonds further away from the aryl platform. This motif leads to higher toxicity and reduces the therapeutic window available to the triamide system, 33b. In this regard, compound 32 represents an optimized PTI and was clearly the most potent PTI evaluated.

II.5 Experimental

II.5.1 Materials.

Silica gel (32-63 µm) and chemical reagents were purchased from commercial sources and used without further purification. All solvents were distilled prior to use. All reactions were carried out under an N$_2$ atmosphere. $^1$H and $^{13}$C spectra were recorded at 400 or 75 MHz,
respectively. TLC solvent systems were listed as volume percents, and \( \text{NH}_4\text{OH} \) referred to concentrated aqueous ammonium hydroxide. All tested compounds provided satisfactory elemental analyses.

**II.5.2 Biological Studies.**

CHO and CHO-MG* cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Note: the media must contain L-proline (2µg/mL) for proper growth of the CHO-MG* cells. L3.6pl cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were grown at 37°C under a humidified 5% \( \text{CO}_2 \) atmosphere. Aminoguanidine (1 mM for CHO and CHO-MG*, and 250 µM for L3.6pl) was added to the growth medium to prevent oxidation of the drugs by the enzyme (bovine serum amine oxidase) present in calf serum. Cells in early to mid-log phase were used.

**IC\textsubscript{50} Determinations.** Cell growth was assayed in sterile 96-well microtiter plates (Costar 3599, Corning, NY, USA). CHO and CHO-MG* cells were plated at 10,000 cells/mL. L3.6pl cells were plated at 5,000 cells/mL. Drug solutions (10µL per well) of appropriate concentration were added after an overnight incubation for each CHO cell line (90 µL of cell suspension used). After exposure to the drug for 48 h, cell growth was determined by measuring formazan formation from 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium, inner salt (MTS) using a SynergyMx Biotek microplate reader for absorbance (490 nM) measurements.\textsuperscript{24}
II.5.3 Synthetic Procedures and Characterization

$N^1,N^3,N^5$-tris(4-((4-aminobutyl)amino)butyl)benzene-1,3,5-tricarboxamide (33a). A solution of Boc-protected 38a (910 mg, 0.67 mmol) was dissolved in absolute ethanol (70 mL) and stirred at 0°C for 10 min. A 4 M HCl solution (40 mL, 160 mmol) was added to the reaction mixture dropwise and stirred for at 0°C for 30 min and then at room temperature overnight. The solution was concentrated in vacuo to give 33a as a white solid (625 mg, 99%); $^1$H NMR (D$_2$O) $\delta$ 8.28 (s, 3H), 3.45 (t, 6H), 3.14 (q, 12H), 3.07 (t, 6H), 1.78 (m, 24H); $^{13}$C NMR (D$_2$O) $\delta$ 172.0, 138.0, 131.8, 50.3, 49.9, 42.4, 41.8, 28.6, 27.0, 26.1, 25.8. HRMS (FAB) $m/z$ calc for C$_{33}$H$_{72}$Cl$_9$N$_9$O$_3$ (M + H)$^+$ 915.4366, found 915.4366.

Elemental Analysis: C$_{33}$H$_{72}$Cl$_9$N$_9$O$_3$·3.5H$_2$O theory C: 43.28, H: 8.37, N: 13.77; found C: 43.47, H: 8.32, N: 13.55.

$N^1,N^3,N^5$-tris(4-((4-aminobutyl)amino)butyl)amino)butyl)benzene-1,3,5-tricarboxamide nonahydrochloride (33b). Light pink solid, 84%. $^1$H NMR (D$_2$O) $\delta$ 8.29 (s, 3H), 3.46 (t, 6H), 3.15 (24 H), 3.05 (t, 6H), 1.79 (m, 36H); $^{13}$C NMR (D$_2$O) $\delta$171.8, 137.8, 131.9, 50.6, 49.6, 42.4, 41.5, 27.1, 28.8, 27.1, 26.8, 25.8. HRMS (FAB) $m/z$ calc for C$_{45}$H$_{99}$Cl$_9$N$_{12}$O$_3$ (M + H)$^+$ 847.7337, found 847.7332. Elemental Analysis: C$_{45}$H$_{99}$Cl$_9$N$_{12}$O$_3$·6 H$_2$O theory C: 42.11, H:8.72, N:13.10; found C:42.16; H: 8.34, N: 12.94.

$N^1,N^{1'}$-(1,3-phenylenebis(methylene))bis(N$_4$-(4-aminobutyl)butane-1,4-diamine) (34a). Compound 40a (174 mg, 0.21 mmol) was dissolved in EtOH (30 mL) and stirred at 0°C for 10 min, and then a 4 M HCl solution (12 mL, 48 mmol) was then added dropwise and stirred for 30 min. The temperature was then allowed to rise to room temperature and the solution was then stirred under N$_2$ for four hours. The solvents were then removed in vacuo and the remaining white solid was washed three times with hexanes to remove BHT. The hexanes were then filtered.
away to give a white solid (80 mg, 24% over 3 steps). $^1$H NMR (D$_2$O) δ 7.57 (s, 3H), 7.55 (s, 1H), 4.26 (s, 4H), 3.20-3.01 (m, 16H), 1.76 (m, 16H); $^{13}$C NMR (D$_2$O) δ 134.7, 133.9, 133.8, 133.0, 53.6, 49.8, 49.5, 41.7, 26.8, 25.7. HRMS (FAB) $m$/z calc for C$_{24}$H$_{48}$N$_6$ (M + H)$^+$ 421.3940, found 421.4013. Elemental Analysis: C$_{24}$H$_{54}$Cl$_6$N$_6$·0.75 H$_2$O theory C: 44.15, H: 8.57, N: 12.87; found C: 44.07, H: 8.57, N: 12.71.

$N^1,N'^1$-(1,3-phenylenebis(methylene))bis($N^4$-(4-((4-aminobutyl)amino)butyl)butane-1,4-diamine) (34b). Compound 40b (130 mg, 0.11 mmol) was dissolved in EtOH (10 mL) and stirred at 0°C for 10 min, and a 4 M HCl solution (6 mL, 24 mmol) was added dropwise and stirred for 30 min. The temperature was then allowed to rise to room temperature and the solution was stirred under N$_2$ for four hours. The solvents were then removed in vacuo to give a white solid (95 mg, 98%). $^1$H NMR (D$_2$O) δ 7.59-7.57 (m, 4H), 4.30 (s, 4H), 3.17-3.04 (m, 24H), 1.79-1.77 (m, 24H); $^{13}$C NMR 187.9, 134.4, 133.8, 133.7, 132.9, 53.4, 49.6, 49.3, 41.5, 26.6, 25.5. HRMS (FAB) $m$/z calc for C$_{32}$H$_{66}$N$_8$ (M + H)$^+$ 563.5410, found 563.5483. Elemental Analysis: C$_{32}$H$_{74}$Cl$_8$N$_8$·0.5 H$_2$O theory C: 42.54, H: 8.37, N: 12.40; found C: 42.57, H: 8.63, N: 12.07.

(S)-2,6-diamino-N-(3-((4-((3-aminopropyl)amino)butyl)amino)propyl)hexanamide (35). $^1$H NMR (D$_2$O) δ 3.92 (t, 1H), 3.38 (m, 1H), 3.27 (m, 1H), 3.12 (m, 10H), 2.91 (t, 2H), 2.18 (m, 2H0), 1.89 (m, 4H), 1.77 (m, 4H), 1.71 (m, 2H), 1.39 (m, 2H); matched literature values.$^{34}$ HRMS (FAB) $m$/z calc for C$_{16}$H$_{43}$Cl$_5$N$_6$O (M + H)$^+$ 331.3180, found 331.3175. Elemental Analysis: C$_{16}$H$_{43}$Cl$_5$N$_6$O·1 H$_2$O theory C: 36.20, H: 8.54, N: 15.83, found C: 36.45, H: 8.66, N: 15.58.
[4-(3,5-Bis-{4-[tert-butoxycarbonyl-(4-tert-butoxycarbonylamino-butyl)-amino]-
butylcarbamoyl}-benzoylamino)-butyl]-{4-tert-butoxycarbonylamino-butyl}-
carbamic acid tert-butyl ester (38a). A solution of (4-amino-butyl)-(4-tert-butoxycarbonylamino-butyl)-
carbamic acid tert-butyl ester 37a (1.80 g, 5.01 mmol) and CH₂Cl₂ (12 mL) was prepared. A
solution of K₂CO₃ (1.65 g, 11.94 mmol) and water (20 mL) was added to the original mixture.
Aliquat (0.10 mL) was added and the mixture was cooled to 0°C. 1,3,5-
Benzenetricarboxylic acid chloride 36 (400 mg, 1.51 mmol) in CH₂Cl₂ (24 mL) was added dropwise to the original
mixture and allowed to gradually warm and stir vigorously at room temperature for 6 hours.
After the reaction was complete (by ¹H NMR), the solvents were removed in vacuo, and the
residue was redissolved in CH₂Cl₂. The organic layer was then washed with 0.1  M HCl (24 mL),
followed by deionized H₂O (24 mL), and saturated aq. Na₂CO₃ (24 mL). The organic layer was
then separated, dried over Na₂SO₄, filtered, and concentrated to give a crude solid which was
then purified by flash column chromatography to afford 38a (1.05 g, 56%) as a white solid
(Rf=0.25; 4% MeOH/96% CH₂Cl₂); ¹H NMR (CDCl₃) δ 8.41 (s, 3H), 4.75 (br s, 3H), 3.49 (m, 6H), 3.23-3.06 (m, 18H), 2.78-1.30 (m, 78H); ¹³C NMR (CDCl₃) δ 166.5, 156.1, 155.7, 135.2,
128.5, 79.3, 78.9, 46.6, 40.1, 39.8, 28.4, 27.3, 26.4. HRMS (FAB) m/z calc for C₆₃H₁₁₁N₉O₁₅ (M
+ Na)+ 1256.8092, found 1256.8105. Elemental Analysis: C₆₃H₁₁₁N₉O₁₅·1 H₂O theory C: 60.41,

