NOVEL COMPOUNDS INHIBITING HIV INFECTION, BREAST CANCER METASTASIS, AND BACTERIAL GROWTH

by

Joyce Brewer Shepard

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Dr. Martin Teintze

Approved for the Department of Chemistry and Biochemistry

Dr. Bern Kohler

Approved for The Graduate School

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DEDICATION

This work is dedicated first and foremost to Karen and Jeffrey Brewer. They deserve more than one dedication can give for being so supportive, listening, giving the best advice, and most of all, for loving so much. This is for you.

This is also dedicated to Dr. Eric Shepard, an *extraordinary* husband. Without Eric, there would be no thesis. His perseverance, confidence, strength, and optimism is catching. His support is unwavering and his love is unequaled. It is finally done.

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NOMENCLATURE

AMD3100 – bicyclam compound that binds to and inhibits CXCR4

AMD3465 – monocyclam compound that binds to and inhibits CXCR4

BEMA – Bisethylaminomelamine

CC_{50} – The concentration at which there is 50% cytotoxicity

CCR5 – a GPCR that HIV uses as a co-receptor

CNBr – Cyanogen Bromide

CXCR4 – a GPCR that the body uses for chemotaxis, angiogenesis, and cell survival

ECL2 – Extracellular Loop 2

GPCR – G-protein coupled receptor

HIV – Human Immunodeficiency Virus

IC_{50} – The concentration where there is 50% binding inhibition

MDA-MB-231 – Human breast cancer cell line

NB325 - Polymeric compound with multiple biguanide compounds

PHMB – Polyhexamethylene biguanide hydrochloride

R5 – HIV virus that utilizes CCR5 for cellular entry

SCID – Severe combined immunodeficiency disease

T140 – 14 amino acid peptide that binds to and inhibits CXCR4

TBAM – Trisbutylaminomelamine

TEAM – Trisethylaminomelamine

THAM – Trishexylaminomelamine

X4 – HIV virus that utilizes CXCR4 for cellular entry

ABSTRACT

We synthesized novel guanide, biguanide, phenylguanide, and naphthyguanide derived compounds on linear, branched, and dendrimer backbones that are effective inhibitors of HIV infection, breast cancer metastasis, and bacterial growth. HIV utilizes CXCR4 as a co-receptor for cellular entry. Blocking CXCR4 inhibits infection with X4 strains of the virus. Initial competition assays demonstrated that some of the phenylguanide compounds bound to CXCR4 with high affinity. The derivatives with high CXCR4 affinity inhibited X4 viral infection, but did not inhibit R5 or X4R5 viruses.

Importantly, many cancers overexpress CXCR4, including breast cancer. CXCR4 activation leads to cellular chemotaxis, angiogenesis, and cell survival, all of which promote cancer survival and proliferation. Compounds with high CXCR4 affinity were evaluated for inhibition of breast cancer metastasis. *In vitro* toxicity of all the derivatives was determined, followed by *in vitro* migration inhibition. Three derivatives with the best selectivity indexes for CXCR4 were examined in an *in vivo* lung colony metastasis assay. Spermidine trisphenylguanide (SI = 1785) was evaluated at 50 μM, 200 μM, and 300 μM and showed increasing inhibition of lung metastases (P = 0.34, 0.3, 0.02, respectively). Spermidine bis-2-naphthylguanide (SI = 1230) and spermine tris-2-naphthylguanide (SI = 191) were evaluated at 100 μM and showed significant reduction of lung metastases (P = 0.1 and 0.04, respectively).

The topical antiseptic biguanide chlorhexidine, is structurally similar to our derivatives. So, the derivatives were tested for antimicrobial activity against drug susceptible and resistant pathogenic *Enterococcus*, *Staphylococcus*, *Acinetobacter*, and *Pseudomonas* strains. THAM trisphenylguanide, DNT2300 biguanide and phenylguanide, and DNT2200 phenylguanide demonstrated broad spectrum bacteriocidal activity similar to chlorhexidine. Preliminary *in vivo* studies on mice treated with THAM trisphenylguanide either immediately after methicillin resistant *Staphylococcus aureus* (MRSA) infection or an hour post MRSA infection showed significant reductions in bacterial burden in the intraperitoneal cavity, heart and kidney in the immediate treatment group and slight reductions of bacterial burden in the one hour treatment group. This data shows the potential for treatment of MRSA infections with the tested compounds. Thus, different subsets of the novel guanide compounds discussed here can inhibit HIV infection, breast cancer metastasis, and bacterial growth.

CHAPTER ONE

INTRODUCTION

Novel Guanide, Biguanide, and Phenylguanide Compounds with Applications in HIV Infections, Breast Cancer Metastasis, and Growth Inhibition of Antimicrobial Resistant Bacteria

This thesis is comprised of four distinct chapters that discuss research projects related to HIV, breast cancer, and antibiotic resistant bacteria (Figure 1.1). The initial research project attempted to identify the amino acids within CXCR4 important for binding the peptide inhibitor T140. The crosslinking studies to discover the CXCR4 binding pocket were ongoing when a collaborator enlisted the Teintze lab to characterize NB325, a heterogeneous polybiguanide compound that bound to CXCR4. The inability to purify an anti-HIV active compound from the polymeric mixture resulted in the synthesis of discrete guanide, biguanide, and phenylguanide compounds on linear, branched, and dendrimeric backbones. The purified guanide compounds were subsequently tested for CXCR4 affinity, and those that were discovered to bind CXCR4 with reasonable binding affinity were then examined for anti-HIV activity. The compounds that bound to CXCR4 with the highest affinity were further examined for their ability to inhibit CXCR4 induced cancer metastasis. Tumor cells expressing CXCR4 have a survival advantage for migration, angiogenesis, and cell survival. The functional application of the guanide, biguanide, and phenylguanide compound family was further extended to antibacterial studies, as

the biguanide derivatives are structurally similar to well characterized antibacterial compounds effective against antibiotic resistant bacteria. Accordingly, all of the discrete guanide compounds that were synthesized were examined for antibacterial activity against strains of antibacterial susceptible and resistant bacteria.

Chapters two, three, and four of this thesis discuss the application of the discrete guanide, biguanide, and phenylguanide compounds in terms of HIV inhibition, inhibition of breast cancer metastasis, and antibacteriocidal activity, respectively (Figure 1.1). The results from the crosslinking study are contained in the appendix.

HIV Infection

Human Immunodeficiency Virus-1 (HIV-1) and HIV-2, lentiviruses in the family Retroviridae, are responsible for Acquired Immunodeficiency Disease (AIDS). HIV infects mostly macrophages and $CD4^+$ T-cells. Both of these cell types express CD4, which is the primary receptor for viral gp120, as well as the necessary co-receptors required for viral entry into the host cell. The two predominant co-receptors for HIV entry into the cell are G-protein coupled receptors (GPCRs) CCR5 and CXCR4 [1-4]. HIV viruses that utilize CCR5 for host entry are termed R5 or M-tropic viruses. Viruses that utilize CXCR4 for host entry are called X4 or T-tropic viruses. There are other co-receptors used by HIV to enter the cell, and these include CCR2b, CCR3, CCR8, and V28 [5], but these

receptors occur with less frequency than CXCR4 or CCR5. Initial infection by HIV is typically with R5 virus. Tropic switch to X4 virus typically happens late in the disease process and corresponds closely to the appearance of opportunistic infections characteristic of AIDS.

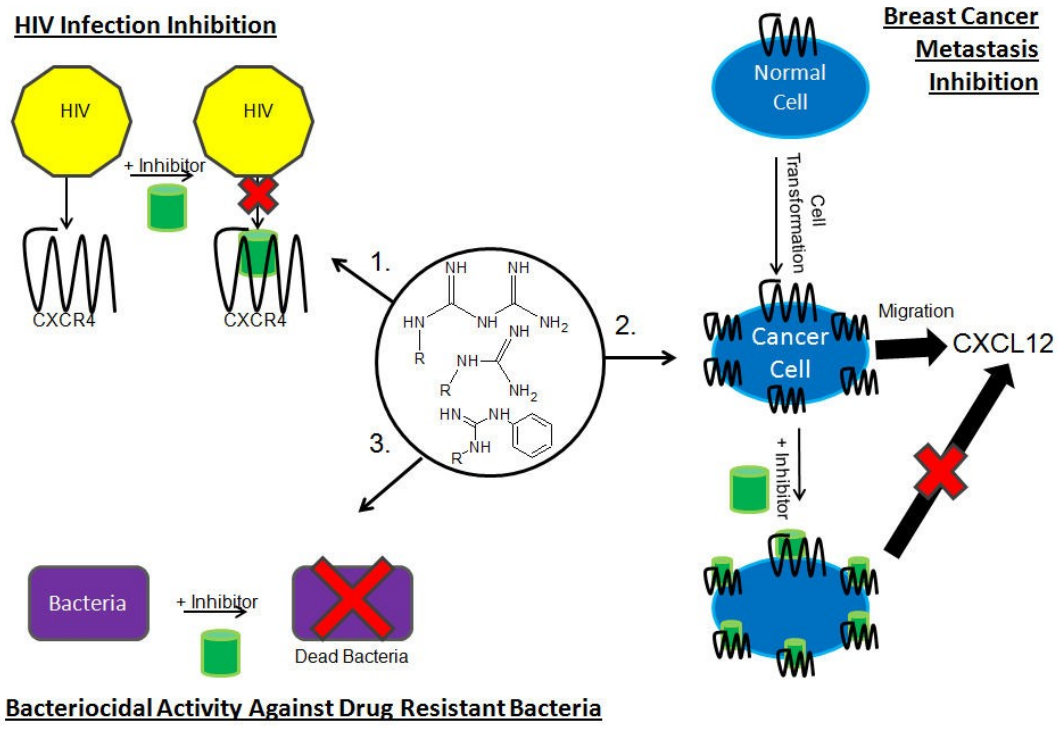


Figure 1.1. Guanide, biguanide, and phenylguanide functional groups (center) were synthesized in the Teinzte laboratory on a variety of backbone chains. These compounds were then tested for their effectivity in (1) inhibition of HIV infection by blocking the CXCR4 receptor, (2) preventing breast cancer metastasis through binding to the CXCR4 receptor, and (3) bacteriocidal activity in multidrug resistant bacteria.

Initial infection with the HIV virus results in non-diagnostic, non-specific symptoms, such as headaches, fever, or fatigue, that last only a short time. $CD4^+$ T-cell numbers initially decrease with a partial rebound after initial symptoms resolve. The intermediate infection phase is called the latent phase. Over time, $CD4^+$ T-cell numbers continue to decrease over the course of years until the absolute number falls below 200 cells per μL, at which point the official diagnosis of the patient changes to AIDS. Viral loads are typically high with initial infection, but then drop to low or, at times, undetectable levels until viral titers start to rise again around the time of AIDS diagnosis. Despite viral titers falling below the clinical detectable limit, there are always replicating HIV virus within the host body.

$CD4^+$ T-cells mostly function as T helper (T_h) cells that are necessary for the human body to mount an immune reaction to the presence of a foreign antigen. After a diagnosis of AIDS, the number of $CD4^+$ T-cells is too low for the body to mount an effective immune response against opportunistic infections that can arise from bacteria, viruses, and many eukaryotic pathogens. These opportunistic pathogens are typically associated with patient mortality.

Without treatment, almost all HIV patients develop AIDS and die within a few years of the AIDS symptoms. For most other viruses, the most effective strategy is use of a preventative vaccine, such as the influenza vaccine or the hepatitis B vaccine, which stimulates the host immune system to fight the virus prior to exposure to the active virus, and this prevents host viral infection. For

these types of viruses, most of the anti-viral vaccines work with high a success rate. However, HIV preventative vaccines have not worked effectively in humans. One reason is because HIV has a very high mutation rate due to poor efficiency at fixing replication errors during replication. The other main reason for the failure of HIV vaccines is the presence of a latent, non-replicating virus within host cells that cannot be targeted by the immune system. Despite the non-productive vaccine history, HIV vaccines are still being tested in clinical trials. One recent study utilized a vaccine of adenovirus type five that expressed the *gag*, *pol*, and *nef* HIV genes [6]. While this study was expected to yield good results, there were equal numbers of infections in the treated and untreated groups, resulting in a failure of the vaccine. The reasons for this vaccine failure are still being explored.

Current HIV treatment is predominantly carried out using anti-retroviral therapy (ART). Highly active ART treatment is a combination of drugs that target different aspects of the HIV lifecycle. There are currently six classes of anti-retroviral drugs. *Nucleoside/nucleotide reverse transcriptase inhibitors* are deoxynucleotide analogs that compete with natural nucleotides for incorporation into the growing strand as the reverse transcriptase transcribes the viral single stranded RNA into double stranded DNA. The analogs cause strand termination from the lack of the 3' hydroxyl group so the next deoxynucleotide cannot form a phosphodiester bond to it, thereby resulting in chain termination. *Non-nucleoside reverse transcriptase inhibitors* are non-competitive inhibitors that bind to the

reverse transcriptase protein and prevent it from continuing making the dsDNA (double stranded DNA). *Protease inhibitors* are drugs that target viral assembly. These inhibit the new virion from being properly assembled by binding to the proteases and preventing them from cleaving the polypeptide precursors into mature proteins. *Integrase inhibitors* prevent the integrase protein from integrating viral DNA into host DNA. *Maturation inhibitors* prevent maturation of the viral capsid by inhibiting the processing of the Gag protein which cleaves into the proteins that provide the basic physical infrastructures of the virus. The final class of antiretroviral treatments is the entry inhibitors. These drugs prevent HIV and cellular fusion or target the co-receptors required for HIV entry into the host cell. Enfuvirtide® by Trimeris (Hoffman-La Roche) is a fusion inhibitor that prevents viral gp41 from fusing with the host cell membrane. This therapy is effective at preventing membrane fusion but is extremely expensive. For this reason, this drug is not included in combination therapy and is typically reserved for patients who fail combination therapy. An example of a co-receptor inhibitor is Maraviroc®, a small hydrophobic molecule that binds to the co-receptor CCR5 and thereby prevents HIV from using it to enter the cell. Maraviroc® will be discussed in more detail in later.

While ART combination therapy has demonstrated an excellent ability to reduce viral load to below detectable levels for longer timeframes than normal, there are multiple drawbacks to ART treatment. The first drawback relates to the number of pills a patient must consume over the course of a 24 hour period. This

results from the fact that combination therapy utilizes not only multiple drugs, but relies on the need to take multiple doses of these drugs per day. If a patient misses a dose, the virus may eventually adapt and become resistant to those drugs being taken. The second drawback is that each of the drugs being consumed as part of the treatment regimen has side effects associated with them. The side effects for these drugs are extensive, and include nausea, diarrhea, pain, jaundice, liver dysfunction, and neurological disorders. These side effects can be multifarious, and leave a patient depressed and choosing between the drugs that keep the virus titers low and an acceptable quality of life. Thirdly, the drugs are very expensive. Combination therapy is unaffordable to many people who are infected with HIV. Even those who are able to afford it initially are facing a lifetime of bills due to the longevity of the typical treatment procedures. Low-income patients cannot afford these costs. The three drawbacks described above are by no means trivial, but the largest drawback to ART treatment relates to the fact that there is still no eradication of the virus from the patient. It is inevitable that the virus will eventually mutate to forms that are drug resistant, resulting in increasing viral titers and the eventual AIDS diagnosis. Due to these significant shortcomings, research is ongoing to attempt to discover new HIV treatments that hopefully will improve upon ART.

The newest approved HIV drug is an entry inhibitor Maraviroc® (Selzentry, Celsentri, UK-427857) developed by Pfizer [7-8]. Maraviroc is a CCR5 inhibitor that blocks M-tropic virus. HIV initially binds the viral protein gp120 trimer to the

CD4 receptor on the host cell surface. This is followed by a protein rearrangement that exposes a new binding site for the CCR5 co-receptor. After gp120 binding to the CCR5 co-receptor, the gp41 protein is exposed, which then facilitates membrane fusion between the cell and virus, allowing the viral nucleocapsid to be released inside the cell. Maraviroc® binds to the CCR5 protein at the same binding site as HIV and in doing so prevents HIV gp120 from binding to the co-receptor, thus inhibiting viral infection before it even begins. All of the drugs used in antiviral therapy except the fusion inhibitors work on stopping HIV after it has already infected the host cell. Maraviroc® is not currently used as part of conventional ART therapy but is used for patients who have failed ART therapy.

While Maraviroc® blocks the CCR5 co-receptor and therefore M-tropic virus, there is no compound to block CXCR4, the co-receptor that T-tropic virus can use to infect the host cell. R5 virus, which uses CCR5 for cellular entry, predominates through initial HIV infections and early in the infection timeline. However, patients who have failed ART therapy are typically late in the disease course when X4 virus predominates instead of R5 virus. Maraviroc® cannot protect the patient from X4 virus that uses CXCR4 for cellular entry. While R5 virus predominates early in infection, it is possible to have infection with a dual-tropic R5X4 virus that is capable of using either CCR5 or CXCR4 as a co-receptor. HIV infection with either R5X4 or X4 virus can still occur with

Maraviroc® treatment blocking only the CCR5 co-receptor. Hence, there is an urgent need for development of a CXCR4 inhibitor.

AMD3100 is an example of a CXCR4-directed inhibitor that successfully passed first and second stage clinical trials, but ultimately failed due to toxicity associated side effects. AMD3100 (Anormed/Genzyme) was found to have an EC_{50} value of 5 μg/mL with a CC_{50} value of >500 μg/mL, giving a therapeutic index (TI) of >100,000 [9-10]. This demonstrated CXCR4 selective binding without associated cytotoxicity. There was also evidence that AMD3100 may have prevented HIV induced cell death when blocking the CXCR4 receptor [11]. Despite these promising attributes, this compound ended up failing clinical trials due to cardiotoxicity and a lack of oral bioavailability. AMD3100 had to be injected intravenously in order to have activity in the body due to the lack of oral bioavailability. Interestingly, an unanticipated side effect was that treatment with AMD3100 resulted in mobilization of stem cells from the bone marrow into the blood circulation [9-10]. While this side effect was undesirable for chronic use in preventing HIV infection due to stem cell vulnerability to HIV infection, AMD3100 did end up passing clinical trials in 2007 for use in combination treatment with G-CSF (granulocyte-colony stimulating factor) in harvesting stem cells from the blood for stem cell transplants in certain diseases that did not include HIV. This AMD3100, G-CSF combination therapy ended up replacing the sometimes ineffective dose of G-CSF alone.

With the failure of AMD3100 in clinical trials partially due to a lack of bioavailability, additional studies were done to try and isolate similar compounds that would retain the ability to bind to CXCR4 at low concentrations, but be orally bioavailable for pill administration instead of requiring intravenous injection. One compound that came out of this work was AMD3465. AMD3465 was also developed by AnorMed and demonstrated that two bicyclam groups were not necessary for anti-HIV activity [12]. AMD3465 was composed of a monocyclam group, a phenyl ring in the center, and a pyridine group. This molecule carried a +3 charge instead of +4 like AMD3100. AMD3465 was ten-fold more effective as a CXCR4 antagonist but equally as effective in anti-HIV activity as AMD3100 [12]. While this compound was also not orally bioavailable, it was an important step in demonstrating that bicyclam structures were not the only compounds capable of inhibiting HIV infection through the CXCR4 receptor.

T140 is a 14 amino acid peptide comprised of N-R R Nal C Y R K $_{D}$-K P Y R Cit C R-COOH (Nal = Napthylalanine, Cit = Citrulline) that was developed from a horseshoe crab 22 amino acid polyphemusin peptide [13]. This peptide binds to CXCR4 at 0.43 nM in HIV infection assays[14]. The structure of this peptide is an anti-parallel beta sheet. There is one disulfide bond that holds the peptide in this conformation. The original horseshoe peptide had two disulfide bonds, but structure optimization led to the realization that only one disulfide was necessary for optimum CXCR4 binding. T140 has five amino acids that are essential for CXCR4 binding. Replacing Arg1, Arg2, Nal3, Tyr5, or Arg14 resulted in a

reduction in CXCR4 binding [15-16]. The center of the peptide is the least critical part of the structure in terms of binding activity, as each amino acid can be swapped out for other amino acids without a corresponding loss in activity. There are five different positively charged arginine groups in the T140 sequence with three of them located at or near the N- and C-terminal regions, while the remaining two are present in the middle of the peptide. There are also two lysine groups that are not important for CXCR4 binding and can be easily replaced with other amino acids [15-16].

In vitro evaluation of this peptide showed excellent potential for *in vivo* inhibition of HIV infectivity [17]. However, after much work characterizing this peptide and optimizing it for the best amino acid sequence for CXCR4 binding [18-19], it was discovered that the peptide rapidly degrades in serum [16]. The C-terminal arginine 14 amino acid, which is essential for binding to CXCR4, is readily cleaved off in serum. Amidating the C-terminus resulted in a more stable peptide structure in serum, but this also added an extra positive charge to the molecule, making it more toxic to cells in general. Further research on this peptide attempting to balance toxicity and activity led to many T140 derivatives but failed to find a single peptide that displayed acceptable toxicity, was stable in serum, and bound with equally high affinity to CXCR4 as T140 [20-22]. T140 still remains one of the top compounds for binding for CXCR4.

Other compounds such as ALX40-4C [23-24], the first co-receptor blocker to be tested in clinical trials, AMD11070 [25], and others that bind to CXCR4

were examined in clinical trials for HIV viral load reduction [23]. None of these peptide derivatives have yet to pass clinical trials.

A different compound, NB325, was a polydisperse PEHMB (polyethylene hexamethylene biguanide) designed by Dr. Mohamed Labib at Advanced BioDevices (Princeton, New Jersey). NB325 has a polymeric repeating unit of biguanide groups separated by alternating two and six (ethyl-, hexyl-) carbon linkages. This sequence is repeated one to four times giving the possibility of four to ten biguanide functional groups [26]. Compounds similar in structure to PEHMB had already been established as disinfectants such as PHMB (polyhexamethylene biguanide) in contact lens solution [27], and chlorhexidine digluconate as a vaginal disinfectant, mouthwash and surgical scrub.

NB325 binds to CXCR4, probably at the negatively charged extracellular loop 2 region [26, 28]. It has been shown to block HIV infection by overlapping the binding site of HIV-1 gp120 on CXCR4 on the second extracellular loop [26] and displays an IC_{50} (50% inhibitory concentration) value of 0.01% (~71μM) [26]. It is not toxic at or below 0.316%, and, therefore, was an excellent candidate for further evaluation as a HIV inhibitor. NB325 also inhibited CXCR12 induced cell migration in the body at concentrations as low as 0.0003%, proving that it may also inhibit gp120 induced apoptosis and chemotaxis [26, 29]. Interestingly, NB325 was also shown to weakly inhibit HIV infection through the other predominant co-receptor CCR5 due to an unidentified mechanism [30-31]. Due to the anti-HIV activity against both of the necessary co-receptors for HIV viral

entry, this compound was further evaluated for broad-spectrum microbicide use. Further evaluation of this compound in a cervicovaginal model showed low (0.5%) percentages of NB325 to not induce any undesirable side effects such as inflammation or epithelial cell disruption, but higher percentages (1%) did exhibit some epithelial cell sloughing which could be detrimental to HIV inhibition [31-32]. Remarkably, NB325 did show persistent X4 antiviral activities for up to eight hours in a cervicovaginal *in vivo* model [28, 31]. Interactions with CD4 positive T-lymphocytes may last up to 24 hours after exposure but inhibition of X4 infection decreases after eight hours [28].

Despite promising activity in preventing HIV infection predominantly via X4 virus, there were multiple drawbacks to NB325 that prevented it from going into clinical trials. One significant drawback to this polybiguanide compound was that it was extremely difficult to separate out discrete molecules from the NB325 amalgam with a defined number of biguanide groups in order to isolate the active compound(s). Dr. Royce Wilkinson (MSU, Bozeman) attempted to separate individual polymeric compounds using many different techniques such as reverse phase chromatography, size exclusion chromatography, and ion exchange chromatography but separation of one active component was not accomplished (data not published). Also, the polymeric mixture did not give an accurate mass for verification of the presence of the expected products. The mixture was examined for molecular weight on multiple mass spectrometers without the various theoretical polymeric masses being detected. NMR was attempted but

due to the heterogeneity of the mixture, no structure could be unambiguously deducted from the NMR spectrum. Side reactions, such as cyclizations, could also take place during the polymerization reaction, which was performed at high temperatures without solvents. This further complicated the attempts to isolate the active anti-HIV compounds from the mixture. Following the failure to separate out one active compound from the mixture, there was an effort to synthesize the same polymer in a stepwise manner under milder conditions in order to generate distinct polymers. These methods were also unsuccessful due to low yields at each successive step (data not published).

For this compound to be approved by the FDA as a topical microbicide that prevents HIV infection by CXCR4-utilizing virus, one individual active biguanide compound needed to be identified. Therefore, synthesis of discrete compounds similar in structure to the hypothesized polymeric products containing biguanide groups from NB325 was the next approach to determine an active biguanide compound that retains anti-HIV activity.

Along these lines, three series of discrete compounds were synthesized by Dr. Royce Wilkinson from a set of twelve starting polyamine compounds by converting some or all of the amine groups on the compounds into guanide, biguanide, or phenylguanide groups. Some of the twelve starting polyamines were linear and some were branched or dendrimer scaffolds [33]. The starting polyamine precursors were purchased from commercial sources with the exception of the melamine derivatives. Dr. Royce Wilkinson synthesized the

melamine precursors and then converted the primary amines on them into guanide, biguanide, or phenylguanide groups. Synthesis completion was verified through mass spectrometry and NMR to evaluate the reaction. The product was then purified using liquid chromatography [33].

These discrete derivatives may allow some insight into the active groups within the polydisperse preparation of NB325. We initially made compounds that separated biguanide groups by ethyl or hexyl groups, similar to the biguanide groups separated by alternating ethyl- and hexyl- chains in the NB325 theorized mixture. Additionally, amine precursor compounds were used that contained three and four carbon chains separating the amino groups [33]. The dendrimer starting compounds had four, six, or eight branches separating the amine groups. The melamine starting compounds were hypothesized from possible side reactions in the NB325 synthesis reaction. These compounds had three separate arms that separated the melamine center from the amine groups by an ethyl, butyl, or hexyl linking chain. The groups on these precursor amine compounds were converted into guanide, biguanide, or phenylguanide groups to try and identify the best compound for blocking HIV infection.

We expected to find compounds that had equal or better activity in inhibiting HIV infection through CCR5 and/or CXCR4 co-receptor binding as the parent NB325 mixture. Interestingly, the results showed that the synthesized compounds only had activity in preventing X4 HIV infection through CXCR4 binding. The hypothesis that the phenylguanide compounds similar in structure to

previously characterized CCR5 inhibitors (not discussed here) would likely bind to CCR5 was found to be incorrect; the phenylguanide derivatives synthesized herein were CXCR4 specific. Regardless, the guanide derived compounds showed better binding activity to the CXCR4 receptor than NB325, thus providing an important first step towards identifying a discrete guanide based derivative to be used in clinical trials.

Breast Cancer and Metastasis

In the field of cancer biology, there are numerous terms to describe similar cell types and cellular processes. While there is no universal definition of terms, there are a few that will be defined for this thesis for clarity purposes. These definitions are based on Henry Pitot's Fundamentals of Oncology [34].

A tumor is defined as a heritably altered, relatively autonomous growth of tissue. There are four independent terms within this definition that need to be highlighted. A tumor is *heritable*, meaning the genetic material will be passed to all progeny. *Relatively autonomous* refers to the fact that a cancer is not subject to all the regulations and rules that normally apply to individual cells, their interactions with each other, and the greater organ the cell is a part of. The qualification of 'relatively' in front of it means that the cancer is not completely autonomous and may only be autonomous in subtle ways such as *growth* regulation. The last qualification for this definition is that tumors originate only in *tissues*. This implies that tumors can only be in multicellular organisms and can

be thought of as the “plague of evolution” [34]. This is the basic definition of a tumor for this thesis. Neoplasia is defined as the basic disease process. Cancer will indicate a disease process that has biological characteristics of a malignant disease process.

I also want to define the difference between benign and malignant neoplasms. Malignant neoplasms are typically non-encapsulated, invasive, poorly differentiated, undergoing rapid growth that is anaplastic with common mitoses, and there are metastases present. Benign neoplasms are typically encapsulated, noninvasive, well differentiated, growing slowly, with no metastases present.

Metastasis occurs typically through cells traveling to adjacent or distant sites using one of three mechanisms, the blood circulation, the lymphatic system, or through trauma such as surgery. Often at the time of primary tumor diagnosis, the metastases are not apparent due to the fact that they form micrometastases that are minute tumor colonies below the detection limit of tumor identification technology.

The series of steps that entail the metastatic cascade is sometimes called the “invasion-metastasis cascade.” This series of steps begins with a fully formed benign primary tumor with a fully functional vasculature system. Benign tumors have to undergo an initial process where they acquire the ability to breach the basement membrane and surrounding stroma. This is typically where benign tumors become classified as malignant or cancerous and is classically called the

EMT (epithelial-mesenchymal transition). Cells going through EMT lose cell adhesion to surrounding cells such as the loss of E-cadherin, allowing the cells mobility. Once on the stroma side of the basement membrane, the malignant cells have better access to both local capillaries and local lymphatics. Cell movement through blood vessel or lymph walls and into the lumen of those vessels is typically termed intravasation. Cell movement out of those vessels is termed extravasation.

Travel through the lymph or blood vascular systems forces any cell to navigate and survive despite a lack of solid substrate attachments, a lack of supply of survival factors, and hydrodynamic shear forces of the circulation. However, once in the blood circulation, the tumor cells eventually make their way to the heart and out to the rest of the body, where they can extravase out of the circulation by using specific cell surface receptors to adhere to the vasculature walls and undergo MET (mesenchymal-epithelial transition). When the cells undergo MET, expression of E-cadherin allows cellular adhesion to the cells around it, anchoring it in place. Once adhered to the cell wall, the tumor cell must invade the endothelial cell lining the vasculature and/or the tissues of the vessel wall in order to enter into the new local environment.

Colonization of the new environment can be difficult for the tumor cells. The new tissue locations most likely express different types and levels of survival factors and/or growth factors required for the tumor cells to multiply and survive in the new environment. The tumor cells may then have one of multiple fates;

they may quickly die, turn dormant as micrometastases, or they may grow into a secondary metastasis capable of causing difficulties with or without symptoms for the patient. Colonization in a new environment is the rate limiting step for secondary metastases. While today's cell staining technology can identify many micrometastases throughout the body, only a few actually grow to macrometastasis size.

There are multiple theories for mechanisms of metastasis that are not necessarily mutually exclusive but are all possibilities that a tumor cell might use to metastasize depending on the type of tumor cell. One theory is the "seed and soil" theory initially proposed by Stephen Paget [35]. The seed and soil theory states that tumor cells traveling through the vasculature will metastasize to the next downstream organ on the blood pathway provided that the seed (tumor cell) can grow in a compatible tissue (soil). For breast tumor cells, the next organs on the blood stream pathway with small capillaries are the lungs. While this theory may explain many sites of secondary metastases, it cannot explain metastases that occur at sites that are not downstream in the blood circulation.

Another theory, and the one of focus for this thesis, is the theory of migration of tumor cells towards chemoattractants (chemokines) [34]. For this theory, target organs of metastasis release specific chemoattractants that actively recruit/attract circulating metastatic cells. This cell homing system can explain the sites of metastasis that are not downstream of the primary tumor. It can also explain the higher percentage of metastases at these less likely sites

than can be expected by the seed and soil theory. The one question still remaining for the chemoattractant theory is determination of whether the chemokine is actively homing the metastasizing tumor cells to them or if it is merely encouraging tumor cell colonization after the cells have already attached to the tissue site.

The third theory for metastasis location is the ZIP code theory [34]. This theory is based on the expression of tissue specific adhesion molecules on the vasculature luminal surface. Each tissue will express different adhesion molecules, such as integrins, that can be thought of as individual homing addresses that bind certain tumor cells better than others. The downfall of this theory is that tumor cells can be coated in platelets that would block the cell adhesion molecules from binding freely to the vasculature. While each of these three theories cannot explain all tumor metastases individually, combined they may explain the vast majority. The metastasis mechanism utilized by one tumor cell may be different than other tumor cells. Each tumor cell is different enough to even use multiple methods to localize to a secondary site to colonize. For example, the downstream organ may express high levels of a chemokine that increases colonization efficiency. The downstream organ for breast tumors is the lungs which also happen to express high levels of CXCL12 which can home breast tumors expressing high levels of the chemoreceptor CXCR4.

Breast cancer is diagnosed in one out of three women diagnosed with cancer in the United States [36]. It is the second most common malignancy

among women, second only to skin cancers, which are by far the most prevalent type of cancer and also happen to be the easiest to diagnose and remove. Increased access to mammograms and better treatment regimens resulted in a 2.2% decrease in the overall number of breast cancer deaths from 1990 to 2007. Projections for 2011 are for over 288,000 new cases of breast cancer diagnoses with the vast majority being invasive [36]. Approximately 39,000 deaths are projected for 2011 from breast cancer alone. Also of significance is that 20-30% of women with breast cancer will develop secondary metastases in their lifetime according to the American Cancer Society [37].

Invasive breast cancers are typically initially categorized into three subtypes based on cellular expression of certain receptors. These are the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2). Survival rates are based on the phenotype expression for each patient. ER+ and PR+ patients tend to have better prognosis versus triple negative patients (ER- PR- HER2-) who have the poorest prognosis [36].

Depending on the phenotypic expression of the breast cancer, there are now many different therapies available beyond the typical chemotherapy/radiation/surgery options. These therapies are specific to the expression profiles of the individual cancers and are considered more personalized therapy than standard chemotherapy; traditional, standard treatments are not personalized to a patient's own tumor. The three that are

discussed in greater detail below are Herceptin, Tamoxifen, and aromatase inhibitors. For the work described herein, we anticipate the new guanide derivative compounds to be classified into this group of personalized therapy based on CXCR4 expression.

Herceptin (trastuzumab), a monoclonal antibody that targets HER2/neu+ breast cancer cells, was approved by the FDA in 2006. This antibody is thought to function by binding to the cancer cell overexpressing the HER2 protein on the cell surface. The NK cells and macrophages target the antibody coated cell and kill the cell using a process coined “antibody dependent cellular cytotoxicity” (ADCC) [38]. Another mechanism that may contribute to Herceptin’s effectiveness is the internalization of the HER2 protein which results in both a sensitivity to apoptosis and deactivation of the oncoprotein nature of HER2 itself. Herceptin also interferes with HER2’s angiogenic effects, helping to reduce vital blood flow to the cancer [38].

Tamoxifen (Fareston, toremifene) made by AstroZeneca acts as a competitive inhibitor towards estrogen binding with the estrogen receptor and is used for treating ER+ breast cancers [39]. The estrogen receptor activation pathway results in TGF-α (transforming growth factor-α) expression and autocrine growth factor responses. Tamoxifen binding to the ER does not stimulate a TGF response and blocks the receptor from responding to estrogen and the resultant growth factors. Tamoxifen binding instead causes the cells to secrete TGF-β, which is a growth inhibitor [39].

Aromatase inhibitors are a class of compounds that are used to treat breast cancer in post-menopausal women [40]. Aromatase is the enzyme which is known to synthesize estrogen by converting androgens to estrogens. In pre-menopausal women, the ovaries are the primary source of estrogen, and this class of inhibitors is ineffective because the resultant stimulation of the hypothalamic-pituitary-ovarian axis overrides their effect. In postmenopausal women all estrogen is produced locally in extragonadal tissues where aromatase inhibitors work well. There are multiple drugs in this class including exemestane, letrozole, and anastrozole [40-41]. Aromatase inhibitors tend to be well tolerated for long term administration due to few side effects associated with their use [41].