[4-(3,5-Bis-{4-[tert-butoxycarbonyl-(4-tert-butoxycarbonyl)-(4-tert-butoxycarbonylamino-butyl)-amino]-butylcarbamoyl}-benzoylamino)-butyl]-{4-tert-butoxycarbonylamino-butyl}-
carbamic acid tert-butyl ester (38b). A similar procedure was
used for 38b. White solid, 75%. ¹H NMR (CDCl₃) δ 8.19 (s, 3H), 3.42 (t, 6H), 3.17 (t, 30H),
1.71-1.41 (m, 117H); $^{13}$C NMR (CDCl$_3$) δ 166.4, 156.2, 155.7, 135.4, 128.9, 79.5, 46.8, 45.8, 41.2, 40.5, 39.5, 29.1, 28.5, 27.6, 26.5. Elemental Analysis: C$_{90}$H$_{162}$N$_{12}$O$_{21}$ theory C: 61.83, H: 9.34, N: 9.61; found C: 61.64, H: 9.37, N: 9.38.

**Di-tert-butyl (((1,3-phenylenebis(methylene))bis(azanediyl))bis(butane-4,1-diyl))bis((4-((tert-butoxycarbonyl)amino)butyl)carbamate) (40a).** In order to maximize the yield, MeOH was dried and distilled prior to this reaction. To a stirred solution of $N^1,N^6$-diboc-homospermidine (566 mg, 1.58 mmol) in 25% MeOH/CH$_2$Cl$_2$ (20 mL) was added a solution of 1,3-benzene dicarboxaldehyde 39 (69.9 mg, 0.52 mmol) in 25% MeOH/CH$_2$Cl$_2$ (15 mL). The reaction was then stirred at room temperature under N$_2$ overnight. After imine formation was complete (by $^1$H NMR), the solvents were removed in vacuo and the residue was redissolved in 50% MeOH/CH$_2$Cl$_2$ (25 mL). The solution was then cooled to 0°C followed by addition of pre-dried NaBH$_4$ (118 mg, 3.13 mmol) in small portions and the mixture was stirred at room temperature under N$_2$ for 2 hours. The solvents were then removed under reduced pressure and the residue was redissolved in CH$_2$Cl$_2$ and washed three times with aqueous Na$_2$CO$_3$. The organic layer was separated, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. After column chromatography ($R_f$=0.25, 25% hexanes/0.1% NH$_4$OH/74.9% THF, see note below), compound 40a was isolated as a mixture with BHT and was deprotected without further purification. $^1$H NMR (CDCl$_3$) δ 7.26-7.15 (m, 4H), 3.62 (s, 4H), 3.14 (t, 12H), 2.58 (t, 4H), 1.58-1.27 (m, 16H); $^{13}$C NMR (CDCl$_3$) δ 155.0, 154.5, 139.5, 126.9, 78.1, 66.9, 53.0, 48.2, 45.6, 39.2, 26.8, 25.8, 24.5. Note: The BHT impurity could be avoided by pre-distilling the THF prior to chromatography.
Di-tert-butyl (((1,3-phenylenebis(methylene))bis(azanediyl))bis(butane-4,1-diyl))bis((4-((tert-butoxycarbonyl)(4-((tert-butoxycarbonyl)amino)butyl)amino)butyl)carbamate) (40b). To a stirred solution of N<sup>1</sup>,N<sup>6</sup>,N<sup>11</sup>-triBoc-homospermine (705 mg, 1.33 mmol) in 25% MeOH/CH<sub>2</sub>Cl<sub>2</sub> (20 mL) was added a solution of 1,3-benzene dicarboxaldehyde 39 (52.3 mg, 0.39 mmol) in 25% MeOH/CH<sub>2</sub>Cl<sub>2</sub> (15 mL). The reaction was then stirred at room temperature under N<sub>2</sub> overnight. After imine formation was complete by <sup>1</sup>H NMR, the solvents were removed in vacuo and the residue was redissolved in 50% MeOH/CH<sub>2</sub>Cl<sub>2</sub> (25 mL). The solution was then cooled to 0°C followed by addition of NaBH<sub>4</sub> (88.5 mg, 2.34 mmol) in small portions and the mixture was stirred at room temperature under N<sub>2</sub> for 2 hours. The solvents were then removed under reduced pressure and the residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> and washed three times with aqueous Na<sub>2</sub>CO<sub>3</sub>. The organic layer was separated, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated in vacuo. After column chromatography (R<sub>f</sub>=0.31 (10% MeOH/1% NH<sub>4</sub>OH/89% CH<sub>2</sub>Cl<sub>2</sub>)), compound 40b was isolated as a colorless oil (240 mg, 53%) <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.32-7.19 (m, 4H), 3.77 (s, 4H), 3.15 (t, 20H), 2.65 (t, 4H), 1.60-1.44 (m, 78H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 156.0, 155.5, 140.5, 128.4, 127.8, 126.7, 79.1, 54.0, 49.3, 46.9, 46.6, 40.2, 28.5, 27.4, 26.1. HRMS (FAB) m/z calc for C<sub>62</sub>H<sub>114</sub>N<sub>8</sub>O<sub>12</sub> (M + H)<sup>+</sup> 1163.8629, found 1163.8629. Elemental Analysis: C<sub>62</sub>H<sub>114</sub>N<sub>8</sub>O<sub>12</sub> theory C: 64.00, H: 9.87, N: 9.63; found C: 64.24, H: 9.96, N: 9.82.
CHAPTER III: INVESTIGATION OF ANTI-METASTATIC AGENTS PREDICATED ON DIHYDROMOTUPORAMINE C AND ITS DERIVATIVES

III.1 Abstract

The motuporamine family of natural products are of biological interest due to their unique anti-migration and anti-angiogenic properties. Previous work has demonstrated some of the key structural aspects of these compounds necessary to maintain their biological activity. These included a preferred macrocycle ring size (15-membered ring), a preferred degree of unsaturation (zero) within the heterocycle and a norspermidine motif. This chapter discusses the development of new motuporamine analogues which modulate the cytotoxicity of this drug class while simultaneously enhancing their anti-migration ability. In summary, by incrementally moving the polyamine chain outside the ring, new systems were discovered which doubled the anti-migration potency and reduced the toxicity by 29-fold. The development of non-toxic anti-metastatic agents would have direct application in the treatment of human cancers.

III.2 Introduction

In 1998, Andersen et al reported the discovery of the motuporamine family of compounds from the sea sponge *Xestospongia exigua* off the coast of Motupore Island in Papua New Guinea. These compounds were of particular interest as they each possessed a large macrocycle tethered to a norspermidine (3,3-triamine) message (Figure III-1). This drug class was structurally similar to the anthryl-polyamines previously reported by Phanstiel et al.\textsuperscript{10,38} Biologically, dihydromotuporamine C (44a) proved to be the most interesting as it was highly cytotoxic to the MDA-231 breast carcinoma cells and had good anti-invasive properties.\textsuperscript{38,39} Due
to the motuporamine compounds exhibiting structural similarities to 5a (Ant44) and other previously made analogues\textsuperscript{10}, it was thought that they may function in a similar manner to the anthryl-polyamine conjugates.