Besides these commonly identified breast cancer markers, more recent studies have identified proteins that are upregulated in cancer cells. One receptor that is upregulated in some cancers is the GPCR CXCR4. Upregulation of CXCR4, however, is not limited merely to breast cancer. Research has found numerous cancers overexpressing CXCR4 that include, but are not limited to, breast [42], lung [43-44], prostate [45-46], renal [47], pancreatic [48], glioblastomas [49], gliomas [50], ovarian [51], B-cell chronic lymphocytic leukemias [52], and melanomas [53]. While CXCR4 is not overexpressed in every cancer patient with these cancers, overexpression of CXCR4 is correlated with malignancy, poor prognosis, and poor outcomes in most patients.

CXCR4 binds one characterized ligand termed CXCL12 (SDF-1). Activation of CXCR4 by CXCL12 ligand binding initiates intracellular signaling of

multiple divergent pathways. These pathways result in cellular chemotaxis, survival, proliferation, calcium fluxes, and gene transcription (Figure 1.2). CXCR4 is normally expressed on multiple different cell types such as T- and B-lymphocytes, monocytes, macrophages, CD34+ hematopoietic stem cells, neutrophils, eosinophils, endothelial cells, epithelial cells, astrocytes, and neuronal cells.

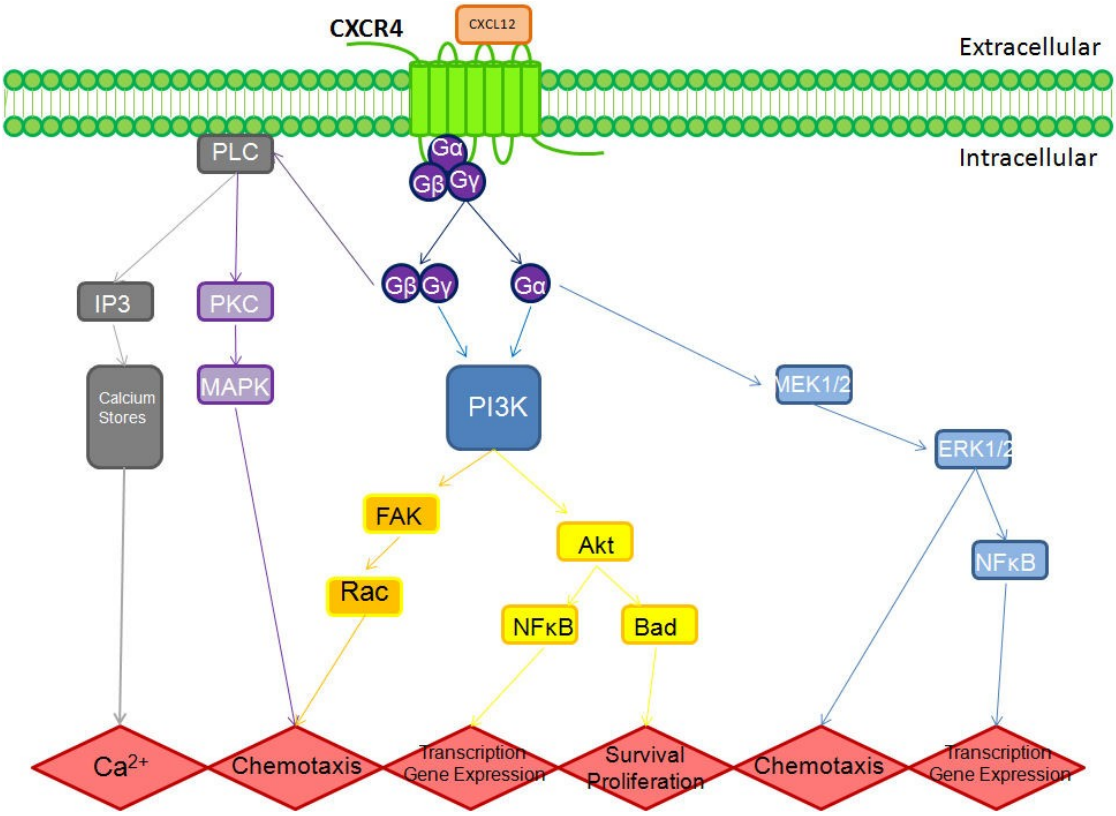


Figure 1.2. Intracellular pathways activated by CXCL12 binding to CXCR4.

CXCL12 binding to CXCR4 can initiate multiple divergent pathways. CXCR4+ chemotaxis is the predominant result of CXCR4 activation and is stimulated through the phosphatidylinositol 3-kinase (PI3 kinase) pathway, the

ERK 1/2 pathway, as well as the PKC (protein kinase C pathway) [54-57]. The body naturally expresses CXCL12 in order to recruit CXCR4 positive cells to areas such as the bone marrow or sites of injury, which commonly express all the factors needed to induce the cells to proliferate. The body upregulates expression of CXCL12 in response to tissue injury or stressors such as ischemia, bleeding, heart infarcts, and other hypoxic events in order to promote healing [54]. Attracting lymphocytes to the sites of injury may also be associated with inflammation and thereby associate CXCR4 expression with the corresponding tissue repair. Vascularization of damaged tissue is also critical andCXCR4+ cells are also recruited to these hypoxic sites to begin vascularization of the ischemic area [54, 58]. Cells that are recruited include hematopoietic cells, smooth muscle progenitors, and endothelial precursors which are capable of differentiating into vascular cells under the correct conditions.

The CXCR4/CXCL12 pathway can also trigger anti-apoptotic cell signaling through the Akt pathway, allowing cell survival [59]. Interestingly enough, the same CXCR4/CXCR12 activation can promote pro-apoptotic cell signaling in neuronal cells or in T cells when Akt is inhibited [59-61]. This data implicates CXCR4 in a pro-survival pathway, as well as a chemotactic pathway. CXCR4 activation can also result in the release of intracellular calcium flux and transcription of specific genes.

As mentioned above, many different tumor cells overexpress the CXCR4 receptor. Examining the normal outcomes of CXCL12 binding to CXCR4, effects

that include chemotaxis, survival, and angiogenesis, it is not hard to understand why tumor cells overexpressing this receptor propagate in the body, as all three of those outcomes are beneficial to tumor cells. Multiple laboratories have demonstrated tumor cell migration (chemotaxis) after exposure to CXCL12 revealing intracellular actin bundles that form pseudopodia followed by the tumor cells migrating directionally up a CXCL12 gradient [42, 62-66].

Inhibiting the CXCR4/CXCL12 interaction in tumor cells that utilize this pathway is an effective way of inhibiting tumor metastasis to secondary sites within the body. It may also have positive effects in reducing vascularization of metastatic neoplasms and cell survival. Tumor cells that do not overexpress the CXCR4/CXCL12 pathway would clearly not benefit from therapy targeting this pathway. Inhibition of the CXCR4 pathway resulting in reduced metastases has been demonstrated repeatedly in many different studies that are summarized below.

The first group to identify CXCR4 overexpression in breast cancer was Müller et al. in 2001 [42]. They identified the coincidence that the common sites of metastasis for breast cancer are the bone marrow, lymph nodes, lungs and the liver, which are all sites expressing high levels of CXCL12.They then proved that not only was CXCR4/CXCL12 the main reason for breast cancer metastases in those organs expressing CXCL12, but that neutralizing the CXCR4 receptor using an anti-human CXCR4 monoclonal antibody significantly impaired lung and lymph node metastases [42].

In 2004, Liang et al. also found high CXCR4 expression in malignant human breast cancers and corresponding low expression levels in normal breast tissue [67]. Liang showed that tumor metastasis could be inhibited by blocking the CXCR4 binding sites using a peptide very similar to T140. The group modified the T140 peptide in order to be more resistant to degradation in the serum by amidating the C-terminus and non-basic residues were substituted for basic residues in the sequence in an attempt to reduce the overall positive charge of the molecule. The *in vivo* studies in this report were performed using MDA-MB-231 human breast cancer cells in mice. The number of lung metastases found in the group treated with the CXCR4 antagonist was significantly lower than the group without the antagonist ($P = 0.0000044$) [67].

A subsequent study in 2006 utilized alexidine dihydrochloride as a potential new anticancer agent for head and neck cancers [68]. Alexidine dihydrochloride is a bisbiguanide compound with a structure similar to NB325 but with longer carbon chains separating the biguanide group. This was the first use of a bisbiguanide compound for anticancer activity. The paper demonstrated preferential killing of cancer cells *in vitro* without interfering with the actions of three other common chemotherapeutic agents. Yip et al. claim that alexidine dihydrochloride functioned by inducing apoptosis through caspase activation and increases in cytosolic calcium levels. *In vivo* data was presented, however, these results only demonstrated a proof of principle that alexidine resulted in killing the cancer cells *in vitro,* and therefore might not be clinically relevant. The

methodology employed in this study involved first incubating alexidine dihydrochloride with the cell culture for 48 hours, then adding an additional dose of drug prior to cell injection into mice. According to the data presented, less than 5% of cells were still capable of colony formation. This experimental design is clearly less than optimal for probing the drug's effects on CXCR4 binding (and subsequently inhibition of metastasis *in vivo*), and was the principal reason we decided to initially test activity of alexidine dihydrochloride using our more stringent *in vivo* model.

Recently, there was another paper published demonstrating reductions in lung metastases after AMD3100 treatment [69]. This study used CXCR4+/+ and CXCR4+/- C57BL/6 mice injected with B16 melanoma cells pretreated with AMD3100 for 30 minutes prior to injection. The mice were also treated twice a day with AMD3100 for five days a week for two weeks after tumor cell injection. The number of lung metastases observed were decreased in both treated groups and the total neoplastic area showed statistical reduction in size of the tumors after treatment with AMD3100 ($p = 0.038$ and $p = 0.021$). Treatment with AMD3100 not only reduced the number of metastases but also the size of the metastases. There are many other studies that show metastasis inhibition using AMD3100 in many tumor models that will not be discussed in detail here [70-77].

There are several publications that use other molecules or compounds to interfere with the CXCR4/CXCL12 induced metastasis cascade in many different cancer models. Other molecules that have shown success in limiting or

preventing metastasis vary from anti-CXCR4 antibodies [42], T140 peptide derivatives [70], siRNA [70, 78], shRNA lentiviral transduction particles [79], and heparinoids [80]. Each of these studies verifies the same premise; that blocking the CXCR4 binding site reduces CXCL12 induced metastasis.

The guanide, biguanide, and phenylguanide compounds developed in the Teintze lab that were discussed in detail in the HIV section of this chapter are also being evaluated as inhibitors of CXCR4/CXCL12 induced metastasis using both *in vitro* and *in vivo* experiments. Results show that these compounds indeed bind to CXCR4 with varying levels of affinity. Based on this attribute, we hypothesized that the high CXCR4 affinity compounds would interfere with the CXCR4-mediated metastasis in a breast cancer model. Accordingly, multiple compounds were chosen for *in vivo* studies in a mouse model injected with MDA-MB-231 human breast cancer cells.

Antimicrobial Compounds and Resistance

Incidences of antibiotic-resistant bacteria are rapidly increasing compared to historical numbers. This comes at a time in our history when the number of new drugs becoming available to treat resistant bacteria is steadily decreasing. The multi-drug-resistant bacteria causing the highest number of hospital infections are aptly named the ESKAPE pathogens for their ability to circumvent or escape the effects of current antimicrobial drugs. ESKAPE stands for: ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumonia*,

***A**cinetobacter baumannii*, ***P**seudomonas aeruginosa*, and ***E**nterobacter* species. The rapid evolution of drug resistance in these species has forced a call from the Infectious Diseases Society of America for a cohort of new drugs to treat them within the next decade [81].

In terms of the research described herein, the *Enterococcus faecalis*, methilicillin resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* bacteria were examined. Neither *Klebsiella* nor *Enterobacter* were examined in this study as we have no access to these species through current collaborators. The guanide derivative compounds were additionally tested for effectiveness against *E. coli*, *Burkholderia*, and the eukaryotic pathogen *Acanthamoeba*.

The mechanism that each of these bacteria use to acquire drug resistance will be discussed in detail at the end of this section. However, the causative events leading to bacterial drug resistance have long been blamed on overuse of antibiotics during patient treatment regimes [82]. These practices ultimately select for drug resistant bacteria through the evolution process. D'Costa et al. determined that antibiotic resistance is not a new phenomena but is a natural process that has long been utilized by organisms [83]. Non-therapeutic use of antibiotics in agriculture and aquaculture further complicate the situation [84]. The problems we are facing today are arising from the overuse of antibiotics, which cause natural selection of the drug resistant phenotype.

One proactive solution to reducing the overuse of antibiotics relates to improved personal hygiene, and the increased use of topical antimicrobial agents is a direct result of this. Topical antimicrobial agents typically have active ingredients such as cationic antiseptics in them, which are effective against a broad-spectrum of bacteria. By reducing bacterial transfer between patients, doctors, and in the community at large, the number of illnesses that require doctor visits and medication can then be decreased and this will place less of a burden on antibiotic treatment.

Cationic antiseptics and disinfectants have been in use in hospitals, clinics, businesses, and personal homes for more than half a century. Topical, not oral or intravenous, use of these compounds remains a safe and effective way to prevent and treat some bacterial infections and reduce bacterial growth [85]. In clinical settings, topical cationic antiseptics and disinfectants are not only the active ingredients in medicated handsoaps and body washes, but are also now present in polymeric materials and coatings that interface between imbedded medical devices and the skin [86]. These types of compounds have shown marked improvement in prevention of localized infections, and they have also proven to reduce the number of nosocomial infections through better hygiene [87]. The biggest advantage is that there is little antimicrobial resistance to some of these cationic compounds [85].

Cationic antiseptics can be classified into multiple categories that include monoquaternary ammonium compounds (QAC), bisbiguanides, and polymeric

biguanides. QAC compounds are amphoteric surfactants that are comprised of a quaternary nitrogen and a hydrophobic substituent. Examples of QACs are cetrimide and benzalkonium chloride. The hydrophobic part of the compound integrates into the lipid membrane and results in disruption of the cell membrane [85]. Biguanide molecules are compounds that have multiple positively charged biguanide groups separated by aliphatic chains of varying lengths. The polymeric biguanides have a repeating unit of an aliphatic chain with a biguanide moiety. Examples of bisbiguanides are chlorhexidine and alexidine. The most predominant example of polybiguanides is PHMB (polyhexamethylene biguanide hydrochloride). Biguanide groups are thought to interact with the cellular membrane, displacing positive divalent cations such as calcium and disrupting the cell membrane [85]. Other mechanisms of action such as energy deprivation are currently under investigation [88]. Biguanide compounds are not limited to uses as antimicrobial agents. There are biguanide compounds approved for lowering blood sugar (metformin), anti-malarial compounds (Proguanil), and anti-protozoal compounds (Proguanil) [85, 89].

The bisbiguanide chlorhexidine has been in use for over 50 years as an antiseptic [85, 87]. It is considered a broad spectrum antimicrobial compound due to the fact that it is active against both gram-positive and gram–negative bacteria. Despite being used for so long, there is little indication that there is any resistance forming against chlorhexidine treamtent [85, 90]. It remains active even against multi-drug resistant isolates of bacteria, implying that the

mechanism of action is not affected by the presence of β-lactamases or efflux pumps [91]. Chlorhexidine is used as a vaginal disinfectant [92-93], in mouthwash [94-96], and as a protective agent against chlamydial infection [97].

Alexidine, a bisbiguanide similar in structure to chlorhexidine, has also been shown to be active against both gram-positive and gram–negative bacteria. In comparison to chlorhexidine, there are indications that it has faster bacteriocidal activity [98]. In practice, alexidine has similar uses to chlorhexidine, mainly in disinfectants and mouthwashes.

PHMB is a polybiguanide compound also marketed as a broad spectrum antimicrobial agent. In general, the longer the polymeric chain length, the better the activity of this compound [99]. The uses of this polybiguanide range from contact lens cleaners [100], *Acanthamoeba keratitis* treatments [101], mouthwashes, and swimming pool disinfectants (Baquacil™, Arch Chemicals).

There are significant drawbacks to the current biguanide compounds available as disinfectants. One of the disadvantages to treatments with these compounds is that they are toxic to mammalian cells as well as to bacteria and are therefore not as effective when given internally. Another area of concern relates to their prevalent use in hospitals and domestic settings that the odds of bacteria gaining resistance to them is more and more likely as they are applied in more settings with such high occurrence.

For these reasons, the Teintze lab tested the guanide, biguanide, and phenylguanide compounds (introduced previously) against the pathogens listed

previously to examine their potential effectivity as broad spectrum disinfectants. We hypothesized that the biguanide molecules similar in structure to chlorhexidine and alexidine would be active as broad spectrum anti-microbials. Another possibility was that the compounds could exhibit less mammalian cell toxicity than chlorhexidine and thus be tolerated better *in vivo*. Surprisingly, it was discovered that the best compounds were not the biguanide derivatives as hypothesized but rather were the phenylguanide derivatives; these compounds were not as toxic as chlorhexidine and preliminary studies show they are well tolerated *in vivo*.

Bacteria are capable of evading current antibiotics through many mechanisms. Most of these mechanisms can be classified into one of three categories [102]. The first category is through antibiotic inactivation. This can occur either through modification of the active antibiotic compound into an inactive compound or by degradation of the compound itself. The second category bacteria use to evade antibiotics is through alteration of the target protein to either preclude binding or to bypass the effects that compound binding causes. The mechanisms for these actions relate to changing the conformation of the target protein itself, altering amino acid sequence, up regulating alternate pathways, and changing the gene expression of the target protein. The third category concerns decreased uptake of the antibiotic itself. This typically arises from increased activity of efflux systems but can also be a consequence of an

alteration of the pathway used by the antibiotic to find the target. Each pathogen evaluated in this study is discussed in the next few paragraphs.