![Chemical structures](image)

**Figure III-1.** Structures of motuporamine natural products (41, 42, 43, 44a) and Dihydromotuporamine C mimics (45a and 45b) and Ant44 (5a)

Recently, it has been shown that dihydromotuporamine C (44a) affects the sphingolipid biosynthetic pathway.\textsuperscript{40} Baetz et al. initially showed through a genome-wide haploinsufficiency screen in yeast that dihydromotuporamine C targets sphingolipid metabolism.\textsuperscript{40a} This was
demonstrated via the recognized sensitivity of genes *LCB1* and *TSC10* to the addition of dihydromotuporamine C. Both are key genes involved in the biosynthesis of sphingosine, which is a sphingolipid precursor.\(^{40a}\) This was the first molecular target described for *44a*. The authors went on to demonstrate that dihydromotuporamine C at 60 µM was able to fully inhibit the growth of yeast cells.\(^{41}\) However, the addition of an intermediate of the sphingosine biosynthesis pathway (dihydrosphingosine) to dihydromotuporamine C-treated yeast cells, was able to rescue this growth inhibition.\(^{41}\) Dihydromotuporamine C was shown to lower the intracellular levels of ceramide and the addition of exogenous ceramide was able to partially rescue yeast growth indicating that dihydromotuporamine C directly targets sphingolipid biosynthesis.\(^{40}\) Despite this finding in yeast, it could not be demonstrated that exogenous ceramide could completely rescue the effects of dihydromotuporamine C.\(^{40a,b}\) This observation could be explained by dihydromotuporamine C having multiple targets in human cells or that the rescue event was limited by the amount of ceramide that could be added as ceramide is known to have pro-apoptotic properties.\(^{40a,b}\) The fact that the less toxic dihydrosphingosine could rescue these cells is consistent with the latter possibility.

Beyond sphingolipid metabolism, Rho GTPase was also identified as a potential target of dihydromotuporamine C (*44a*).\(^{40b}\) An increase in Rho signaling has been associated with many types of cancer.\(^{42}\) This increase in Rho signaling has also been seen to play a part in the anti-invasive action of dihydromotuporamine C.\(^{40b,43}\) This has been partly explained by a loss of cell polarity and an increase in adhesion strength caused by Rho activation.\(^{40b}\) Another key player in the anti-migration ability of dihydromotuporamine C is the activation of integrin signaling, which in turn leads to the inhibition of tumor migration by activated Rho signaling.\(^{40b,44}\)
A series of structure activity relationship studies has also helped determine which aspects of the dihydromotuporamine C (44a) structure provides for its unique biological activity. The structure of the motuporamines can be broken into two main components: the macrocyclic ring, and the norspermidine tail. The 15-membered macrocyclic ring is favored over other size rings as it demonstrated the highest levels of cytotoxicity and invasion inhibition in MDA-MB-231 cells. Williams et al. also showed that the degrees of unsaturation in the ring also dramatically affected the biological activity of the series, where a saturated ring (completely void of unsaturation) proved to be the most potent design in terms of anti-migration properties. A carbazole ring substitute also showed promising anti-invasion properties, although it was not as potent as the parent, dihydromotuporamine C.

The other key structural characteristic of the motuporamines is the norspermidine tail. It was demonstrated that acetylation of the terminal amino group had no effect on the biological activity of motuporamine C (43), however, acetylation of both amine groups saw a complete loss of activity. This finding suggested the importance of the central secondary amine for high biological activity.

Previous efforts by Phanstiel et al. explored the conformational preferences of dihydromotuporamine C (44a) by computer modeling. These experiments demonstrated that the saturated ring system was conformationally mobile as expected. The orientation of the polyamine chain off the ring was shown to be critical and helped the authors design new motuporamine mimics. These mimics were predicated upon anthryl-polyamines. Specifically, AntNEt33 (45a) had similar preferred molecular conformations as dihydromotuporamine C.
and was shown to mimic the cytotoxicity of dihydromotuporamine C in CHO and CHO-MG cells. Although AntNEt33 (45a) was a good mimic of dihydromotuporamine C in vitro, it failed to mimic the anti-metastatic properties of dihydromotuporamine C in vivo.

Efforts to append a cell-targeting homospermidine message to this macrocycle unfortunately resulted in a non-PTS targeting drug with lower cytotoxicity.\textsuperscript{25} Additional work demonstrated that this was due to the tertiary amine center at the $N'$ position. In short, $N'$-ethylation provided a compound with a similar cytotoxicity profile as dihydromotuporamine C. However, the $N'$-ethyl group was also shown to dramatically reduce the ability of the drug to access the PTS for cell entry.\textsuperscript{45}

During studies to explore how 5a and its analogues\textsuperscript{10} compare in their biological function to dihydromotuporamine C (44a), 44a was evaluated for its PTS selectivity. In previous studies compounds that possessed the 3,3-triamine (i.e. norspermidine) message (as seen in 44a) exhibited poor PTS selectivity while compounds with a 4,4-triamine (i.e. homospermidine) message exhibited the highest PTS selectivity.\textsuperscript{10} This led to the synthesis of the parent compound 44a as well as the homospermidine analogue 44b in order to determine if the optimized polyamine message would provide increased PTS selectivity over the parent. When both of these compounds were subjected to the CHO/CHO-MG screen, neither demonstrated PTS selectivity as their CHO-MG/CHO IC\textsubscript{50} ratio was found to be 1.\textsuperscript{25} Due to the presence of the tertiary amine at the $N'$ position for 44a and 44b, a structural analogue using anthracene as the macrocycle (45a) was synthesized with a tertiary amine at the $N'$ position, and bioevaluation showed an identical CHO-MG/CHO IC\textsubscript{50} ratio of 1.\textsuperscript{25} This follow-up study using $N'$-alkylated anthryl-
polyamines demonstrated that PTS selectivity was dependent upon the $N^1$-substitution pattern. Evidently, tertiary amine centers at the $N^1$ position inhibit the PTS selectivity of the drug conjugate, whereas a secondary amine center facilitates PTS selectivity.

Compounds **44a** and **44b** each possess an amine at the $N^1$ position that is not only a tertiary amine but also is sterically encumbered by the macrocyclic ring. As stated earlier, a similar phenomenon was seen when the $N^1$-substitution pattern of anthryl-polyamines is altered (e.g. Ant44 (5a) vs AntNEt44 (45b)), and poor PTS selectivity was seen upon $N^1$-ethylation. When the amine is moved further away from the anthracene core, the PTS selectivity dramatically increased. However, moving it too far away from the anthracene core decreased PTS selectivity. This relationship prompted the question: would moving the polyamine message of **44a** and **44b** away from the macrocyclic core subsequently increase the PTS selectivity? It was also of interest to see if moving the polyamine message also affected the cytotoxicity and the anti-migration ability of the compound. Chapter 3 addresses these questions through the synthesis and bioevaluation of compounds **46-47** and their comparison to compounds **44a** and **44b**. Note that compounds **46** and **47** retain the secondary amine center at the $N^1$ position, which was shown to be an essential element for PTS-targeting. Dramatic improvements were observed. These structural changes resulted in a 29-fold decrease in toxicity and a doubling of the anti-migration potency of the drug platform.
III.3 Results and Discussion

**Synthesis.** The synthesis of 44\textsubscript{a} was previously described by Goldring *et al.*\textsuperscript{46}, Fürstner *et al.*\textsuperscript{47}, Williams *et al.*\textsuperscript{39a}, and Kaur *et al.*\textsuperscript{25}, while the synthesis of 44\textsubscript{b} was previously described by Kaur *et al.*\textsuperscript{25}.

The syntheses of the four remaining compounds began with the synthesis of 46\textsubscript{a} and 46\textsubscript{b}. As shown in Scheme III-1, reductive amination of commercially available ketone 48 with Boc-protected polyamines (49\textsubscript{a} or 49\textsubscript{b})\textsuperscript{36, 48} gave the respective compounds 50\textsubscript{a} and 50\textsubscript{b} in moderate yield (54\% and 75\%, respectively). These two compounds were then each treated with 4 $M$ HCl in EtOH to remove the Boc groups and provided 46\textsubscript{a} and 46\textsubscript{b} in high yield (98\% and 99\%, respectively). The overall yields for compounds 46\textsubscript{a} and 46\textsubscript{b} were 53\% and 74\%, respectively, from ketone 48.
Scheme III-1. Synthesis of 46a and 46b

Reagents: (a) NaBH(OAc)$_3$, AcOH, CH$_2$Cl$_2$; (b) 4 M HCl, EtOH

In contrast, the synthesis of the extended motifs 47a and 47b proved to be more challenging as the generation of aldehyde 53 was more difficult than initially thought (Scheme III-2). The first attempt at generating aldehyde 53 was through the use of (methoxymethyl)triphenylphosphonium chloride in a classic Wittig reaction to generate the vinyl ether, followed by hydrolysis. Generation of the vinyl ether failed while trying several base sources (BuLi, NaHMDS, NaH) where only the starting ketone 48 was recovered. The second attempt utilized Vilsmeier-Haack conditions with POCl$_3$ in DMF to generate (Z)-2-chlorocyclopentadec-1-enecarbaldehyde followed by reduction with H$_2$ and Pd/C. The alkene intermediate was generated fairly smoothly, albeit at 70% conversion. The subsequent reduction method was unsuccessful at reducing the alkene intermediate to the final desired aldehyde 53. While it was unclear why these methods failed, steric crowding may have played a role.

After two unsuccessful attempts at generating aldehyde 53, a longer synthetic route was chosen. This route began with commercially available ketone 48 and a standard Wittig reaction.
with methyltriphenylphosphonium iodide, to generate alkene 51 in 69% yield. Alkene 51 was then subjected to hydroboration conditions, followed by oxidation of the resultant alcohol with PCC to generate aldehyde 53 which was used without further purification. Reductive amination of aldehyde 53 with Boc-protected polyamines 49a and 49b generated 54a and 54b in 36% and 51%, respectively. These Boc-protected compounds were then deprotected with 4 M HCl to give 47a and 47b in 92% and 95% yields, respectively. The overall yields of 47a and 47b were 17% and 21% respectively from ketone 48.

**Scheme III-2.* Synthesis of 47a and 47b**

![Scheme III-2. Synthesis of 47a and 47b](image)

**Reagents:** (a) Ph₃PCH₃I, BuLi, THF, 0°C; (b) BH₃·THF, 0°C then H₂O₂, 3 M NaOH, RT; (c) PCC, CH₂Cl₂; (d) 49a₄⁸ or 49b₆⁶, NaBH(OAc)₃, AcOH, CH₂Cl₂; (e) 4 M HCl, EtOH

**Biological Evaluation.** Once synthesized, the conjugates were screened for toxicity in L3.6pl, CHO, and CHO-MG* cells. L3.6pl cells were selected as a human metastatic pancreatic cancer cell line. CHO cells were chosen along with a mutant PTS-deficient line (CHO-MG*) in order to comment on polyamine transport selectivity.₃c-e, ₇
**CHO and CHO-MG* Studies.** *IC*₅₀ and *MTD Determinations.* CHO cells were chosen along with a mutant cell line (CHO-MG*) to comment on how the synthetic conjugates gain access to the cells. As discussed earlier, the CHO-MG* cell line is polyamine-transport deficient and represents a model for alternative modes of entry (other than PTS) including passive diffusion or use of another transporter. The CHO cell line on the other hand, represents cells with high polyamine transport activity. A comparison of toxicity in these two CHO cell lines allowed for a screen that would detect selective use of the polyamine transporter. High utilization of the PTS would be very toxic to CHO cells. However, the CHO-MG* cells should be less sensitive to drugs which target the PTS. Ultimately, a CHO-MG*/CHO IC₅₀ ratio was determined to assess PTS selectivity. A high IC₅₀ ratio (>>2) would be observed for PTS selective compounds. The results obtained with compounds 44, 46, and 47 are shown in Table III-1.
Table III-1. Biological Evaluation of Motuporamine Derivatives (44, 46, and 47) in CHO and CHO-MG* Cells$^{a,b,c,d}$

<table>
<thead>
<tr>
<th>Compound</th>
<th>CHO-MG* IC$_{50}$ (µM)</th>
<th>CHO IC$_{50}$ (µM)</th>
<th>IC$_{50}$ ratio$^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>44a (Motu33)</td>
<td>2.96 (±0.1)</td>
<td>2.90 (±0.2)</td>
<td>1</td>
</tr>
<tr>
<td>44b (Motu44)</td>
<td>4.67 (±0.9)</td>
<td>4.38 (±0.3)</td>
<td>1</td>
</tr>
<tr>
<td>46a (MotuN33)</td>
<td>5.95 (±0.5)</td>
<td>2.84 (±0.2)</td>
<td>2.1</td>
</tr>
<tr>
<td>46b (MotuN44)</td>
<td>5.18 (±0.9)</td>
<td>4.53 (±0.2)</td>
<td>1</td>
</tr>
<tr>
<td>47a (MotuCH$_2$33)</td>
<td>87.3 (±11)</td>
<td>82.9 (±20)</td>
<td>1</td>
</tr>
<tr>
<td>47b (MotuCH$_2$44)</td>
<td>45.5 (±1.4)</td>
<td>47.8 (±2.4)</td>
<td>1</td>
</tr>
</tbody>
</table>

$^{a}$CHO and CHO-MG* cells were incubated with 1 mM AG for 24 h prior to drug addition. $^{b}$The ratio denotes the (CHO-MG*/CHO) IC$_{50}$ ratio, a measure of PTS selectivity. $^{c}$Cells were incubated for 48 h at 37°C with the respective conjugate. $^{d}$All experiments were run in triplicate.

As reported earlier, dihydromotuporamine C (44a) and its analogue 44b did not exhibit PTS selectivity as their IC$_{50}$ ratio in the original CHO-MG line and CHO were both 1.25 This was verified again to be the case in CHO and CHO-MG* as both 44a and 44b exhibited a CHO-MG*/CHO IC$_{50}$ ratio of 1. Low CHO-MG*/CHO IC$_{50}$ ratios were observed for analogues 46a, 46b, 47a, and 47b demonstrating a general lack of PTS selectivity.

Despite this apparent lack of PTS selectivity, an interesting general trend in cytotoxicity was observed in both CHO and CHO-MG*. As the amine at the N$^1$ position was moved away from the macrocyclic core, the cytotoxicity decreased. This was most dramatic for 47a and 47b where a methylene spacer was present between the amine at the N$^1$ position and the macrocyclic
core. It is also interesting to note that the 4,4-analogue 47b (CHO IC$_{50}$: 47.8 µM) was roughly
twice as toxic as the 3,3-analogue 47a (CHO IC$_{50}$: 82.9 µM).

**L3.6pl Studies. IC$_{50}$ and MTD Determinations.** Compounds 44, 46 and 47 were also
tested in the metastatic human pancreatic cancer cell line, L3.6pl.$^{32b, 37}$ This cell line was chosen
due to its $K$-$RAS$ mutation which has been linked to increased polyamine uptake.$^{32b, 37}$ This
increase in polyamine uptake lends itself to being exploited by polyamine-based drug conjugates.
These two features made this cell line an ideal candidate for evaluating potentially anti-
metastatic polyamine-based drug conjugates.

When compounds 44, 46, and 47 were initially tested in L3.6pl their IC$_{50}$ values (Table
III-2) were nearly identical to the IC$_{50}$ values found in CHO and CHO-MG*. These IC$_{50}$ results
were again interesting as they showed that as the polyamine message was moved away from the
macroyclic core, the cytotoxicity decreased, and was most evident for 47a and 47b. The MTD
was determined for each of these compounds (Table III-2). This dosing information was
important as it allowed each compound’s anti-migration properties to be assessed at its own
MTD and also at the MTD of the parent compound, 44a (0.6 µM) in subsequent wound healing
assays.
Table III-2. Biological Evaluation of Motuporamine Derivatives (44, 46, and 47) in L3.6pl Cells<sup>a,b,c</sup>

<table>
<thead>
<tr>
<th>Compound</th>
<th>L3.6pl IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>L3.6pl MTD (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>44a (Motu33)</td>
<td>0.99 (±0.07)</td>
<td>0.6</td>
</tr>
<tr>
<td>44b (Motu44)</td>
<td>1.8 (±0.2)</td>
<td>1.0</td>
</tr>
<tr>
<td>46a (MotuN33)</td>
<td>2.8 (±0.2)</td>
<td>2.0</td>
</tr>
<tr>
<td>46b (MotuN44)</td>
<td>2.8 (±0.1)</td>
<td>2.0</td>
</tr>
<tr>
<td>47a (MotuCH&lt;sub&gt;2&lt;/sub&gt;33)</td>
<td>89.4 (±5.4)</td>
<td>80</td>
</tr>
<tr>
<td>47b (MotuCH&lt;sub&gt;2&lt;/sub&gt;44)</td>
<td>48.7 (±2.8)</td>
<td>40</td>
</tr>
</tbody>
</table>

<sup>a</sup>L3.6pl cells were incubated with 250 µM AG for 24 h prior to drug addition. <sup>b</sup>Cells were incubated for 48 h at 37°C with the respective conjugate. <sup>c</sup>All experiments were run in triplicate.