MRSA are gram positive cocci under the microscope and are considered facultative anaerobes. MRSA has historically been associated with nosocomial or hospital acquired infections in healthcare settings. Recently, however, there has been a significant increase in numbers of both hospital acquired infections as well as community associated infections that cannot be traced back to healthcare settings. Importantly, MRSA is not only resistant to methicillin but also all the beta-lactam antibiotics such as penicillins, cephalosporins, and monobactams. There are also increasing numbers of MRSA strains that are acquiring resistance to the macrolide, quinolones, and vancomycin families of treatments. Beta-lactams are considered excellent drugs since they have no correlating targets in the mammalian cell. Staphylococci have acquired resistance to β-lactam antibiotics through three different mechanisms. The first is inactivation through hydrolysis by β-lactamases [102]. The second mechanism is recruitment and expression of altered PBPs (penicillin binding proteins) that have low affinity for the β-lactam antibiotics. For MRSA, this is the mechanism of action. The *mecA* gene encodes for the PBP2 protein that has low affinity for antibiotics such as methicillin[102]. The third mechanism is through decreased uptake of the molecules as a consequence of increased efflux of the antibiotic out of the cell [102]. Staphylococci can also have resistance to antibiotic aminoglycosides from adenylating hydroxyl groups present on the antibiotic [102]. Adenylation is also

the source of resistance to lincosamides. Efflux pumps and methylation of the active site are in turn responsible for resistance to macrolides.

Enterococcus faecalis are gram positive cocci also considered to be facultative anaerobes. This bacterium normally resides in the human and animal intestinal tract. *Enterococcus* is typically considered an opportunistic pathogen but can cause urinary tract infection (UTI), endocarditis, and meningitis. It is considered to be intrinsically resistant to antibiotics such as the β-lactams and aminoglycosides. It carries an extra PBP that has low affinity for β-lactam antibiotics. When the other PBPs are inhibited by the presence of the β-lactam antibiotics, the extra PBP takes over transpeptidase function [102]. Enterococci can also have some β-lactamases. Enterococci can inactivate chloramphenicol through acetyltransferases. Acetyltransferases are also responsible for resistance to lincosamides, although efflux pumps may also cause resistance.

Acinetobacter baumannii are gram negative aerobic cocci (rod like during active growth) that can lead to opportunistic infections. Recent increases of Acinetobacter infections in both nosocomial and community acquired infections have not been explained. There has also been a large incidence in war-related wounds in Iraq and Afghanistan veterans [103-105]. The remarkable ability of Acinetobacter baumannii to adhere to and survive for extended periods of time on biological and abiotic surfaces make drug resistant strains of Acinetobacter difficult to eliminate [106]. It has natural multidrug resistance due to efflux pumps and β-lactamases. It also has a remarkable ability to take up DNA and acquire

resistance to new drugs [103, 105]. With up to 30% of ICU Acinetobacter baumannii cases resistant to at least three classes of drugs, the development of new compounds to treat Acinetobacter is vital [103].

Pseudomonas aeruginosa is also an opportunistic pathogen with intrinsic multi-drug resistance. It is a gram-negative, aerobic rod-shaped bacterium. It can be found in pulmonary infections, uterine tract infections, burn wounds, and bloodstream infections. Its intrinsic resistance stems from constitutive expression of multidrug efflux systems, which provide low levels of resistance to all antibiotics. Further upregulation of the efflux systems result in high levels of antibiotic resistance [102]. This bacterium also has acetyltransferases that prevent aminoglycoside antibiotics and chloramphenicaol from binding to the ribosome at the target tRNA site. Chloramphenicol resistance occurs through acetyltransferases that inactivate this antibiotic.

Burkholderia cepacia are gram-negative aerobic rod shaped bacteria. This opportunistic virulent pathogen is commonly found in and transferred between cystic fibrosis patients[107]. Infections can also be in the urinary tract or respiratory tract, resulting in bacteremia or peritonitis [108]. Similar to *Pseudomonas*, *Burkholderia* are known to have constitutive expression of efflux systems that confer general multidrug resistance and an easy ability to acquire new resistance [102, 109]. It is considered one of the most antimicrobial-resistant organisms identified in laboratories [108].

Interestingly, many of the bacteria listed in this study are common microbial colonizers in wound infections [110]. Wound infections treated with commonly used antiseptics such as the biguanide chlorhexidine are less likely to select for antibiotic resistant bacteria populations while retaining broad spectrum antimicrobial activity [110]. Topical applications such as wound dressings for the guanide, biguanide, and phenylguanide compounds discussed herein may perform very well as topical broad spectrum wound treatments.

Different bacteria use distinct mechanisms for the various antibiotics that they have developed resistance to. Interestingly, chlorhexidine has been in use for over fifty years without bacterial resistance becoming prevalent [85, 90]. The compounds synthesized in the Teintze lab may ultimately have resistance developed to them through some of these mechanisms. However, due to the fact that drug resistant strains are currently susceptible to these guanide derivatives leads us to believe that the current mechanism of antibiotic resistance for the examined pathogens will not apply to our compounds. The guanide derivative compounds synthesized in the Teintze lab may certainly help the growing problem of treating multi-drug resistant bacteria.

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CHAPTER TWO

NOVEL COMPOUNDS CONTAINING MULTIPLE GUANIDE GROUPS THAT BIND THE HIV CORECEPTOR CXCR4

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

Author: Royce A. Wilkinson

Contributions: Synthesized and purified the guanide, biguanide, and phenylguanide derivative compounds, tested CXCR4 affinity of the compounds, and contributed to the experimental design and writing the manuscript.

Co-Author: Seth H. Pincus

Contributions: Designed the HIV efficacy study. Tested nine compounds for anti-HIV activity against four HIV viral strains and determined cellular cytotoxicity for the same nine compounds.

Co-Author: Joyce B. Shepard

Contributions: Tested CXCR4 affinity of the compounds, determined the cellular cytotoxcity of all the compounds.

Co-Author: Sarah K. Walton

Contributions: Tested CXCR4 affinity of the compounds.

Co-Author: Edward P. Bergin

Contributions: Synthesized and purified a couple of derivative compounds.

Co-Author: Mohamed Labib

Contributions: Provided NB325, discussed the results and implications of the study, and contributed to the manuscript.

Co-Author: Martin Teintze

Contributions: Discussed the results and implications and wrote the manuscript.

Manuscript Information Page

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Novel compounds containing multiple guanide groups which bind the HIV co-receptor CXCR4

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Royce A. Wilkinson,[1] Seth H. Pincus,[2] Joyce B. Shepard,[1] Sarah K. Walton,[1] Edward P. Bergin,[1] Mohamed Labib,[3] and Martin Teintze[1*]

Department of Chemistry & Biochemistry, Montana State University, Bozeman, Montana 59717[1]; The Research Institute for Children, Children's Hospital, New Orleans, Louisiana 70118[2]; Novaflux Biosciences, Inc., Princeton, New Jersey 08540[3]

* Corresponding Author. Mailing address: 155 Chemistry & Biochemistry Building, Montana State University, Bozeman, MT 59717. Phone: (406) 994-4411. Fax: (406) 994-5407. E-mail: mteintze@montana.edu.

ABSTRACT

The G-protein coupled receptor CXCR4 acts as a co-receptor for HIV-1 infection, as well as being involved in signaling cell migration and proliferation. Compounds that block CXCR4 interactions have potential uses as HIV entry inhibitors to complement drugs like maraviroc that block the alternate co-receptor CCR5 or in cancer therapy. The peptide T-140, which contains five arginine residues, is the most potent antagonist of CXCR4 developed to date. In a search for non-peptide CXCR4 ligands that could inhibit HIV entry, three series of compounds were synthesized from 12 linear and branched polyamines with 2, 3, 4, 6, or 8 amino groups which were substituted to produce the corresponding guanidines, biguanides, or phenylguanides. The resulting compounds were tested for their ability to compete with T-140 for binding to the human CXCR4 receptor expressed on mammalian cells. The most effective compounds bound CXCR4 with an IC_{50} of 200 nM, and all of the compounds had very low cytotoxicity. Two series of compounds were then tested for their ability to inhibit the infection of TZM-bl cells with X4 and R5 strains of HIV-1. Spermine phenylguanide and spermidine phenylguanide inhibited infection by X4 strains, but not by R5 strains, at low micromolar concentrations. These results support further investigation and development of these compounds as HIV entry inhibitors.

INTRODUCTION

New therapeutics with novel mechanisms of action are needed for treatment of human immunodeficiency virus type 1 (HIV-1) infections. Maraviroc, a small molecule antagonist of the CCR5 receptor was the first drug of its type to be approved, and it has proven to be effective against “R5” HIV that uses the CCR5 co-receptor (9). However, most patients that have failed conventional therapies also harbor “X4” HIV that uses CXCR4 or dual-tropic viruses that can use either CCR5 or CXCR4. The appearance of the X4 viruses is associated with progression to AIDS symptoms (4), and drug resistance is more often linked to CXCR4-utilizing than to CCR5-utilizing HIV (39). Therefore, there is also a need to develop effective drugs that block CXCR4. So far, none of the CXCR4 antagonists in development have shown promise in HIV clinical trials, though some are being pursued as cancer therapeutics because CXCR4 may be involved in metastasis. Some CXCR4 inhibitors, such as the bicyclam AMD3100 and the monocyclam AMD3465, bind to a site located in the transmembrane domain of the receptor (13, 15, 26, 27, 30, 40). Like the CCR5-specific small molecule inhibitors, these compounds are believed to block viral entry and chemokine signaling by allosteric mechanism(s). In contrast to CCR5 inhibitors, however, many of the CXCR4-specific inhibitors contain multiple positive charges which can interact with additional acidic residues in the extracellular loops of CXCR4 that are not conserved in CCR5 (2, 10, 27, 28, 35).

T-140 is a 14-residue peptide antagonist of CXCR4 that blocks binding of X4 strains of HIV-1 *in vitro* and displaces the natural agonist ligand SDF-1α with nanomolar affinity but does not bind to other chemokine receptors, such as CCR5 (30-32). It is much smaller than SDF-1α (14 residues vs 65 residues), but is rapidly degraded by proteolysis in serum (30) and has not been pursued as an HIV or cancer therapeutic. Recently, smaller cyclic peptides based on the structure of T-140 (25, 37), as well as non-peptide analogs (36), have been reported. However, the latter have IC_{50}s at least two orders of magnitude higher than that of T-140. The structure of T-140 contains 5 Arg and 2 Lys residues; the latter can be substituted with uncharged sidechains without loss of activity. ALX40-4C (N-alpha-acetyl-nona-D-arginine amide acetate) is another peptide antagonist of CXCR4 (8). An early clinical trial showed that it was well tolerated (7) and provided an important proof of principle that CXCR4 activity can be safely inhibited in humans. KRH-1636 is a CXCR4 antagonist which exhibits strong activity against X4 strains of HIV and competitively displaces SDF-1 from the receptor, and can be absorbed from the duodenum of rats (17). Like T-140 and ALX40-4C, KRH-1636 contains an Arg residue. An analog of this compound (KRH-2731) was reported to have enhanced activity and was claimed to be orally bioavailable in rats (24), but neither its structure nor any clinical trial results have been published. AMD3100 (plerixafor) is a bicyclam with 8 secondary and tertiary amine groups (5, 6). Clinical trials for HIV have been abandoned because this compound showed poor efficacy, cardiotoxicity, and it was not orally bioavailable

(16). The structurally similar monocylam AMD3465 has higher affinity for CXCR4, and is potent against X4 HIV strains in vitro (IC_{50}: 1–10 nM) (19), but is also not orally bioavailable (14). AMD070 (a.k.a. AMD11070), which has two aromatic rings in addition to a primary and a tertiary amine, has an IC_{50} of 2-26 nM against an X4 HIV strain and is orally bioavailable (22, 29).

Previous studies at Novaflux and Drexel University have led to the identification and characterization of a new HIV-1 inhibitor, NB325 (20). This is a biguanide-based, low molecular weight oligomer (~800-1400 Daltons) that inhibits infection via specific interactions with CXCR4 and has been shown to be a potent HIV-1 inhibitor in a number of *in vitro* assays (3, 34). NB325 functions by specifically preventing the interaction of HIV-1 gp120 with the extracellular loop 2 (ECL2) of the CXCR4 co-receptor. Persistent interactions between NB325 and CXCR4 result in prolonged inhibition of HIV-1 infection (33). NB325 blocks SDF-1α signaling through CXCR4, but not SDF-1α attachment (34). The specificity of this new class of compounds as CXCR4 antagonists has also been confirmed by competitive binding with the peptide antagonist T-140 (described below) and inhibition of Ca2+-flux induced by the natural chemokine agonist SDF-1α (CXCL12) [T. Sakmar, unpublished]. However, because NB325 is a polydisperse preparation consisting of a homologous series of compounds with 4 to 10 biguanide groups, its approval for clinical use is unlikely. We therefore developed a series of discrete, small molecule inhibitors containing multiple guanide or biguanide groups to test their ability to inhibit HIV-1 binding to

CXCR4. The compounds were initially screened for their ability to inhibit the binding and cross-linking of a fluorescently labeled photoactive analog of T140 to CXCR4. The most effective inhibitors of T140 binding and cross-linking were subsequently tested for their ability to inhibit HIV infection. Some of these compounds inhibited infection by X4, but not R5 or X4/R5 strains of HIV. The results demonstrate the potential of these compounds to be leads in the development of X4 HIV entry inhibitors, but they will have to be improved upon substantially to match the inhibition of R5 HIV by maraviroc.

MATERIALS AND METHODS

Starting material amine compounds. Putrescine dihydrochloride and spermine tetrahydrochloride were purchased from Sigma (St. Louis, MO). 1,4-diaminobutane, 1,6-hexanediamine dihydrochloride, 1,6-hexanediamine, diethylenetriamine, and spermidine were purchased from Acros (Morris Plains, NJ). Starburst®PAMAM G(0.0) was purchased from Dendritech Inc. (Midland, MI). Priostar™ dendrimers DNT-2200 and DNT-2300 were purchased from Dendritic Nanotechnologies, Inc. (Mt. Pleasant, MI).

(i)Trishexylaminomelamine (THAM) synthesis.

Trishexylaminomelamine was synthesized following the method of Kaiser et al. (18). Cyanuric chloride (TCI America, Tokyo) was reacted with a 3.3 molar ratio of N-BOC-1,6-diaminohexane (Alfa Aesar, Ward Hill, MA) in refluxing water for 1.25 hours. The pH of the solution was monitored with phenolphthalein and the

pink color maintained by gradual addition of a 0.4M sodium carbonate solution. The reaction was lyophilized and the BOC groups removed by treating the residue with 100% trifluoroacetic acid (TFA) for 2h at room temperature (rt). The deprotected product was purified by reverse-phase HPLC.

(ii)Trisethylaminomelamine (TEAM) synthesis. The synthesis was begun as above with N-BOC-1,2-diaminoethane produced as previously described (23). The initial reaction gave the bis addition product (after deprotection) with one unreacted chlorine. Treatment of this compound with a concentrated ammonia solution in water resulted in the formation of a dimeric compound (BEMA dimer). Treatment of the bis addition product before deprotection with excess ethylenediamine, followed by deprotection, gave the desired trisethylamino product which was purified by reverse-phase HPLC.

(iii)Trisbutylaminomelamine (TBAM) synthesis. The synthesis was similar to the TEAM synthesis. The initial bis addition product, after deprotection, was treated with excess 1,4-butanediamine and refluxed for 2h in water. Purification by HPLC under acidic conditions did not result in separation of the desired product from the excess diamine. However, under basic conditions the product bound and was not eluted. Subsequent washing with 0.1% TFA in water eluted the desired product without the contaminating diamine.

Synthesis of guanide derivatives. The reaction of polyamine starting materials was carried out using 10% excess O-methylisourea sulfate salt (MP Biomedicals) in water at basic pH. Free amine compounds were added to the O-

methylisourea in water and incubated at 37-42ºC for 1-2 hours. Reactions with acidified amine starting materials (HCl or TFA salts) were neutralized with triethylamine (TEA) before being allowed to react. Products were purified by reverse phase HPLC and characterized by MALDI mass spectrometry.

Synthesis of biguanide derivatives. S-methyl-guanylisothiouronium iodide was prepared as previously described (11). Briefly, iodomethane (Acros) was added dropwise to a suspension of 2-imino-4-thiobiuret (Aldrich) in 100% ethanol while stirring at rt. The reaction flask was shaken at 37ºC for 2 hours. The solvent was removed by evaporation and the residue was washed with cold diethyl ether to give a white, crystalline solid which was collected by filtration and used without further purification.

Addition of the biguanide reagent (S-methyl-guanylisothiouronium iodide, in excess) to the amine compounds was done in 100% EtOH as previously described (38) at rt or 60ºC, or in water at rt, 37ºC, or 65ºC. The pH of the reactions were monitored and adjusted with TEA or sodium carbonate when it fell below pH~8. Reaction times varied between 5h and five days. Products were purified and characterized as above.

Synthesis of phenylguanide derivatives. S-methyl-N-phenylisothiouronium iodide was prepared as above by addition of methyl iodide to 1-phenyl-2-thiourea (Acros). Addition to the amine compounds was accomplished by refluxing 10% excess of the S-methylthiourea compound with the amine in 50% acetonitrile/water overnight. Reactions with acidified amine

starting materials (HCl or TFA salts) were neutralized with triethylamine (TEA) as above. Products were purified and characterized as above.

Synthesis of a photoactive, fluorescent T140 peptide. A fluorescent, photoactive cross-linkable derivative of the CXCR4 binding peptide T140 (Ac-RRNalCYRBpaD-KPYRCitCR, Nal=naphthylalanine, Cit=citruline, Bpa=4-benzoylphenylalanine) (30, 32) was synthesized on a solid support using standard Fmoc chemistry. Fmoc amino acids with appropriate side chain protecting groups were purchased from Advanced Chemtech (Louisville, KY). After cleavage from the resin and side chain deprotection, the crude peptide was purified by reverse-phase HPLC. A dilute solution of the peptide in 10 mM ammonium bicarbonate, pH=8, was stirred while bubbling air through the solution at 4ºC until oxidation of the intrapeptide disulfide bond was complete as analyzed by MALDI mass spectrometry. The solution was lyophilized and the oxidized peptide purified by reverse phase HPLC.

The purified peptide was fluorescently labeled on the ε-amine of the D-lysine residue by reaction with N-hydroxysuccinimide activated 6-(fluorescein-5-(and-6)-carboxamido)hexanoic acid (Invitrogen, Carlsbad, CA) in dry DMF with 10% pyridine overnight at 4ºC. The labeled peptide was purified by reverse phase HPLC.

CXCR4-T140 cross-link inhibition assay. The ability of the guanide, biguanide, and phenylguanide compounds to interact with CXCR4 was tested by a cross-link inhibition experiment. Serial dilutions of the inhibitor compounds

were incubated with approximately 1.5×10^5 CXCR4 expressing cells (syn-CXCR4-C9 in Cf2Th) (1) for 30 minutes on ice. The photoactive, fluorescent T140 peptide was added to a final concentration of 75 nM, and incubated on ice for an additional 30 minutes. Cross-linking of the T140 peptide to the receptor was carried out by irradiation at 365 nm in a Rayonet Photochemical Reaction chamber (Southern New England Ultraviolet Company, Hamden, CT) 4 times for 5 minutes with 5 minutes of cooling on ice between irradiations. The cell pellets were spun down, washed with 1× PBS, and resuspended in PAGE sample buffer with 40 mM dithiothreitol and run on an SDS-PAGE gel. The gels were imaged on a Bio-Rad Molecular Imager FX (Hercules, CA) and the fluorescent CXCR4 bands were quantified with the Bio-Rad Quantity One software.

CCR5-maraviroc analog cross-link inhibition assay. A maraviroc analog containing a benzophenone cross-linking group and a fluorescein tag were prepared (synthesis to be published later). This compound was used to test the guanide, biguanide, and phenyguanide compounds for interaction with CCR5 in an assay similar to the CXCR4-T140 inhibition assay above.

Assay for Anti-HIV Activity. Cell-free virus supernatants were prepared in PHA blast cultures as described elsewhere (21). TZM-bl cells (41) were obtained from the NIH AIDS Research and Reference Reagent Program; 4×10^4 cells were plated per well. CXCR4 inhibitors were added to the cells for 1 hour at 37°. Predetermined dilutions of the virus supernatants in 15 µg/ml DEAE-dextran (Sigma) were then added to the wells containing the cells plus inhibitor. Three

days later, the cells were lysed and luciferase activity measured (Bright-Glo Luciferase assay, Promega, Madison WI).

Toxicity Assays. Compound cytotoxicity was evaluated using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) following the manufacturer's instructions (Promega, Madison, WI) using the human breast cancer cell line MDA-MB-231 or the TZM-bl cell line used in the HIV infectivity assay.

RESULTS

Guanide, biguanide, and phenylguanide synthesis. The results of the compound syntheses are summarized in Table 2.1. Starting material compounds (see Fig. 2.1 for structures) are listed in the first column with the number of amines available for addition of guanide, biguanide, or phenylguanide groups (see Fig. 2.2) in parentheses. The results for each column show the number of amine groups derivatized. In cases labeled with 'or', two fractions were isolated with each of the respective products (e.g. both the monophenylguanide and bisphenylguanide additions to putrescine were isolated and tested separately). In cases labeled with a dash, the designated products were inseparable by HPLC (e.g. DNT2300 yielded a mixture of compounds with either 5 or 6 guanide groups per molecule).

Other deviations from the expected products are noted in Table 2.1. In three of the guanide addition reactions, additional +15 and sometimes +30 amu

products were co-purified with the isolated products. For these compounds, 20-50% of the isolated product was observed to be the +15 product, with a small amount (<10%) of the +30 product. These compounds were tested for activity as isolated; later, it was discovered that these products were the result of loss of ammonia during the substitution reaction instead of the expected loss of methanol. Treatment of these compounds with concentrated ammonium hydroxide can replace the O-methyl groups and yield the desired products, (see Supplemental Fig. 9.1), but the effect of this reaction on the activity of the compound was not tested.

The reaction of diethylenetriamine (DETA) with the phenylguanide reagent resulted in some cyclic byproducts (see Supplemental Fig. 9.2) that can be encountered when the reactive amine groups are closely spaced. These same mechanisms are probably involved in the formation of the products labeled 'cyanoamine and biguanides' in the reaction of DETA with S-methyl-guanylisothiourea. This reaction proceeds through the loss of urea and the formation of a cyclic product, as seen with the phenylguanides, or to the formation of a cyanoamine. However, mass spectrometry is unable to differentiate between the two byproducts. Finally, the addition of biguanides to the dendrimer DNT2300 resulted in an unexplained deficit of 4 amu from the isolated products regardless of the number of biguanide groups added.

CXCR4-T140 cross-link inhibition assay. The compounds were tested for CXCR4 binding by inhibiting the cross-linking of a photoactive, fluorescent

derivative of the known CXCR4 binding peptide T140. Initially, this assay was tested by using NB325 as the inhibitor. Fig. 2.3 shows the results for four separate experiments. While the relative fluorescence density (signal strength) varies from gel-to-gel, the IC_{50} values are quite reproducible, varying between 0.0006% and 0.001%. Assuming a median molecular weight of 800-1400 for NB325 gives an IC_{50} of ~10 μM.

A representative gel and analysis are shown in Fig. 2.4 for the dendrimer derivative DNT2200 octaguanide. The amount of fluorescence was quantified as the fluorescence density of a box encompassing the doublet band (CXCR4) in the middle of the gel.

The results of the CXCR4-T140 cross-link inhibition assay are shown in Table 2.2. IC_{50} values are given for the starting material amines and the respective guanide, biguanide, and phenylguanide derivatives. Most of the starting amines had IC_{50} values greater than or equal to the highest concentration tested (200 μM), with the exception of spermine, which inhibited the T140 cross-linking with an IC_{50} of 75 μM.

As a general rule, the inhibition activity increased in the order amines < biguanides < guanides < phenylguanides. However, there are a couple of notable exceptions. The butanediamine was only active as the guanide derivative, with an IC_{50} of 50 μM. For trishexylaminomelamine (THAM), the guanide and biguanide derivatives were active, but interestingly, the phenylguanide compound showed no inhibition. The derivatives of linear

polyamines (butanediamine, hexanediamine, diethylenetriamine, spermidine, and spermine) generally increased in activity as they increased in size. As a group, the spermidine and spermine derivatives were the most active, and the most inhibition was exhibited by spermidine bisphenylguanide and spermidine trisphenylguanide with IC_{50} values of 200 nM.

The melamine core guanide derivatives (TEAM, TBAM, THAM) increased in activity as the chain length increased (ethyl < buty < hexyl). Conversely, the corresponding phenylguanides showed the opposite trend, with the THAM bisphenylguanide actually showing no inhibition at the highest concentration tested (200 mM). The dendrimer derivatives (PAMAM-G0, DNT2300, DNT2200) were generally equally active as the number of reactive amines varied from 4 to 6 to 8.

Inhibition of HIV infection. Six of the most active compounds, and the amines from which they were derived, were selected for preliminary testing as HIV infection inhibitors. Spermidine, spermine, DNT2300, and their guanide and phenylguanide derivatives were tested for their ability to inhibit HIV infection at 10μM in the TZM-bl assay. The compounds were screened against CXCR4, CCR5, and R5/X4 tropic virus strains. The spermidine and spermine phenylguanides were effective against both X4 strains, with no inhibition of the R5/X4 strain and limited inhibition of the R5 strain (see Fig. 2.5).

The underivatized amine spermine appeared to be active against both R5 and X4 strains at this concentration. However, in a second TZM-bl assay run to

investigate the dose-response of the active phenylguanides and their respective amine starting materials, the phenylguanides were active at approximately 10-fold lower concentrations than the corresponding amines against three X4 strains (Fig. 2.6 & 2.7A). None of the compounds showed significant activity (>2-fold reduction) against an R5 virus. At 10μM, spermine did not significantly inhibit fluorescent T-140 binding to CXCR4 (see above) or binding of a fluorescent maraviroc analog to CCR5, nor was it cytotoxic to TZM-bl cells (data not shown). Therefore, the reason for the observed inhibition of X4 and R5 infection by the underivitized amines is unclear.

Cytotoxicity. The toxicity of the compounds was evaluated using an MTS dye reduction assay in a CXCR4 expressing human breast cancer cell line for all of the compounds (Supp. Table 1) and in TZM-bl cells for spermidine phenylguanide and the spermidine control (Fig. 2.7B). The majority of the compounds showed no toxicity at the highest concentrations tested against the MDA-MB-231 cells. Spermidine and spermidine phenylguanide both had CC_{50} values of approximately 1000 μM against the TZM-bl cells while showing HIV inhibition at <5 μM (Fig. 2.7A).

DISCUSSION

We have synthesized a novel series of compounds containing multiple guanide, biguanide, or phenylguanide groups on linear aliphatic or dendrimer scaffolds. These compounds bear some resemblance to the

polyethylenehexamethylene biguanide NB325 as well as to the CXCR4 antagonist peptide T-140, which has five guanide groups on the sidechains of arginine residues. Among the new compounds, the bis- and trisphenylguanide derivatives of spermidine exhibited the highest affinity for CXCR4, with an IC_{50} of 200 nM in competition assays with T-140. This is a 50-fold higher affinity than found for NB325 in this assay, and is comparable to that of other non-peptide analogs of T-140 that have been reported (36), though it does not approach the nanomolar affinity of T-140 itself.

The best T140 inhibitors were also tested for their ability to inhibit HIV infection. The spermine and spermidine derivatives and amines were tested against a panel of X4, R5, or X4/R5 isolates. Spermine phenylguanide and spermidine phenylguanide were effective at inhibiting the infection of TZM-bl cells by X4, but not R5, HIV strains, consistent with the observation that they bound to CXCR4 but not CCR5. In fact, none of the compounds in Table 2.2 inhibited the binding of a fluorescent maraviroc analog to CCR5 (data not shown). Spermidine phenylguanide had an IC_{50} value of 3 μM when tested for inhibition of three X4 strains (NL4-3, 92HT599, and MN). The HIV inhibition data were also consistent with the T140 inhibition results in that the phenylguanides were more active than the related guanide or biguanide compounds. However, the underivatized parent amines appeared to show inhibition against both X4 and R5 strains of HIV, even though they were not effective in inhibiting T140 binding

and cross-linking to CXCR4, suggesting that they were acting via another mechanism.

In experiments using primary human CD4+ T lymphocytes as the target cells, NB325 inhibited HIV-1 IIIB infection with IC_{50} concentrations of approximately 0.01% (~ 71 μM) (34). This is about 50-fold higher than the concentrations of spermine phenylguanide and spermidine phenylguanide required to inhibit X4 HIV infection, consistent with the higher affinity for CXCR4 relative to NB325. Since NB325 is a mixture, it is of course possible that it contains a component with greater activity. We attempted to fractionate NB325 by reverse phase, ion exchange, and size exclusion chromatographies, but were unable to isolate a fraction with higher affinity for CXCR4 (data not shown).

The optimal structure for a non-peptide CXCR4 antagonist cannot be readily predicted from the data available. Attempts to develop small cyclic peptide and non-peptide analogs from T-140 (12, 36, 37) have pointed to the importance of both a guanide functional group (in the form of an arginine sidechain) and an aromatic group (napthyl). This is consistent with our observation that the phenylguanides were more active than either the guanides or biguanides and suggests that using a larger aromatic moiety such as naphthalene may increase the observed CXCR4 binding compared to our initial phenylguanide derivatives.

The positioning of the aromatic group also appears to be important. The most active phenylguanides (spermidine & spermine derivatives), with the

aromatic groups on the ends and sides of the main chain of the molecule have at least an order of magnitude better binding than the best melamine derivative (THAM trisguanide), which has the aromatic group in the center of the molecule with the charges surrounding it. There also appears to be a necessary balance between charge and hydrophobic character. This is exemplified in the series of melamine derived phenylguanides in which increasing the length of the spacers between the melamine and phenylguanide groups, which increases overall hydrophobicity, led to a progressive loss of activity.

The spacing between the positively charged groups, at least in the linear compounds, also appears to be important. Experiments in the development of NB325 indicated that optimal efficacy was found when an alternating 2:6 (ethyl-, hexyl-) spacing between biguanides was present compared to evenly spaced ethyl-, butyl-, or hexyl-based polybiguanides (20). In the experiments reported here, the most active core molecules (spermine and spermidine) have spacings of three or four carbons between the reactive amine groups, whereas the less active diethylenetriamine and hexanediamine cores have two and six carbons respectively. However, the flexibility of the polyamine backbones versus those of the polybiguanides makes comparison of the distances between the charged groups in the two types of molecules difficult.

The compounds reported here, especially the phenylguanides, have significant advantages over the polybiguanides in that they exhibit higher affinity for CXCR4 and are discrete compounds rather than heterogeneous mixtures;

therefore, they represent more promising leads for developing non-peptide inhibitors of X4 HIV infection. Their very low cytotoxicity makes them promising candidates for future animal and clinical studies and their non-peptide structures may give them greater serum stability than T140.

Table 2.1: Compounds Synthesized

Starting Amine	**Guanide**	**Biguanide**	**Phenylguanide**
Butanediamine (2)	2	2	1 or 2
Hexanediamine (2)	2*	1 or 2	1 or 2
Diethylenetriamine (3) (DETA)	2	3†	3‡
Spermidine (3)	2 or 2-3	1-2	2 or 3
Spermine (4)	2	2	3-4
Trisethylaminomelamine (3) (TEAM)	3	1	3
Trisbutylaminomelamine (3) (TBAM)	3	1-2	2 or 3
Trishexylaminomelamine (3) (THAM)	3	1-3	2
Bisethylaminomelamine dimer (2) (BEMA dimer)	2	ND	1-2
PAMAM G0 (4)	4*	3-4	4**
DNT2300 (6)	5-6*	3-6¶	6
DNT2200 (8)	8	1-5	8

* +15 mass also (O-Me replace NH_2), ** small amount of +5 phenylguanide groups, † cyanoamines & biguanides, ‡ cyclic byproducts, ¶ masses 4 amu too low, ND synthesis not done.

Table 2.2: CXCR4/T-140-Fl Inhibition Data				
	Starting Amine	**Guanide**	**Biguanide**	**Phenylguanide**
Butanediamine	> 200	50	> 200	> 200
Hexanediamine	≥ 200	> 200	> 200	150 (1) 20 (2)
Diethylenetriamine (DETA)	≥ 200	50	> 200	15 (1) 6 (2)
Spermidine	> 200	8 (2) 10 (3)	65 (1-2)	0.2 (2) 0.2 (3)
Spermine	75	10 (2)	4-8 (2)	0.5 (3-4)
Trisethylaminomelamine (TEAM)	> 200	20	> 200 (1)	1.5 (3)
Trisbutylaminomelamine (TBAM)	≥ 100	10	> 200 (1-2)	6 (2) 20 (3)
Trishexylaminomelamine (THAM)	> 200	2	45 (1-3)	> 200 (2)
BEMA dimer	> 200	50	ND	15 (1) 70 (2)
PAMAM GO	> 200	4	> 50 (3-4)	3
DNT2300	> 200	8	7.5 (3-6)	1.5
DNT2200	≥ 200	15	≥ 200 (1-5)	3.5
All data are given as IC_{50} values in μM. For comparison, NB325, assuming an average MW ≈ 1000, has an IC_{50} = 10 μM. Numbers in parentheses indicate the number of groups added.				

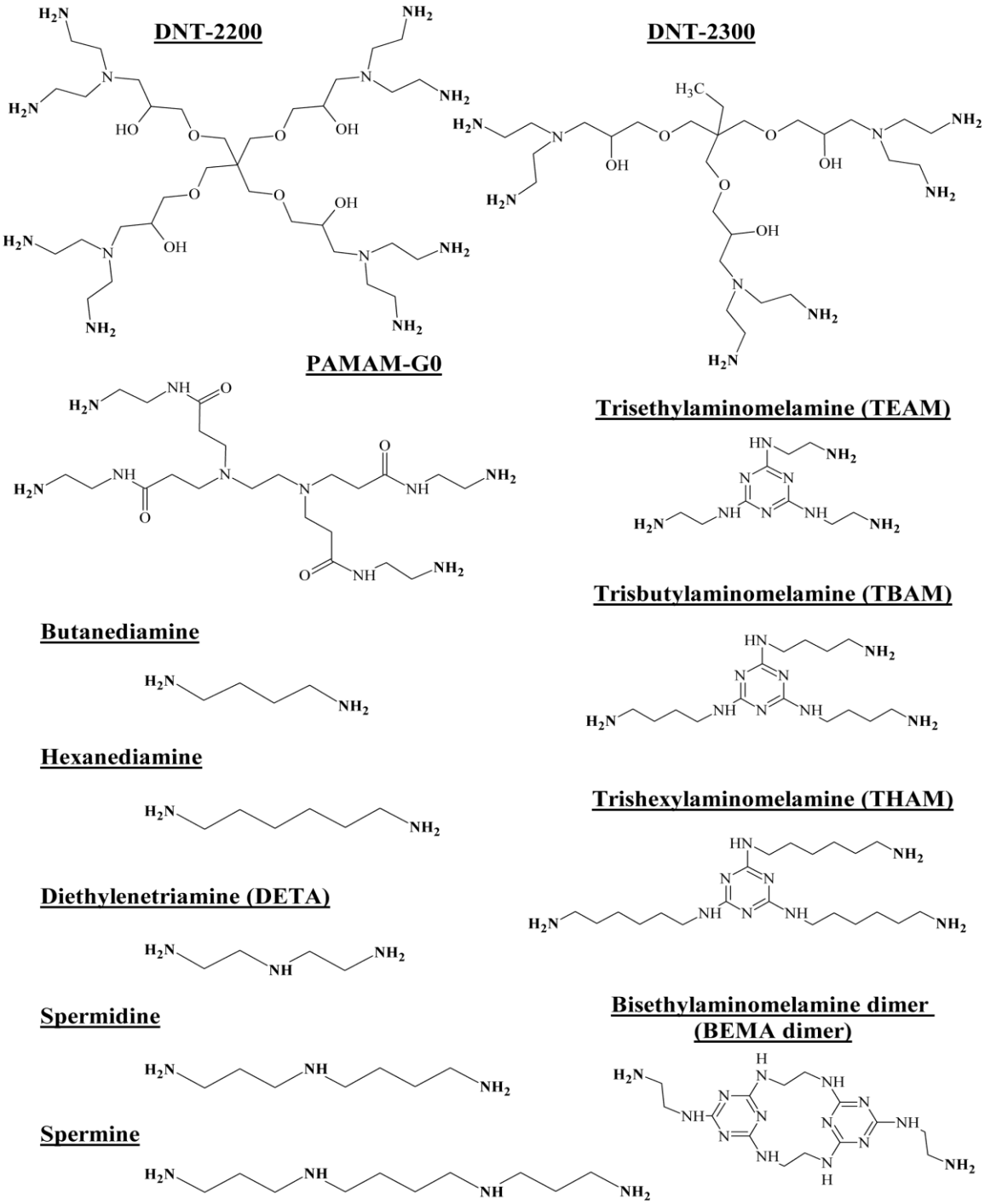


Fig. 2.1. Synthetic starting materials. The structure of starting material polyamines used to synthesize guanide, biguanide, and phenylguanide derivatives. Reactive amines are noted in bold.