Note: Steep cytotoxicity curves were observed and as a result the MTD was often near the respective IC<sub>50</sub> values.

Wound Healing Assay. A wound healing assay was employed to evaluate the anti-migration properties of compounds 44, 46, and 47 in L3.6pl cells. This involved growing a monolayer of L3.6pl cells and then scraping the monolayer with a sterile pipet tip to create a cell free channel in the middle of the well.<sup>49</sup> The experiment was run in 96-well plate format. Wound healing was measured by time-course imaging of each well using a computer-controlled microscope with an x,y,z stage which allowed for reproducible imaging over time. Each drug was evaluated for its dose-dependent inhibition of cell migration. Each compound was evaluated at its own MTD in order to avoid cytotoxic effects which are well known to bias migration studies.<sup>39a</sup> In addition, each compound was also tested at the MTD of 44a (Motu33) to directly
compare the potency of these compounds at the same concentration. These findings are reported in Table III-3.

**Table III-3. Inhibition of L3.6pl Cell Migration by Compounds 44, 46, and 47**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µM)</th>
<th>% Cell Migration Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Untreated</td>
<td>0 (±1.5)</td>
</tr>
<tr>
<td><strong>44a</strong> (Motu33)</td>
<td>0.6</td>
<td>20.3 (±1.8)</td>
</tr>
<tr>
<td><strong>44b</strong> (Motu44)</td>
<td>0.6</td>
<td>16.7 (±0.8)</td>
</tr>
<tr>
<td><strong>46a</strong> (MotuN33)</td>
<td>0.6</td>
<td>19.3 (±1.3)</td>
</tr>
<tr>
<td></td>
<td>2 (±0.1)</td>
<td>32.9 (±0.1)</td>
</tr>
<tr>
<td><strong>46b</strong> (MotuN44)</td>
<td>0.6</td>
<td>31.4 (±4.1)</td>
</tr>
<tr>
<td></td>
<td>2 (±0.5)</td>
<td>33.4 (±4.1)</td>
</tr>
<tr>
<td><strong>47a</strong> (MotuCH₂33)</td>
<td>0.6</td>
<td>38.4 (±4.2)</td>
</tr>
<tr>
<td></td>
<td>80 (±3)</td>
<td>44.8 (±2.2)</td>
</tr>
<tr>
<td><strong>47b</strong> (MotuCH₂44)</td>
<td>0.6</td>
<td>29.6 (±1.6)</td>
</tr>
<tr>
<td></td>
<td>40 (±3)</td>
<td>45.3 (±3.7)</td>
</tr>
</tbody>
</table>

*a*L3.6pl cells were incubated with 250 µM AG for 24 h prior to drug addition. *b*Cells were incubated for 48 h at 37°C with the respective conjugate. *c*MTD of the respective conjugate. *d*% cell migration was determined with ImageJ software by determining the area not occupied by cells (white space upon microscopic examination: WS) and calculated by \[\frac{(\text{WS at 0 h})-(\text{WS at 48 h})}{\text{WS at 0 h}}\] x 100%. *e*% cell inhibition was calculated by \[1-(\% \text{ cell migration at 48 h})/(\% \text{ cell migration at 0 h})\] x 100%. The untreated control cells migrated 48% of the t=0 wound space. *f*The microscope was equipped with a computer controlled x,y,z pneumatic stage (Zeiss Stemi Microscope) which allowed for reproducible imaging of each well over time. *g*All experiments were run in triplicate and averaged to provide the data above.

When the 3,3-triamine containing systems **44a**, **46a**, and **47a** were compared at 0.6 µM, it was seen that as the N1 amine center was moved away from the macrocyclic ring
(44a→46a→47a), an increase in cell migration inhibition was observed. Compound 47a (MotuCH₂33) had the highest inhibition observed in the series. The corresponding 4,4-triamine series at 0.6 μM showed a dramatic increase in anti-migration ability upon moving the polyamine outside the ring (44b→46b≈47b). This finding was especially interesting for compounds 47a and 47b, which both showed MTDs that were much higher than 0.6 μM (>130-fold and >60 fold, respectively). In this regard these compounds are much less toxic than the parent 44a. This large therapeutic window makes these compounds ideal candidates to pursue in further studies in vivo. All compounds tested in this assay also demonstrated a dose-dependent response. However, dramatic increases in cell migration inhibition were not observed for higher doses, suggesting that the target of these compounds may be easily saturated.

The significant difference in cytotoxicity between 47a and 47b suggested that the polyamine tail, especially norspermidine, provides a means to modulate the cytotoxicity of future analogues. From a synthetic standpoint, this finding is advantageous as the Boc-protected norspermidine tail can be produced in fewer steps than its homospermidine counterpart.

Taking pictures of each wound needed to be done under a 2.5x objective in order to view ~95% of each well. Unfortunately, this objective led to some dark areas as seen in Figures III-3 through III-8. This problem was alleviated through the use of the ImageJ software. This software allowed for enhancing the contrast between areas which were occupied by cells versus areas devoid of cells. In this regard, the software was able to accurately define the edges of each boundary. These capabilities then allowed for accurate area calculations which are seen in Table III-3.
Figure III-3. L3.6pl Cell Migration with 44a (Motu33) via Wound Healing Assay\textsuperscript{a,b}

\textsuperscript{a}L3.6pl cells were incubated with 250 µM AG for 24 h prior to drug addition. \textsuperscript{b}Cells were incubated for 48 h at 37°C with the respective conjugate.
Figure III-4. L3.6pl Cell Migration with 44b (Motu44) via Wound Healing Assay$^{a,b}$.

$^a$L3.6pl cells were incubated with 250 µM AG for 24 h prior to drug addition. $^b$Cells were incubated for 48 h at 37°C with the respective conjugate.
Figure III-5. L3.6pl Cell Migration with 46a (MotuN33) via Wound Healing Assay\textsuperscript{a,b}

\textsuperscript{a}L3.6pl cells were incubated with 250 µM AG for 24 h prior to drug addition. \textsuperscript{b}Cells were incubated for 48 h at 37°C with the respective conjugate.
Figure III-6. L3.6pl Cell Migration with 46b (MotuN44) via Wound Healing Assay\textsuperscript{a,b}

\textsuperscript{a}L3.6pl cells were incubated with 250 µM AG for 24 h prior to drug addition. \textsuperscript{b}Cells were incubated for 48 h at 37°C with the respective conjugate.
Figure III-7. L3.6pl Cell Migration with 47a (MotuCH$_2$33) via Wound Healing Assay$^{a,b}$

$^a$L3.6pl cells were incubated with 250 µM AG for 24 h prior to drug addition. $^b$Cells were incubated for 48 h at 37°C with the respective conjugate.
Figure III-8. L3.6pl Cell Migration with 47b (MotuCH₂44) via Wound Healing Assay$^{a,b}$

$^{a}$L3.6pl cells were incubated with 250 µM AG for 24 h prior to drug addition. $^{b}$Cells were incubated for 48 h at 37°C with the respective conjugate.
III.4 Summary

The IC$_{50}$/MTD determinations coupled with the wound healing assay showed that moving the polyamine message away from the macrocyclic ring had a profound effect on the biological properties of the motuporamine compounds. As stated earlier, compounds 44, 46, and 47 did not demonstrate PTS selectivity in the CHO-MG*/CHO screen. Biological evaluation in L3.6pl, CHO, and CHO-MG* showed that a carbon spacer between the $N^1$-amine and the macroyclic ring (i.e. 47a and 47b) dramatically reduced the cytotoxicity of this architecture. It was also demonstrated that a change in polyamine tail from norspermidine to homospermidine showed a profound effect on cytotoxicity and the magnitude and specificity of this effect depended on the system investigated. For example, the norspermidine derivative 44a was more toxic than its homospermidine derivative 44b. The opposite trend was observed for 47a and 47b. The fact that 46a and 46b had nearly identical toxicities suggests that the type and location of the appended polyamine chain influences the toxicity of the compound.

The wound healing assay demonstrated that moving the $N^1$-amine away from the macrocyclic ring not only reduced the cytotoxicity, but also greatly increased the anti-migration properties. When 47a (MTD: 80 µM) and 47b (MTD: 40 µM) were both dosed at the MTD of 44a (i.e., 0.6 µM), a dramatic increase in anti-migration ability was seen for 47a and 47b relative to the parent system, 44a. This large therapeutic window suggests that 47a and 47b are promising drug candidates for inhibiting the migration of aggressive metastatic cancers. These new leads are relatively non-toxic and are twice as potent as the parent 44a. In summary, new anti-metastatic drugs were identified for evaluation in future in vivo models of metastatic cancers.
III.5 Experimental

III.5.1 Materials.