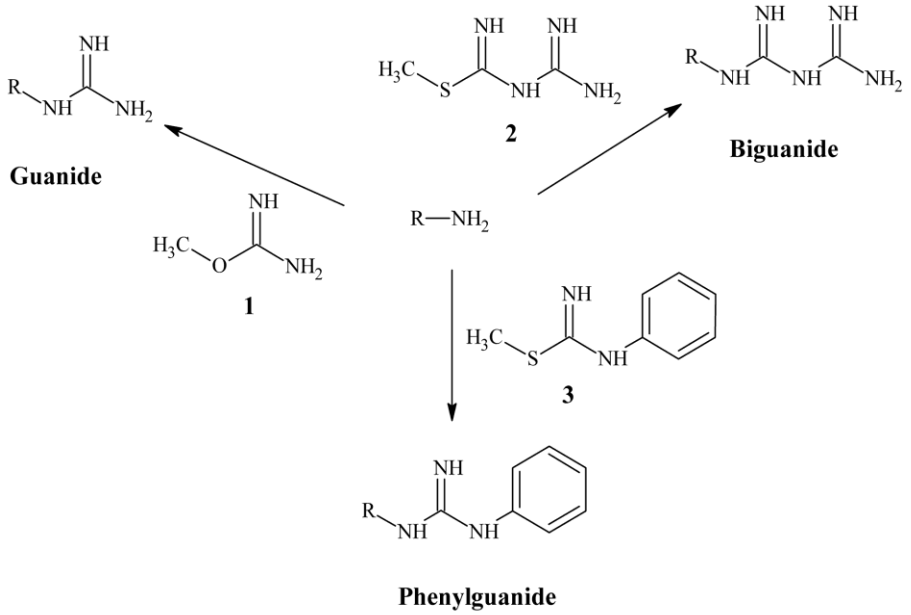


Fig. 2.2. Generic synthetic reaction scheme. Primary and secondary amines treated with the reactive compounds O-methylisourea (1), S-methyl-N-guanylisothiourea (2), or S-methyl-N-phenylisothiourea (3) give the desired guanide, biguanide, or phenylguanide derivatives.

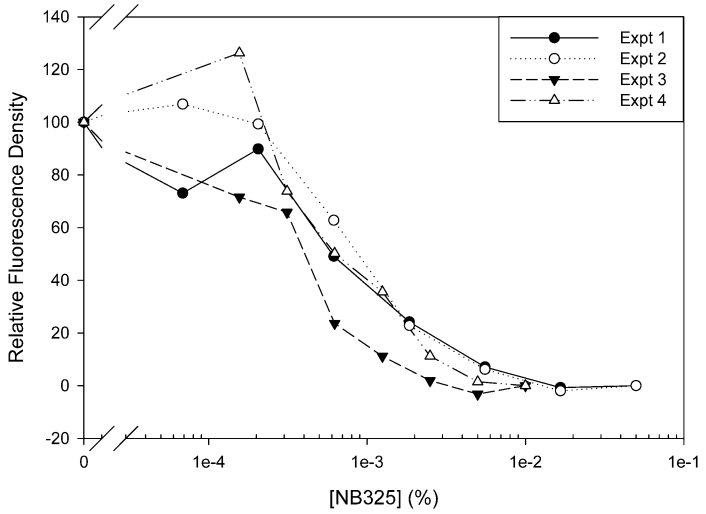


Fig. 2.3. Inhibition of T140-fluorescein cross-linking to CXCR4. CXCR4 expressing cells were incubated with serial dilutions of the polybiguanide NB325, and then allowed to bind the fluorescent, photoactive T140 derivative. After irradiation at 365 nm, the samples were dissolved in SDS and run on an SDS-PAGE gel and imaged with a fluorescent gel imager. Inhibition curves for four separate experiments with one reading per data point are shown.

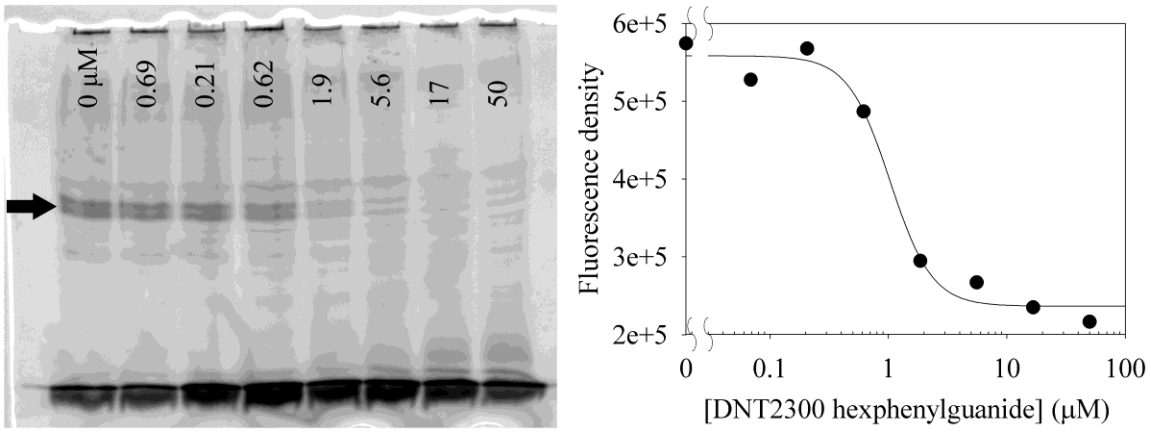


Fig. 2.4. Representative inhibition experiment gel and graphical analysis. Serial dilutions of the inhibitor (DNT2300 hexaphenylguanide) were incubated with CXCR4 expressing cells. The fluorescent, photo-active derivative of T140 was added, and then irradiated at 365 nm to induce cross-linking between the peptide and receptor. Samples were dissolved in SDS, run on an SDS-PAGE gel, and imaged with a fluorescent gel reader. The concentration of inhibitor is marked at the top of each lane. The intensity of the fluorescent CXCR4 bands (doublet indicated by arrow) were quantified and plotted with Sigma Plot to determine an approximate IC_{50} value (~ 1 µM). A Western blot of the gel incubated with MAb 1D4 was used to confirm the identity of the CXCR4 bands and their uniformity across the gel (not shown).

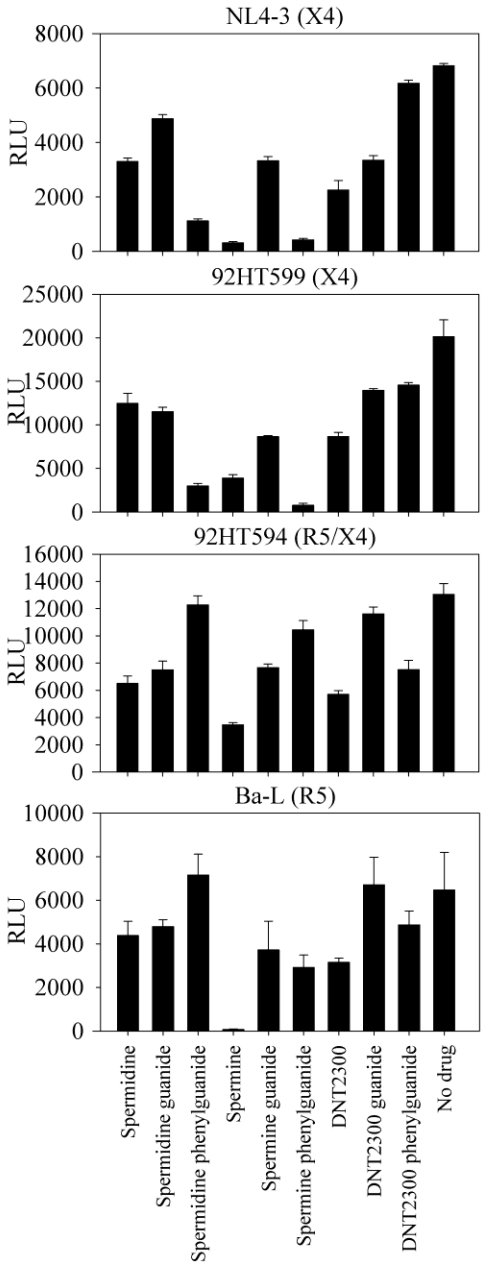


Fig. 2.5. Initial anti-HIV activity screen. The series (amine, guanide, and phenylguanide) of the most active derivatives (spermidine, spermine, and DNT2300) in the T140 cross-link inhibition assay were screened for anti-HIV activity. TZM-bl cells were plated and pre-incubated with the inhibitors at a final concentration of 10 μM. Previously titered viral supernatants were added and the cells incubated for 3 days. Cells were lysed and the TAT-driven reporter luciferase activity measured with the Bright-Glo Luciferase assay. Experiments were performed in triplicate with errors shown as ± SEM. RLU = relative luminescence units

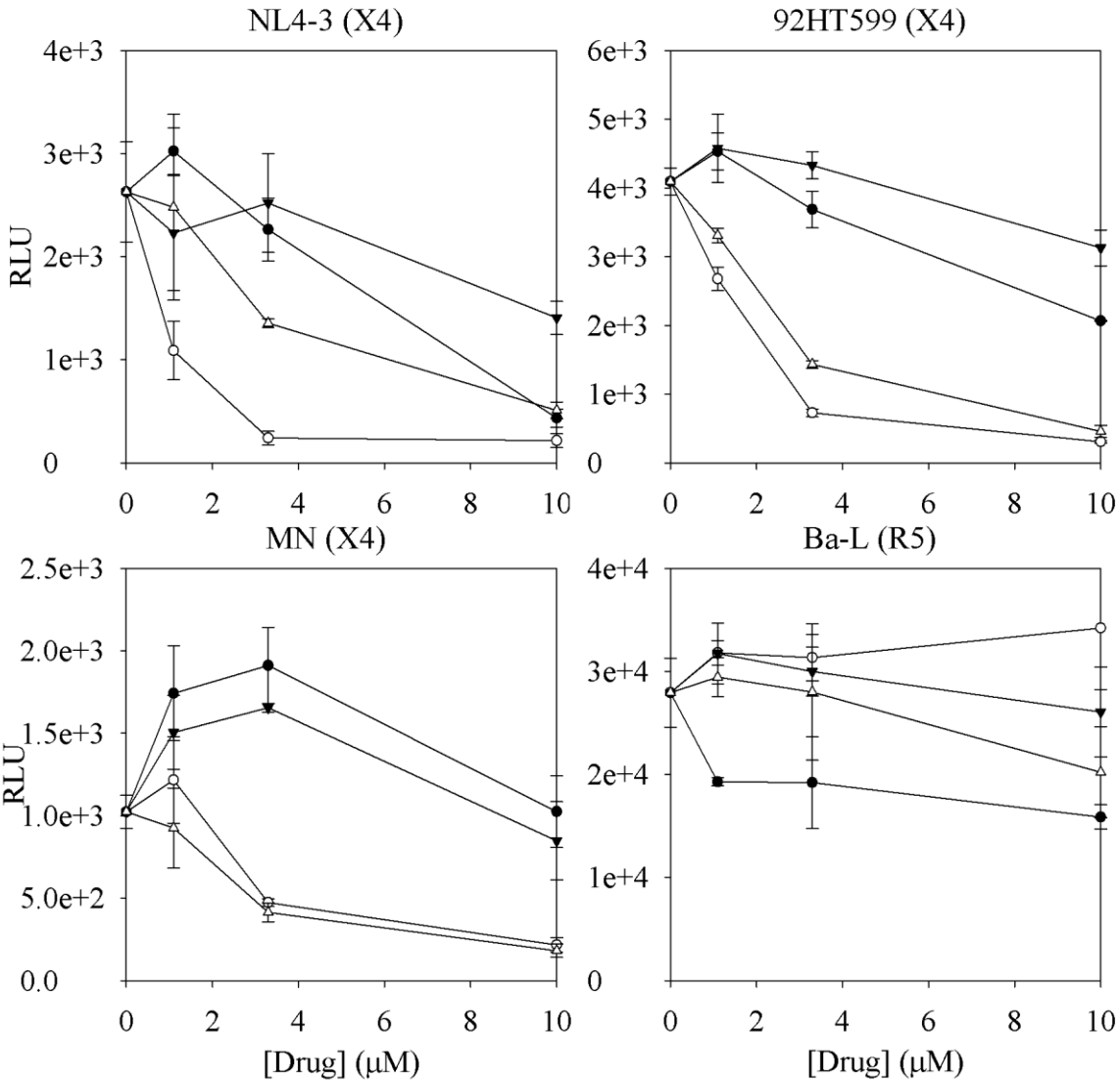


Fig. 2.6. HIV inhibition dose-response trials. Spermine (●), spermine phenylguanide (○), spermidine (▼), and spermidine phenylguanide (∆) were tested for dose-response activity in the TZM-bl luciferase anti-HIV assay. Increased activity against CXCR4-using viral strains were observed with the phenylguanides compared to the underivatized parent amines. Experiments were performed in triplicate with errors shown as ± SEM. RLU = relative luminescence units.

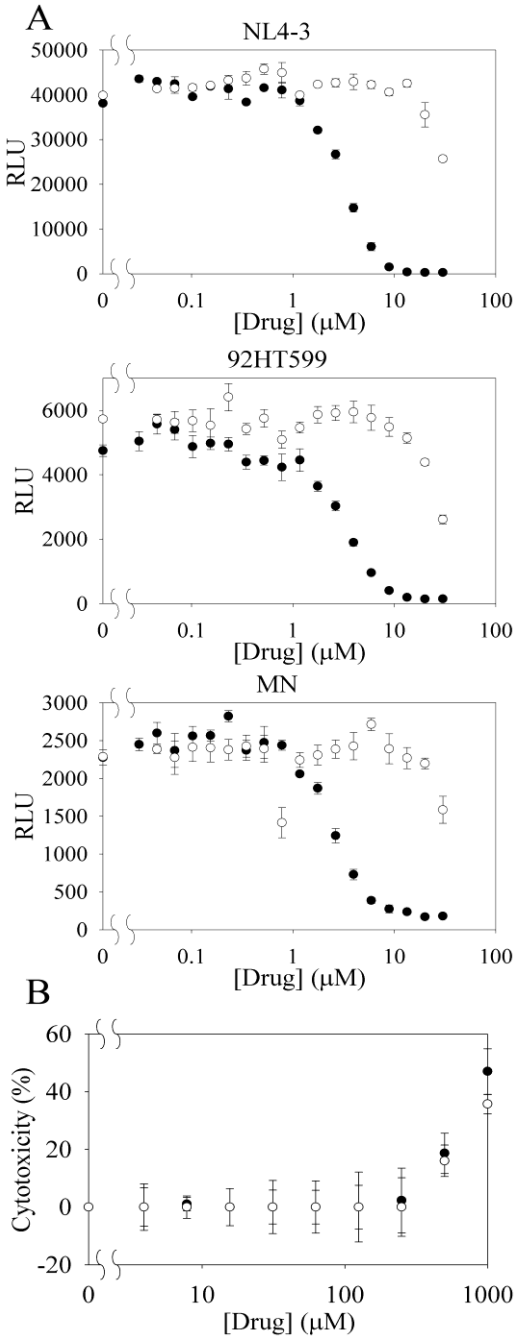


Fig. 2.7. Spermidine phenylguanide activity and cytotoxicity. Panel A: HIV inhibition activity of spermidine phenylguanide (●) and spermidine (○) against three CXCR4 specific HIV clones (NL4-3, 92HT599, and MN) using the TZM-bl assay. Experiments were performed in triplicate with errors shown as ± SEM. RLU = relative luminescence units.Panel B: An MTS assay was used to measure the cytotoxicity (% of control cells) for spermidine phenylguanide (●) and spermidine (○) against the TZM-bl cells. Experiments were performed in duplicate with errors shown as ± SEM.

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CHAPTER THREE

NOVEL COMPOUNDS CONTAINING MULTIPLE GUANIDE GROUPS THAT INHIBIT BREAST CANCER METASTASES

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Joyce B. Shepard

Contributions: Identified CXCR4 expression on cell lines, tested the compounds for CXCR4 affinity, determined cellular toxicity on two cell lines, determined calcium flux inhibition, performed *in vitro* migration assays, *in vivo* toxicity assays, *in vivo* lung colony assays, and wrote the manuscript.

Co-Author: Royce A. Wilkinson

Contributions: Synthesized and purified the guanide, biguanide, phenylguanide, and naphthylguanide compounds and tested the compounds for CXCR4 affinity.

Co-Author: Jean R. Starkey

Contributions: Instructed assay protocols and mouse handling techniques, assisted on all *in vivo* toxicity assays and *in vivo* lung colony assays, discussed the results and implications, contributed to the manuscript, and provided mentorship.