Silica gel (32-63 µm) and chemical reagents were purchased from commercial sources and used without further purification. All solvents were distilled prior to use. All reactions were carried out under an N₂ atmosphere. ¹H and ¹³C spectra were recorded at 400 or 75 MHz, respectively. TLC solvent systems were listed as volume percents, and NH₄OH referred to concentrated aqueous NH₄OH. All tested compounds provided satisfactory elemental analyses.

III.5.2 Biological Studies.

CHO and CHO-MG* cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Note: the media must contain L-proline (2 µg/mL) for proper growth of the CHO-MG* cells. L3.6pl cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were grown at 37°C under a humidified 5% CO₂ atmosphere. Aminoguanidine (1 mM for CHO and CHO-MG*, and 250 µM for L3.6pl) was added to the growth medium to prevent oxidation of the drugs by the enzyme (bovine serum amine oxidase) present in calf serum. Cells in early to mid-log phase growth were used.

IC₅₀ Determinations. Cell growth was assayed in sterile 96-well microtiter plates (Costar 3599, Corning, NY, USA). CHO and CHO-MG* cells were plated at 10,000 cells/mL. L3.6pl cells were plated at 5,000 cells/mL. Drug solutions (10 µL per well) of appropriate concentration were added after an overnight incubation for each CHO cell line (90 µL of cell suspension). After exposure to the drug for 48 h, cell growth was determined by measuring
formazan formation from 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium, inner salt (MTS) using a SynergyMx BioTek microplate reader for absorbance (490 nM) measurements. A 4 h incubation period was used for the MTS assay in accordance with the manufacturer’s protocol.

**Wound Healing Assay.** Anti-migratory properties were assayed in 96-well microtiter plates (Costar 3599, Corning, NY, USA). A stock suspension of L3.6pl cells (5.5x10^5 cells/mL) was prepared. L3.6pl cells (90 µL of stock/well) were plated and allowed to grow to a confluent monolayer (48 h) at 37°C and 5% CO₂. Each well was then scraped with a 1 µL sterile pipet tip, photographed with a Zeiss Stemi Microscope (2.5x objective) (time=0), and then allowed to incubate for an additional 48 h period (time=48h). Over this 48 h period, the untreated control migrated to fill approximately 50% of the original wound. At time zero, the respective drug solutions (10 µL) were added and cell migration monitored after 48 h incubation at 37°C with 5% CO₂. After drug exposure for 48 h, respective time=48 h photographs were taken by the Zeiss Stemi Microscope. These pictures (time=0 and time=48 h) were then compared and analyzed through the use of ImageJ software by calculating the average area invaded by L3.6pl cells. The results are tabulated in Table III-3.

**III.5.3 Synthetic Procedures and Characterization**

N1-(3-Aminopropyl)-N3-cyclopentadecylpropane-1,3-diamine (46a). Compound 50a (450 mg, 0.8 mmol) was first dissolved in EtOH (60 mL) and stirred at 0°C for 10 min. A 4 M HCl solution (35 mL, 140 mmol) was then added dropwise and stirred for 30 min at 0°C under N₂. The reaction was then slowly warmed to room temperature and stirred under N₂ for 4 h. Upon completion, the solvent was removed in vacuo to give 46a as a white solid (365 mg, 98%)
$^1$H NMR (D$_2$O) $\delta$ 3.25 (m, 1H), 3.15 (t, 6H), 3.10 (t, 2H), 2.09 (m, 4H), 1.72 (m, 4H), 1.39-1.35 (m, 26H); $^{13}$C (D$_2$O) $\delta$ 58.6, 44.7, 41.9, 36.5, 28.7, 26.6, 26.3, 26.2, 26.0, 25.9, 23.7, 22.9, 22.4. HRMS (FAB) m/z calc for C$_{21}$H$_{45}$N$_3$ (M + H)$^+$ 340.3613, found 340.3693. Elemental Analysis: C$_{21}$H$_{48}$Cl$_3$N$_3$·0.05 H$_2$O theory C: 55.57, H: 10.68, N: 9.26; found C: 55.77, H: 10.65, N: 8.98.

N$^1$-(4-Aminobutyl)-N$^4$-cyclopentadecylbutane-1,4-diamine (46b). (1.53 g, 99%). $^1$H NMR (D$_2$O) $\delta$ 3.22 (m, 1H), 3.11 (t, 6H), 3.04 (t, 4H), 1.76-1.71 (m, 12H) 1.46-1.28 (m, 26H) $^{13}$C NMR (D$_2$O) $\delta$ 60.8, 60.5, 49.4, 46.9, 41.3, 31.4, 29.2, 28.9, 28.8, 28.6, 28.5, 26.4, 25.5, 25.1. HRMS (FAB) m/z calc for C$_{23}$H$_{49}$N$_3$ (M + H)$^+$ 368.3926, found 368.3983. Elemental Analysis: C$_{23}$H$_{52}$Cl$_3$N$_3$·0.05 H$_2$O theory C: 57.32, H: 10.90, N: 8.72; found C: 57.31, H: 10.95, N: 8.52.

N$^1$-(3-aminopropyl)-N$^3$-(cyclopentadecylmethyl)propane-1,3-diamine (47a).

Compound 54a (250 mg, 0.5 mmol) was first dissolved in EtOH (35 mL) and stirred at 0°C for 10 min. A 4 M HCl solution (17 mL, 68 mmol) was then added dropwise and stirred for 30 min at 0°C under N$_2$. The reaction was then slowly warmed to room temperature and stirred under N$_2$ for 4 h. Upon completion, the solvent was removed in vacuo to give 46a as a white solid (191 mg, 93%) $^1$H NMR (D$_2$O) $\delta$ 3.16 (t, 4H), 3.09 (t, 4H), 2.93 (d, 2H), 2.10 (m, 4H), 1.82 (m, 1H), 1.32 (m, 30H); $^{13}$C NMR (D$_2$O) $\delta$ 54.7, 47.3, 46.9, 38.7, 36.5, 31.6, 29.2, 28.8, 28.7, 28.6, 28.4, 26.0, 25.8, 24.8. HRMS (FAB) m/z calc for C$_{22}$H$_{47}$N$_3$ (M + H)$^+$ 354.3770, found 354.3846. Elemental Analysis: C$_{22}$H$_{50}$N$_3$Cl$_3$·0.15 H$_2$O theory C: 55.32, H: 10.61, N: 8.80; found C: 55.59, H: 10.85, N: 8.47.

N$^1$-(4-aminobutyl)-N$^4$-(cyclopentadecylmethyl)butane-1,4-diamine (47b). Compound 47b was generated using a procedure similar to that described for 47a. (80 mg, 95%). $^1$H NMR
(D$_2$O) $\delta$ 3.07 (t, 4H), 3.04 (t, 4H), 2.93 (d, 2H), 1.76 (m, 9H), 1.33 (m, 28H); $^{13}$C NMR (D$_2$O) $\delta$ 54.6, 49.8, 49.3, 41.2, 36.5, 31.8, 29.3, 29.9, 29.8, 28.7, 28.5, 26.3, 26.0, 25.3, 25.2, 25.0. HRMS (FAB) $m/z$ calc for C$_{24}$H$_{51}$N$_3$ (M + H)$^+$ 382.4083, found 382.4159. Elemental Analysis: C$_{24}$H$_{54}$N$_3$Cl$_3$·0.2 H$_2$O theory C: 56.45, H: 10.74, N: 8.23; found C: 56.48, H: 10.78, N: 8.29.