Co-Author: Martin Teintze

Contributions: Discussed the results and implications and contributed to the manuscript.

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NOVEL GUANIDE SUBSTITUTED COMPOUNDS INHIBIT BREAST CANCER LUNG METASTASES

Joyce B. Shepard[1], Royce A. Wilkinson[1], Jean R. Starkey[2], Martin Teintze[1]

[1]Department of Chemistry and Biochemistry, [2]Department of Microbiology, Montana State University, Bozeman, MT 59717 USA

Abstract

We have synthesized numerous guanide, biguanide, and phenylguanide compounds that bind to CXCR4 and may prevent CXCR4-facilitated metastasis. CXCR4 has already been demonstrated to be overexpressed on some breast cancer cells including the human MDA-MB-231 cell line. Cancer cells overexpressing the CXCR4 receptor are capable of undergoing metastasis to organs expressing high levels of CXCL12 and subsequently proliferating. The novel compounds presented herein were tested for CXCR4 affinity, toxicity, receptor activation, and for their ability to prevent breast cancer metastases. The results showed four compounds bound to CXCR4 at IC_{50} values of 0.5-1.5 μM with no associated cell toxicity or receptor activation at these values. These compounds were then tested for *in vitro* wound migration inhibition and the compound with the best selectivity index value that showed *in vitro* migration inhibition. Spermidine trisphenylguanide, was then tested in an *in vivo* mouse breast cancer lung metastasis model. Initial *in vivo* results in mice treated with alexidine and spermidine trisphenylguanide showed reductions in the number of MDA-MB-231 lung metastases compared to mock-treated control mice. Following these results, new compounds were designed with naphthylguanide functional groups on two backbones (spermidine and spermine) and then were tested for efficacy. The results show an order of magnitude better binding (nM vs μM range) to the CXCR4 receptor without correspondingly increased toxicity. *In vivo* lung colony experiments with the napthylguanide compounds also showed reductions in MDA-MB-231 lung metastases without cardiac, liver, or kidney toxicity.

Introduction

The second most common neoplasm in women is breast cancer, second only to skin cancer. In 2011, an estimated 288,000 people in the United States were diagnosed with breast cancer [2]. Roughly 30% of breast cancer patients will ultimately go on to develop metastatic disease in their lifetimes. The majority of deaths from breast cancer are from recurrent or metastatic breast cancer [3].

Metastases can be defined as secondary growths of a tumor in locations distant from the primary tumor [4]. Metastases pose multiple problems for oncologists and one of the most significant obstacles relates to size. At the time of initial diagnosis, most metastases are below the detection limit of current analytical instruments and are too small to cause traceable symptoms. The second problem with metastases is that they can be located anywhere in the body, both relatively close and distant to the primary tumor. To address this latter difficulty, research has identified the typical pathways utilized by cancer cells when they mobilize from the primary tumor. Some pathways can be predicted based on nearby location, such as the draining lymph node which drains the tissue where the tumor is located [5]. Other tumor locations can be theorized given the expression of tissue specific adhesion molecules [6] while even more locations can be predicted based on chemokine expression. Chemokines represent a signaling system the cells use to metastasize to other sites within the body. For example, sites of metastases that express high levels of the natural

CXCR4 ligand, CXCL12, induce cancer cells expressing the CXCR4 receptor to migrate to those specific sites.

Many tumors overexpress CXCR4 and this overexpression can lead to cell chemotaxis, angiogenesis, and enhanced tumor cell survival. Several different types of tumors overexpress CXCR4, and among these various options our research focuses on human breast cancer [7]. Sites that normally express CXCL12 in the human body are the bone marrow, lungs, lymph nodes, and liver; these sites also happen to be the most common areas for breast cancer metastasis [7]. Several publications during the last decade have validated this connection. These research papers have demonstrated that interfering with the CXCR4: CXCL12 pathway can reduce or stop cancer metastasis. Antibodies [7, 8], RNAi [9], siRNA [10, 11], peptide inhibitors such as T140 [12], or other small peptides [13], organic molecules such as AMD3100 [8, 9, 14], and heparinoids [15] have all demonstrated the ability to reduce CXCR4 cellular metastasis by binding to CXCR4 and preventing CXCL12 from binding.

Our lab developed three series of twelve compounds containing positively charged guanide, biguanide, or phenylguanide groups that bind to CXCR4 and could potentially interfere with the CXCR4-facilitated cell metastasis [1]. The distribution and total number of the positively charged groups varies among the compounds. Half of the compounds are derived from linear backbone chains while the other half are derived from multi-armed compounds, including dendrimers [1]. These compounds were tested for activity in blocking CXCR4-

directed tumor metastasis. Initial assessment was done to find which compounds displayed the best binding to CXCR4 at non-toxic doses, followed by evaluation of individual compound ability to retard migration of breast cancer cells in a tissue culture wound model. The effective compounds were further tested in a mouse lung colonization assay to determine their capacity in reducing the number of metastases *in vivo* compared to untreated controls. Collectively, the results highlighted spermidine trisphenylguanide as the most generally effective compound synthesized in our initial study. Following the success of the first generation compounds in reducing *in vivo* metastases, a series of new compounds were designed with naphthylguanide groups on the same backbone chains. These compounds showed even better binding to CXCR4 at non-toxic concentrations. *In vivo* testing showed that two naphthylguanide compounds (spermidine bis-2-naphthylguanide and spermine tris-2-naphthylguanide) also reduced lung metastases to a significant extent.

Materials and Methods

Cell Lines

The following cell lines were used in this study; Human breast cancer cell line MDA-MB-231 (ATCC HTB-26), mouse lewis lung carcinoma cell line 3LL (ATCC CRL-1642), mouse mammary carcinoma cell line 4T1 (ATCC CRL-2539), human T-lymphoblastic-like cell line CCRF-CEM (ATCC CCL-119) , human

keratinocyte cell line HaCaT [1], canine thymocyte cell line Cf2Th-CXCR4 [16], and canine thymocyte cell line Cf2Th-CCR5 [17].

CXCR4-Inhibitor-T140 Binding Assay

This assay was performed as previously described in Chapter 2 of this thesis on page 9 [1].

Cell Viability Assay

All compounds were tested for cell toxicity on 1 x 10^5 MDA-MB-231 cells using the Promega Corporation CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]). Nine concentrations spanning up to six orders of magnitude were used for each compound unless there were limitations due to only small amounts of compound being available. Each concentration was performed in six replicates. Cells were incubated with compound for 48 to 72 hours. MTS was added and incubated for one to five hours prior to absorbance reading at 490 nm. The same assay was done on HaCaT cells.

CXCR4 Expression Using Flow Cytometry

All cell lines were harvested and resuspended to ~2 x 10^5 cells/mL in PBS (phosphate buffered saline, 0.137 M NaCl, 0.016 M phosphate, pH 7.2). T140 with a fluorescein tag [1] was added to the cells at a final concentration of 75 μM. The mixture was incubated for 30 minutes on ice. Fluorescent data was collected

on a BD Biosciences FACSCalibur flow cytometer and plotted as histograms of fluorescence intensity verses number of events.

Calcium Flux Assay

Assays were done using Molecular Probes® Fluo-4 ™ Calcium Indicators on the Molecular Devices Flexstation II Scanning Fluorometer using CCRF-CEM cells responding to exposure of CXCL12. Cells were loaded with the Fluo-4™ intracellular calcium binding dye for one hour to allow dye to permeate the cell membrane. Initial experiments determined the optimum amounts of CXCL12 for fluxes by analyzing the total calcium flux for varying concentrations. Subsequently, three compound concentrations were added to CCRF-CEM in separate wells with the dye and allowed to incubate for one hour in duplicate, followed by addition of CXCL12 in the Flexstation II to all the wells and monitored the resultant calcium flux through fluorescence.

Tissue Culture Wound Assay

MDA-MB-231 cells were grown to confluency in DME:F12 media supplemented with 10% fetal calf serum (FCS). The media was removed and a straight line carved into the monolayer using a pipette tip. Fresh media was added with or without compound to each culture at one of multiple compound concentrations. Measurements of the width of the wound (width of cleared zone) were taken initially at time of the addition of compound to the wound, and then twice daily until the ‘wound’ closed or four to five days post wounding.

Matrigel Assay

Guidelines were followed for use of the BD BioCoat Matrigel ™ Invasion Chamber. Briefly, 2.5 x 10^4 cells/mL MDA-MB-231 cells in DME: F12 media plus 10% FCS in the presence or absence of compound were placed in the top chamber, and DME: F12 plus 10% FCS, 0.4 µg CXCL12 were placed in the bottom chamber. Chambers were incubated at 37 °C in the presence of 5% CO_2 for 22 to 24 hours, and then unmigrated cells were removed from the top chamber by scraping the top of the membrane with a Q-tip. The lower side of the chamber separation membrane was fixed with methanol, stained with acid hematoxylin, and the number of migrated cells enumerated under a microscope.

Mouse Toxicity

A. Alexidine Dihydrochloride Alexidine Dihydrochloride in Tyrode's calcium magnesium free saline solution (CMF) was injected i.v. (intravenous) (100 µL of 17 µM) into seven *SCID* mice. Reinjections were carried out on days one, two, five, nine, and twelve. Heart punctures were carried out under anesthesia to recover blood from the mice prior to euthanization on day twenty eight. Blood was allowed to coagulate, and then spun down to separate the blood clot from serum. Serum was submitted to the Montana Veterinary Diagnostic Lab for analysis. Serum was obtained concomitantly to the lung colony assay at the time the mice were sacrificed.

B. Spermine Bisguanide Twelve Balb/C mice were injected with 100 µl of 20 µM spermine bisguanide in Tyrodes' CMF on day one, three, and eleven. Heart punctures under anesthesia were carried out to recover blood from the mice prior to euthanization on day fourteen. Blood was allowed to coagulate and was then centrifuged to separate blood clot from serum. The serum was submitted to the Montana Veterinary Diagnostic Lab. The blood collection was performed concurrently with the mice on the lung colony assay at the time that they were sacrificed.

C. Spermidine Trisphenylguanide,
Spermidine Bis-2-Napthylguanide, or
Spermine Tris-2-Napthylguanide Toxicities Three Balb/c mice were injected intravenously with 100 µL of 300 µM spermidine trisphenylguanide, 100 µL of 100 µM spermidine bis-2-naphthylguanide, or spermine tris-2-naphthylguanide in Tyrode's CMF saline solution. The compound was injected on day one, two, and five. On day six, heart punctures were carried out under anesthesia to recover blood from the mice prior to euthanization. Blood was allowed to coagulate and spun down to separate blood clot from serum. Serum was submitted to Montana Veterinary Diagnostic Lab for analysis.

Lung Colony Metastasis Assays

A. Alexidine and MDA-MB-231 Female SCID mice were injected i.v. (intravenous) with 1.4 x 10^6 MDA-MB-231 cells in 100 µl of Tyrode's CMF. Alexidine in CMF (100 µL of 17 µM) was injected i.v. 24 hours prior to the cell

injection and again immediately after injection with cells. Follow up injections of compound were carried out on day five, nine, and twelve after injection of the tumor cells. Thirty days post tumor cell injection, the mice were euthanized and lungs harvested. Lung sets were stained with Bouins fixative [18], and lungs weighed to determine the tumor mass resulting from cells that extravasated out of the circulation and proliferated in the lungs.

B. Spermine Bisguanide and 4T1 Female Balb/C mice were injected i.v. with 1.2 x 10^5 4T1 cells in 100 µL of Tyrode's CMF saline solution. Spermine bisguanide in CMF (100 µL of 20 µM) was injected i.v. three hours prior to injection with the tumor cells. Follow up injections of spermine bisguanide were done two and ten days post tumor cell injections. Fifteen days post injection, the mice were euthanized and lungs removed. The lungs were stained with Bouins fixative and lung colonies counted to determine the relative numbers of cells that extravasated from the circulation and proliferated in the lungs.

C. Spermidine Trisphenylguanide and MDA-MB-231 Female SCID mice were injected i.v. with 1 x 10^5 MDA-MB-231 cells in 100 µl of Tyrodes CMF saline solution. Spermidine trisphenylguanide in CMF (100 µL of 50 or 200 µM) was injected 24 hours prior to injection with MDA-MB-231 cells and again immediately after injection with cells. Follow up injections of compound were carried out on day five after injection of the tumor cells. Thirty days post injection, the mice were euthanized and the lungs removed. The lungs were stained with Bouins fixative

and lung colonies counted to determine the relative numbers of cells that extravasated out of the circulation and proliferated in the lungs.

D. Spermidine trisphenylguanide, spermidine bis-2-naphthylguanide or spermine tris-2-naphthylguanide and MDA-MB-231 Female SCID mice were injected i.v. (intravenous) with 1.2 x 10^5 MDA-MB-231 cells in 100 µl of Tyrodes CMF saline solution. 100 µL of 100 µM spermidine bis-2-napthylguanide, 100 µM spermine tris-2-napthylguanide, or 300 µM spermidine trisphenylguanide in CMF was injected i.v. 24 hours prior to injection with MDA-MB-231 cells and again immediately after injection with the cells. Follow up injections of compound were done on day five after injection of the tumor cells. Thirty days post injection, the mice were euthanized and lungs removed. The lungs were stained with Bouins fixative and lung colonies counted to determine the number of cells that extravasated from the circulation and proliferated in the lungs.

Results

Cellular Expression of CXCR4

Flow cytometry was performed on three different cancer cell lines to verify that they expressed CXCR4 on the cell surface. A cell line that did not express CXCR4 was used to establish the parameters for a negative cell line (Cf2Th-CCR5, Figure 3.1A). A positive cell line which overexpresses the CXCR4 receptor, Cf2Th-CXCR4, was used to set the parameters for positive CXCR4 expression (Figure 3.1B). The MDA-MB-231 human breast cancer cell line was

used in most of our breast cancer studies. This cell line is known to express CXCR4 on the cell surface [7] and indeed displayed positive results in the flow cytometry assay (Figure 3.1C). Perhaps to be expected, the expression level appeared to be lower than that of the genetically modified Cf2Th-CXCR4 cell line.

The other two cell lines tested in this study were the mouse Lewis Lung carcinoma cell line, 3LL, and the mouse mammary carcinoma, 4T1. The flow cytometry assay showed 3LL to only have a very small population of CXCR4 positive cells (Figure 3.1D). Literature searches revealed that 3LL expresses CXCR4 at very low levels within the cell population [19]. The 4T1 cell line was used as the mouse breast carcinoma equivalent model to human breast carcinoma. There are multiple reports that utilize this cell line in their breast cancer studies, including CXCR4 studies performed using the 4T1 cell line [9, 20-22]. Given the literature precedence, we used 4T1 in one of our *in vivo* studies evaluating the effectiveness of spermine bisguanide. However, no reduction in lung tumor load was observed using this cell line *in vivo*. Following these negative results, a flow cytometry experiment was performed and only a sub-population of CXCR4 positive cells were observed (Figure 3.1E). This prompted the requirement for a more thorough literature search which revealed that the sub-population of CXCR4 positive 4T1 cells were hypothesized to be a small population of non-adherent cancer stem cells overexpressing CXCR4 in the normally adherent cell populace [21]. The isolation of the CXCR4 expressing

non-adherent population of cells through tissue culture passaging on the non-adherent surface coating polyhema was then attempted. Polyhema selects for a non-adherent cell phenotype and would therefore select for the hypothetical CXCR4 positive non-adherent stem cell population. However, following multiple passages with increasing concentrations of polyhema, the flow cytometry results showed a failure to isolate a CXCR4 expressing population (Figure 3.2), clearly indicating that a more specific selection was needed.

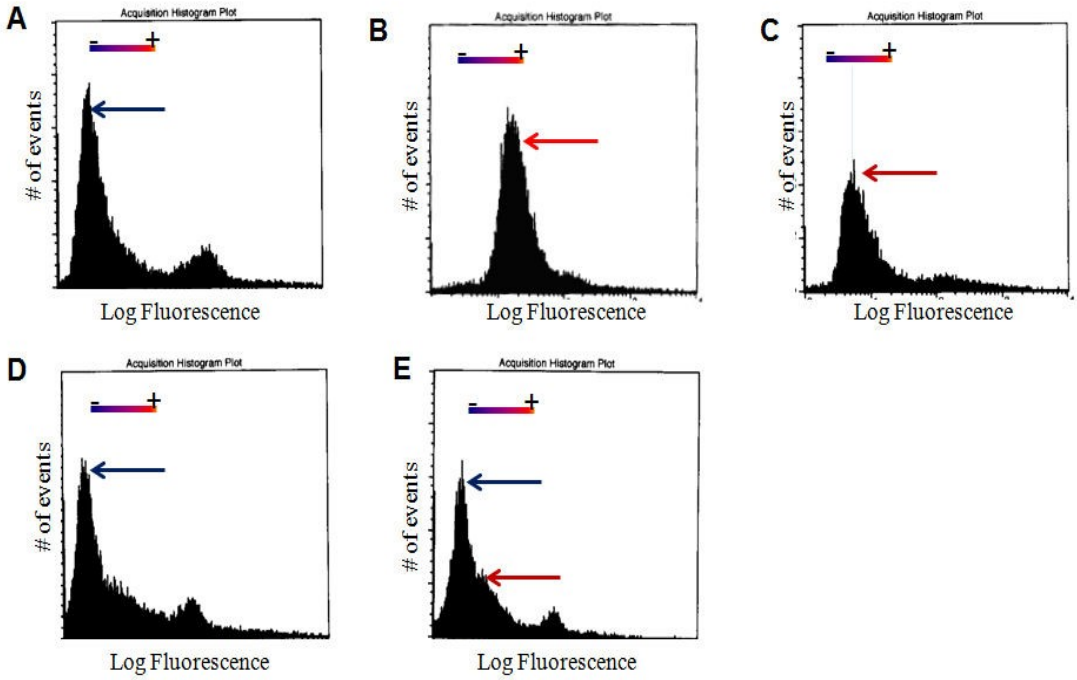


Figure 3.1. Histogram evaluation of CXCR4 expression on three unknown cancer cell lines. The purple to red bar indicates the parameters of the negative cell line (Cf2Th-CCR5) that does not express CXCR4 in purple and the positive cell line (Cf2Th-CXCR4) that overexpresses CXCR4 in red. Arrows designate the peak of interest. Purple arrows indicate negative cell populations and red arrows likely indicate positive cell populations. The peaks at 10^2 are false positive peaks from non-specific binding of the T140 peptide to the cell surface. A) Negative Control (Cf2Th-CCR5), B) positive control (Cf2Th-CXCR4), C) MDA-MB-231, D) 3LL, and E) 4T1.