tert-Butyl (3-((tert-butoxycarbonyl)amino)propyl)(3-(cyclopentadecylamino)propyl)carbamate (50a). A solution of diBoc-norspermidine 49a (410 mg, 1.2 mmol) in CH$_2$Cl$_2$ (10 mL) was added dropwise to a solution of cyclopentadecanone 48 (250 mg, 1.1 mmol) in CH$_2$Cl$_2$ (10 mL) and allowed to stir at room temperature for 10 minutes. Sodium triacetoxyborohydride (1.2 g, 5.6 mmol) and acetic acid (67 mg, 1.1 mmol) were then added to the solution and allowed to stir at room temperature overnight. Once the reaction was complete by $^1$H NMR, the solution was then washed with aq. Na$_2$CO$_3$, and the organic layer was separated, dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated in vacuo. After column chromatography ($R_f=0.28$ (70% hexanes/24% CHCl$_3$/5.9% EtOH/0.1% NH$_4$OH)), 50a was isolated as a pale yellow oil (450 mg, 75%). $^1$H NMR (CDCl$_3$) $\delta$ 3.25 (t, 4H), 3.10 (t, 2H), 2.62 (m, 3H), 1.67 (m, 4H), 1.47-1.26 (m, 46H); $^{13}$C NMR (CDCl$_3$) $\delta$ 156.0, 80.3, 79.2, 57.4, 56.9, 44.2, 43.7, 37.7, 36.7, 32.6, 31.7, 28.9, 28.5, 28.3, 27.5, 26.9, 26.8, 26.6, 26.5, 23.5. HRMS (FAB) $m/z$ calc for C$_{31}$H$_{61}$N$_3$O$_4$ (M)$^+$ 540.4662, found 540.4734. Elemental Analysis: C$_{31}$H$_{61}$N$_3$O$_4$·0.05 H$_2$O theory C: 68.35, H: 11.31, N: 7.71; found C: 68.42, H: 11.24, N: 7.86.

tert-Butyl (4-((tert-butoxycarbonyl)amino)butyl)(4-(cyclopentadecylamino)butyl)carbamate (50b). (1.54 g, 54%). $^1$H (CDCl$_3$) $\delta$ 3.18 (t, 6H), 2.59 (t, 2H), 2.52 (m, 1H), 1.55 (m, 4H), 1.51-1.22 (m, 46H); $^{13}$C NMR (CDCl$_3$) $\delta$ 156.0, 155.5, 79.1, 77.3, 56.8, 47.1, 46.5,
40.2, 32.7, 28.5, 28.4, 27.7, 27.5, 27.4, 26.8, 26.6, 26.5, 26.2, 25.9, 25.5, 23.6. HRMS (FAB) m/z: calc for C_{33}H_{65}N_{3}O_{4} (M)^{+} 568.4975, found 568.5048. Elemental Analysis: C_{33}H_{65}N_{3}O_{4} theory C: 69.80, H: 11.54, N: 7.40; found C: 69.58, H: 11.64, N: 7.31.

**Methylenecyclopentadecane (51).** To a solution of methyltriphenylphosphonium iodide (12 g, 30 mmol) in THF (200 mL) was added dropwise n-BuLi (18.5 mL, 30 mmol) at 0°C. The suspension was stirred at 0°C until a yellow color persisted, and then cyclopentadecanone, 48, (2.21 g, 9.8 mmol) was added as a solution in THF (20 mL). The mixture was allowed to warm to room temperature and stirred overnight. Upon completion, H_{2}O was added to quench any remaining n-BuLi and any precipitates were filtered off. The filtrate was concentrated in vacuo and the residue was redissolved in CH_{2}Cl_{2}, washed three times with deionized H_{2}O. The organic layer was separated, dried over anhydrous Na_{2}SO_{4}, filtered, and then concentrated under reduced pressure to give a pale red oil. After column chromatography (R_{f}=0.9, 10% CH_{2}Cl_{2}/90% hexanes) 51 was isolated as a pale yellow oil (1.5 g, 69%). \(^1\)H NMR (CDCl_{3}) \(\delta\) 4.65 (s, 2H), 1.96 (t, 4H), 1.37 (m, 4H), 1.27-1.25 (m, 20H).\(^{51}\)

**Cyclopentadecylmethanol (52).** A solution of the starting alkene 51 (1.5 g, 6.7 mmol) in THF (8 mL) was added dropwise to a cooled 1 M solution of BH_{3}-THF (20.2 mL, 20.2 mmol). The reaction was stirred at 0°C for 1 h and then allowed to stir for 2 h at room temperature. Excess diborane was decomposed by dropwise addition of water (10 mL). A 3 M solution of NaOH (10 mL) was then added, followed by dropwise addition of 30% H_{2}O_{2} (10 mL). The reaction was stirred for 1 h at room temperature and K_{2}CO_{3} (100 mg) was then added. The layers were then separated and the aqueous layer was extracted three times with CH_{2}Cl_{2}, the organic
layers were pooled, dried over Na$_2$SO$_4$, then filtered and concentrated. After column chromatography ($R_f=0.31$, 50% hexanes/50% CH$_2$Cl$_2$) 52 was isolated as a yellow oil (1.2 g, 74%). $^1$H NMR (CDCl$_3$) $\delta$ 3.49 (t, 2H), 1.53 (m, 1H), 1.41-1.22 (m, 28H); $^{13}$C NMR (CDCl$_3$) $\delta$ 67.0, 39.5, 29.6, 27.5, 27.0, 26.7, 26.6, 26.5, 25.0.

**Cyclopentadecanecarbaldehyde (53).** To a solution of alcohol 52 (300 mg, 1.25 mmol) in CH$_2$Cl$_2$ (20 mL) was added PCC (404 mg, 1.87 mmol) at room temperature. The reaction was monitored by TLC (50% hexanes/50% CH$_2$Cl$_2$) until complete (60 min). Upon reaching completion, the mixture was then diluted with Et$_2$O, filtered through Celite and concentrated in vacuo to give aldehyde 53 as a yellow oil that was used without further purification (250 mg crude). $^1$H NMR (CDCl$_3$) $\delta$ 9.61 (s, 1H), 2.43 (m, 2H), 2.29 (m, 1H), 1.62 (m, 4H), 1.49 (m, 4H), 1.44-1.31 (m, 20H). This aldehyde was found to be unstable and was best used immediately in subsequent steps.

**tert-Butyl (3-(((tert-butoxycarbonyl)amino)propyl)(3-((cyclopentadecylmethyl)amino)propyl)carbamate (54a).**

A solution of diBoc-norspermidine 49a (496 mg, 1.5 mmol) in CH$_2$Cl$_2$ (12 mL) was added dropwise to a solution of the crude cyclopentadecanecarbaldehyde 53 (310 mg crude) in CH$_2$Cl$_2$ (12 mL) and allowed to stir at room temperature for 10 min. Acetic acid (72 µL, 1.26 mmol) was then added and the solution was stirred for 20 min, followed by the addition of sodium triacetoxyborohydride (1.33 g, 6.3 mmol) and stirred at room temperature overnight. Upon completion, the reaction mixture was washed three times with aq. 10% Na$_2$CO$_3$, the organic layer was separated, dried over anhydrous Na$_2$SO$_4$, filtered and concentrated in vacuo. After
column chromatography ($R_f$=0.25; 94.5% CH$_2$Cl$_2$/5% MeOH/0.5% NH$_4$OH), 54a was isolated as a pale yellow oil (250 mg, 36% over 2 steps). $^1$H NMR (CDCl$_3$) $\delta$ 3.24 (t, 4H), 3.11 (t, 2H), 2.73 (d, 2H), 2.62 (m, 4H), 2.48 (t, 2H), 1.92 (m, 1H), 1.70 (m, 4H), 1.48-1.26 (m, 46H); $^{13}$C NMR (CDCl$_3$) $\delta$ 156.1, 80.1, 79.5, 79.2, 54.6, 54.0, 46.2, 46.1, 44.2, 43.7, 37.4, 36.1, 30.6, 29.0, 28.7, 28.4, 28.2, 27.6, 27.1, 26.9, 26.8, 26.6, 26.5, 24.7, 24.5. HRMS (FAB) $m/z$ calc for C$_{32}$H$_{63}$N$_3$O$_4$ (M + H)$^+$ 554.4819, found 554.4894. Elemental Analysis: C$_{32}$H$_{63}$N$_3$O$_4$·0.05 H$_2$O theory C: 68.79, H: 11.38, N: 7.52; found C: 68.74, H: 11.30, N: 7.27.

tert-Butyl (4-((tert-butoxycarbonyl)amino)butyl)(4-((cyclopentadecylmethyl)amino)butyl)carbamate (54b). Compound 54b was synthesized using a similar procedure as 54a. (100 mg, 45% over 2 steps). $^1$H NMR (CDCl$_3$) $\delta$ 3.15 (t, 6H), 2.60 (t, 2H), 2.47 (d, 2H), 1.52 (m, 9H), 1.51-1.42 (s, 18H), 1.39-1.23 (m, 28H); $^{13}$C NMR (CDCl$_3$) $\delta$ 156.0, 156.6, 79.2, 54.8, 49.8, 46.9, 46.7, 40.3, 36.9, 31.0, 28.5, 28.4, 27.6, 27.4, 27.0, 26.8, 26.7, 26.5, 24.8. HRMS (FAB) $m/z$ calc for C$_{34}$H$_{67}$N$_3$O$_4$ (M + H)$^+$ 582.5132, found 582.5213. Elemental Analysis: C$_{34}$H$_{67}$N$_3$O$_4$·0.05 H$_2$O theory C: 69.59, H: 11.53, N: 7.16; found C: 69.59, H: 11.24, N: 7.22.
$^1$H NMR Spectrum of 6b in D$_2$O. (Scheme I-1)
$^1$H NMR Spectrum of 6b in D$_2$O.

(Scheme I-2)
$^1$H NMR Spectrum of 6b in D$_2$O.

(Scheme I-4)

$\bullet$ 6 HCl
$^{13}$C NMR Spectrum of 6b in D$_2$O.

(Scheme I-4)
$^1$H NMR Spectrum of 7b in D$_2$O.

$\cdot 6$ HCl
$^{13}$C NMR Spectrum of 7b in D$_2$O.

•6 HCl
$^1$H NMR Spectrum of 8b in D$_2$O.

-6 HCl
$^{13}$C NMR Spectrum of 8b in D$_2$O.

![Chemical structure and NMR spectrum](image-url)
$^1$H NMR Spectrum of 10 in CDCl$_3$. 

\[
\text{HN} \quad \text{Boc} \quad \text{OH}
\]
$^1$H NMR Spectrum of 11 in CDCl$_3$. 
\(^1\)H NMR Spectrum of 12 in CDCl\(_3\).
$^1$H NMR Spectrum of 13 in CDCl$_3$. 

\[ 	ext{HN} \rightarrow \text{Boc} \quad \text{Boc} \rightarrow \text{N} \rightarrow \text{OH} \]
$^1$H NMR Spectrum of 14 in D$_2$O.

$\text{H}_2\text{N} - \text{H} - \text{O} \text{H}$

$\cdot 2 \text{HCl}$
$^1$H NMR Spectrum of 15 in CDCl$_3$. 
$^{13}$C NMR Spectrum of 15 in CDCl$_3$. 
$^{1}$H NMR Spectrum of 16 in CDCl$_3$. 
$^{13}$C NMR Spectrum of 16 in CDCl$_3$. 

![NMR Spectrum Image]
$^1$H NMR Spectrum of 17 in CDCl$_3$. 
$^1$H NMR Spectrum of 18 in CDCl$_3$. 

![NMR spectrum of 18 in CDCl$_3$.]
$^1$H NMR Spectrum of 19 in CDCl$_3$. 

\[
O\overset{\makebox[0.5cm]{N}}{\makebox[0.5cm]{\textbullet}}\overset{\makebox[0.5cm]{\textbullet}}{\makebox[0.5cm]{\textbullet}} \text{H}_{\text{OH}} 
\]
$^1$H NMR Spectrum of 20 in CDCl$_3$. 

![NMR Spectrum Image]

Chemical shifts: 1.09, 0.89, 0.85.
$^1$H NMR Spectrum of 21 in CDCl$_3$. 

![NMR Spectrum Image]
$^1$H NMR Spectrum of 22 in CDCl$_3$. 

Boc

\[ \text{N} \quad \text{OMs} \]
$^1$H NMR Spectrum of 23 in CDCl$_3$
\(^1\)H NMR Spectrum of 24 in CDCl\(_3\)
$^1$H NMR Spectrum of 25 in CDCl$_3$. 

[Chemical structure image]
$^1$H NMR Spectrum of 26 in CDCl$_3$. 

![H NMR Spectrum of 26 in CDCl$_3$.](image)
$^1$H NMR Spectrum of 27 in CDCl$_3$. 

![NMR Spectrum Image]
\(^1\)H NMR Spectrum of 28 in CDCl\(_3\).

(Scheme I-3)
$^1$H NMR Spectrum of 28 in CDCl$_3$.

(Scheme I-4)
$^{13}$C NMR Spectrum of 28 in CDCl$_3$.

(Scheme I-4)
$^1$H NMR Spectrum of 29 in CDCl$_3$.

(Scheme I-4)
$^{13}$C NMR Spectrum of 29 in CDCl$_3$.

(Scheme I-4)
$^1$H NMR Spectrum of 30 in CDCl$_3$. 
$^{13}$C NMR Spectrum of 30 in CDCl$_3$. 
$^1$H NMR Spectrum of 31 in CDCl$_3$. 
$^{13}$C NMR Spectrum of 31 in CDCl$_3$. 
APPENDIX B: CHAPTER TWO NMR SPECTRA
$^1$H NMR Spectrum of 33a in D$_2$O.

$^6$HCl
$^{13}$C NMR Spectrum of 33a in D$_2$O.

\begin{center}
\includegraphics[width=0.5\textwidth]{structure.png}
\end{center}

\begin{center}
$\cdot 6$ HCl
\end{center}
$^1$H NMR Spectrum of 33b in D$_2$O.

H$_2$NH$_2$NH$_2$NH$_2$NH$_2$NH$_2$H$_2$NH$_2$NH$_2$NH$_2$NH$_2$NH$_2$
$^{13}$C NMR Spectrum of 33b in D$_2$O.

•9 HCl
$^1$H NMR Spectrum of 34a in D$_2$O.

\*6 HCl
$^{13}$C NMR Spectrum of 34a in D$_2$O.

-6 HCl
$^1$H NMR Spectrum of 34b in D$_2$O.

$\bullet$8 HCl
$^{13}$C NMR Spectrum of 34b in D$_2$O.

- 8 HCl
$^1$H NMR Spectrum of 35 in D$_2$O.

H$_2$N-<chemistry>
  \begin{center}
  \begin{tabular}{c}
  \hline
  O \\
  H - H \\
  H - \text{NH}_2 \\
  \hline
  \end{tabular}
  \end{center}

•5 HCl
$^1$H NMR Spectrum of 38a in CDCl$_3$. 

![NMR Spectrum Image]
$^{13}$C NMR Spectrum of 38a in CDCl$_3$. 
$^1$H NMR Spectrum of 38b in CDCl$_3$. 

![NMR Spectrum Image]
$^{13}$C NMR Spectrum of 38b in CDCl$_3$. 

![NMR Spectrum Image]
$^1$H NMR Spectrum of 40a in CDCl$_3$. 
$^{13}$C NMR Spectrum of 40a in CDCl$_3$. 

![C NMR Spectrum of 40a in CDCl3.](image)
$^1$H NMR Spectrum of 40b in CDCl$_3$. 

![Chemical Structure]
$^{13}$C NMR Spectrum of 40b in CDCl$_3$. 
APPENDIX C: CHAPTER THREE NMR SPECTRA
$^1$H NMR Spectrum of 46a in D$_2$O.

- $\cdot 3$ HCl
$^{13}$C NMR Spectrum of 46a in D$_2$O.

• 3 HCl
$^1$H NMR Spectrum of 46b in D$_2$O.

$\cdot 3$ HCl
$^{13}$C NMR Spectrum of 46b in D$_2$O.

$\cdot$3 HCl
$^1$H NMR Spectrum of 47a in D$_2$O.

$\cdot 3$ HCl
$^{13}$C NMR Spectrum of 47a in D$_2$O.

\[
\begin{align*}
\text{H} & \quad \text{H} \\
\text{N} & \quad \text{N} \\
& \quad \text{NH}_2
\end{align*}
\]

•3 HCl
$^1$H NMR Spectrum of 47b in D$_2$O.

•3 HCl
$^{13}$C NMR Spectrum of 47b in D$_2$O.

$\cdot$3 HCl
$^1$H NMR Spectrum of 50a in CDCl$_3$. 

![NMR Spectrum Diagram]
$^{13}$C NMR Spectrum of 50a in CDCl$_3$. 

![Carbon NMR Spectrum](image-url)
$^1$H NMR Spectrum of 50b in CDCl$_3$. 

![Chemical Structure](image)

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$^{13}$C NMR Spectrum of 50b in CDCl$_3$. 
$^1$H NMR Spectrum of 51 in CDCl$_3$. 
$^1$H NMR Spectrum of 52 in CDCl$_3$. 

![NMR Spectrum Image]
$^{13}$C NMR Spectrum of 52 in CDCl$_3$. 

![NMR Spectrum Diagram]
$^1$H NMR Spectrum of 53 in CDCl$_3$. 

![NMR Spectrum](image-url)
$^1$H NMR Spectrum of 54a in CDCl$_3$.
$^{13}$C NMR Spectrum of 54a in CDCl$_3$. 
$^{1}$H NMR Spectrum of 54b in CDCl$_3$. 

![NMR Spectrum of 54b in CDCl3]
$^{13}$C NMR Spectrum of 54b in CDCl$_3$. 
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