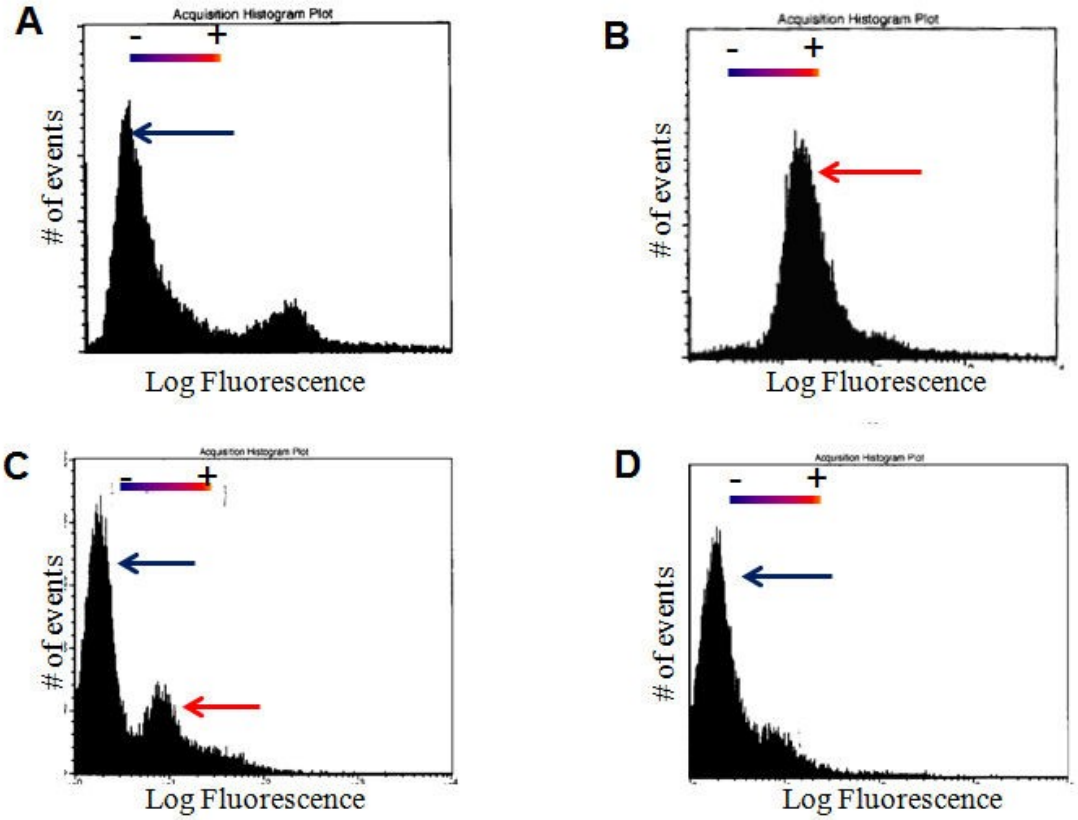


Figure 3.2. Histogram of the attempt to isolate 4T1 CXCR4 positive cells. Purple to red bar indicates the negative cell population in purple and the positive cell population in red. Purple arrows are the negative cell population and the red arrows are the positive cell population. The peak at 101.2 in the negative control is likely from non-specific binding of the fluorescent T140 peptide on the cell surface. A) Negative control that does not express CXCR4 (Cf2Th-CCR5), B) positive control that overexpresses CXCR4 (Cf2Th-CXCR4), C) wild-type 4T1 cells, and D) non-adherent 4T1 cells after 6% polyhema selection.

Binding to CXCR4

All guanide, biguanide, and phenylguanide derivatives synthesized in our lab, as well as the parent underived amines, were initially tested for binding to CXCR4 as discussed in Chapter two of this thesis [1]. Figure 3.3A and B show a

sample gel and corresponding graph of fluorescence intensity versus concentration. Reductions in fluorescence were indicative of compound binding to CXCR4 (Figure 3.3 B). In these plots, the value at which there is a 50% reduction of fluorescence is the 50% inhibitory concentration (IC_{50}). The IC_{50} values for each of the compound derivatives are listed in Table 3.1, adapted from Chapter 2 [1]. The compounds listed as “Starting Amines” are the underivatized parent amine compound prior to derivatization with guanide, biguanide, or phenylguanide groups.

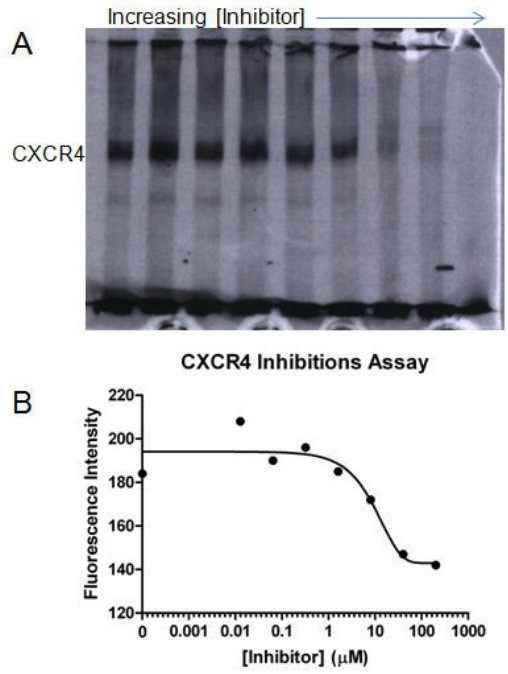


Figure 3.3. CXCR4/T140/Inhibitor Assay. A) Example of SDS-Page gel of CXCR4-inhibitor binding assay. The dark band in the center is CXCR4 and is denoted as such in the figure. Fluorescent quantification was performed on the band. Increasing concentrations of inhibitor are represented in lanes moving from left to right. (B) Example graph of the percent of maximum fluorescence vs inhibitor concentration from the gel in A.

CXCR4-Inhibitor Binding Assay Results

	Starting Amine	Guanide	Biguanide	Phenylguanide
Butanediamine	> 200	50	> 200	> 200
Hexanediamine	≥ 200	> 200	> 200	150 (1) 20 (2)
Diethylenetriamine (DETA)	≥ 200	50	> 200	15 (1) 6 (2)
Spermidine	> 200	8 (2) 10 (3)	65	**0.2 (2) 0.2 (3)**
Spermine	75	10	4-8	**0.5**
Trisethylaminomelamine (TEAM)	> 200	20	> 200	1.5
Trisbutylaminomelamine (TBAM)	≥ 100	10	> 200	6 (2) 20 (3)
Trishexylaminomelamine (THAM)	> 200	2	45	> 200
BEMA dimer	> 200	50	ND	15 (1) 7(2)
PAMAM GO	> 200	4	> 50	3
DNT2300	> 200	8	7.5	**1.5**
DNT2200	≥ 200	15	≥ 200	3.5

Table 3.1. CXCR4 Binding Assay Results. All data are given as IC_{50} values in μM. Numbers in parentheses indicate the number of functional groups added. Highlighted in **red** are the top 4 compounds. The greater than sign (>) designates the highest concentration tested that did not bind to CXCR4. ND = Not Done. Reproduced with permission from [1].

The four compounds that bound to CXCR4 with the highest affinity were spermidine bis & trisphenylguanide, spermine tetraphenylguanide, and DNT2300 phenylguanide (Table 3.1). These four compounds have IC_{50} values between 0.2-1.5 μM. While none of these guanide derivatives bound to CXCR4 with as high of affinity as the peptide T140, which binds at the low nanomolar level (0.43 nM) [23], they were the most potent of the synthesized compounds tested. However, since T140 is not a viable therapeutic possibility due to the fact that it is rapidly degraded *in vivo* [24], there was significant value in further testing the four synthetic compounds. While the clearance rates of our top four compounds are unknown, it is reasonable to hypothesize that they would not be degraded as fast

as the T140 peptide. The remaining compounds synthesized had binding affinities ranging between 3 μM to > 200 μM (Table 3.1). The highest concentration tested was 200 μM unless designated otherwise. Any compound activity listed as > 200 μM is considered inactive and not a candidate for further evaluation.

The only parent amine that bound to CXCR4 was spermine with activity at 75 μM (Table 3.1). The rest of the parent amine compounds did not bind CXCR4 at up to 200 □M, which was the highest level tested. Therefore, there is significant evidence that it is the derivatization scheme that is resulting in the activity of the compounds in terms of their selective binding to CXCR4. The phenylguanide derivatives were generally the most active. The THAM, PAMAM, DNT2300, and DNT2200 guanide derivatives showed activity but the IC_{50} values were not as low as for the phenylguanide derivatives. Only two biguanide derivatives showed any activity, the spermine biguanide and the DNT2300 biguanide. While these two structures are very different, they had similar IC_{50} values of 4-8 μM and 7.5 μM, respectively. The shortest carbon chain derivatives showed the least activity in general, demonstrated by butanediamine, while the longer chains, spermidine and spermine, and the dendrimers showed generally better activity.

Toxicity Data

The toxicity of each of these compounds was tested on the human breast cancer cell line MDA-MB-231 (Table 3.2). The concentrations listed are the CC_{50}

values, which are defined as the concentration values which yield 50% cell death. Any toxicity value listed with a ‘dagger’ sign (†) indicates that the concentration listed was the highest concentration tested and that 50% cell death had not been observed.

Most compounds did not display any significant toxicity, but there were a few exceptions. The spermine parent compound and all of its derivatives were more toxic to the cells than most other compounds, with CC_{50} values ranging from 20 μM to 200 μM. Surprisingly, the spermine derivative that binds the best to CXCR4, spermine phenylguanide (IC_{50} = 200 μM), was only toxic at ten-fold higher concentration than the less active guanide and biguanide derivatives.

Two of the other three compounds that had the highest affinity (low IC_{50}) for CXCR4 had better CC_{50} values. Spermidine bis- and trisphenylguanide had very high CC_{50} levels (> 1 mM and 500 μM, respectively), meaning that they are less toxic to cells than spermine phenylguanide, while still retaining active binding to CXCR4. The fourth compound that had the best affinity for CXCR4 was DNT2300 phenylguanide. DNT2300 phenylguanide was toxic at 50 μM, making it the most toxic of the top four compounds displaying affinity for CXCR4.

Toxicity Data on MDA-MB-231

	Starting Amine	Guanide	Biguanide	Phenylguanide
Butanediamine	† 1 mM	† 100	† 100	† 100
Hexanediamine	† 1 mM	†100	† 1 mM	†50
Diethylenetriamine (DETA)	† 1 mM	†100	†100	(1)1 mM (2) 500 µM
Spermidine	200	500	100	(2) †1 mM (3) 500 µM
Spermine	20	†10	25	200
Trisethylaminomelamine (TEAM)	†1 mM	†1 mM	†1 mM	200
Trisbutylaminomelamine (TBAM)	x	1 mM	†1 mM	†10
Trishexylaminomelamine (THAM)	†1 mM	100	†100	†100
BEMA dimer	† 1 mM	200	x	(1) 400 (2) †100
PAMAM GO	†1 mM	†100	†100	†100
DNT2300	40	†50	20	†100 & 50
DNT2200	100	†50	500	†100

Table 3.2. The listed CC_{50} values for all compounds are in µM unless designated otherwise. Numbers in parentheses indicate the number of functional groups added. The top four compounds from the CXCR4 binding assay are highlighted in **red**. "X" indicates that the compound was not tested due to incomplete synthesis or not enough material to test. The "†" sign indicates that the highest concentration tested (value listed) did not result in 50% cell death.

The three compounds showing high affinity for CXCR4 (table 3.1) were further evaluated for toxicity on a second cell line. The cell line selected for these additional studies was the human keratinocyte cell line, HaCaT (Table 3.3). Most of the CC_{50} values were quite comparable between the two cell lines. The spermidine bisphenylguanide was not retested on the HaCaT cell line due to limiting amounts of available compound.

Toxicity Data on HaCaT

Compound	HaCat CC_{50} (μM)
Spermidine	> 1000
Spermidine - 3ΦG	480
Spermine	> 1000
Spermine - 4ΦG	96
DNT2300	22
DNT2300 - 6ΦG	200

Table 3.3. CC_{50} values for HaCat keratinocyte cells.

With the IC_{50} and CC_{50} data completed, it was then possible to calculate the selectivity index (SI) value for the top four compounds by simply taking the CC_{50} value and dividing it by the IC_{50} value (Table 3.4). High selectivity index numbers indicate that the compound has low toxicity to cells while also requiring low concentrations to be effective.

Selectivity Index

	IC50 (μM)	CC_{50} (μM)	SI	CC_{50} (μM)	SI
Compound		HaCat	HaCat	MDA-MB-231	MDA-MB-231
Spermidine	>200	> 1000	>5	200	>1
Spermidine - 2ΦG	0.2	X	X	>1 mM	>5000
Spermidine - 3ΦG	0.2	480	2400	500	2500
Spermine	75	> 1000	>13.3	20	0.26
Spermine - 4ΦG	0.5	96	192	200	400
DNT2300	>200	22	0.11	40	>0.2
DNT2300 - 6ΦG	1.5	200	133.3	>100 & 50	>33.3

Table 3.4 Calculated SI values. High SI values mean that the compound has low toxicity while needing low concentrations to be effective. The best SI is for spermidine bisphenylguanide in this chart. Blocks marked with an “X” mean the compound was not tested.

Calcium Flux Assay

Given the results from the competition assays which indicated that some compounds were binding to CXCR4 in the same manner as T140, it was necessary to further classify this binding as being either antagonistic or agonistic. To verify antagonistic versus agonistic activity, we evaluated the CXCL12/CXCR4 induced calcium flux response to ensure it was inhibited by the presence of the test compound (Figure 3.4).

CCRF-CEM cells which endogenously express CXCR4 [25] (Figure 3.5) were loaded with the Molecular Probes™ Fluo-4 intracellular calcium fluorescent indicator and incubated with one of our compounds at multiple concentrations. Difficulties with excessive cell death occurring during the course of this assay interfered with getting optimal results but preliminary results were completed successfully on some compounds. The reason for the occurrence of cell death was not determined.

The initial experiment for each calcium flux was to determine how much of the CXCL12 ligand was necessary for inducing the maximum intracellular release of calcium (Figure 3.4A). The concentration of CXCL12 that resulted in the highest intracellular calcium (highest curve on the graph) was then halved and used in the experiments done in the presence of one of the compounds made in our lab. The concentration of CXCL12 was halved to ensure that saturation of receptors was not occurring. If the compound was an inhibitor, a reduction in the amount of released intracellular calcium would be noted. If the compound was an

agonist, there would not be a reduction in the amount of released intracellular calcium but rather an increase in the calcium flux.

These experiments demonstrated that the highest CXCR4 binding compounds spermidine bis and trisphenylguanide, DNT2300 phenylguanide, and T140 (without the fluorescent tag) showed inhibition of the calcium flux response, as seen in Figure 3.4B. As the concentration of the compounds was increased, the intracellular calcium levels decreased further as well. Therefore, these compounds were able to be classified as antagonists of the CXCR4 receptor due to the reduction of intracellular calcium when the compounds were present. Spermine (underivatized), which did not demonstrate binding to CXCR4 in the CXCR4 inhibition assay, was also tested to see if it interfered with the release of intracellular calcium (Figure 3.4B). It did not illustrate a reduction in intracellular calcium like the other three compounds which bound to CXCR4 showed. This further verified that the compounds identified in the binding assay were CXCR4 antagonists and compounds that did not bind to CXCR4 were not capable of activating it.

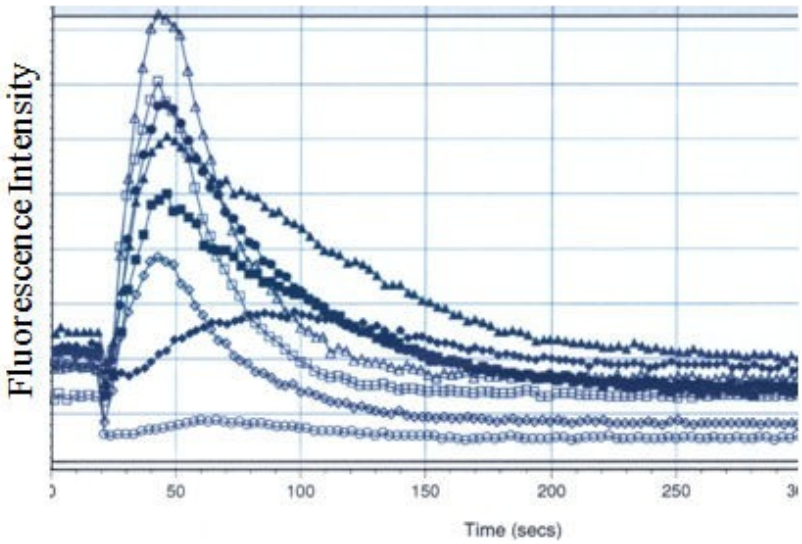


Figure 3.4A. Determination of the concentration of CXCL12 that results in the highest calcium flux. The optimum concentration value to use results in the highest curve on the graph divided by two to ensure that oversaturation does not occur. The symbols in the figure represent: O (negative control, no CXCL12); □ (625 nM CXCL12); Δ (208 nM CXCL12); ◊ (64 nM CXCL12); ● (23 nM CXCL12); ■ (7.7 nM CXCL12); ▲ (2.5 nM CXCL12); ♦ (0.85 nM CXCR4). The best flux was obtained with 208 nM CXCL12 in this graph.

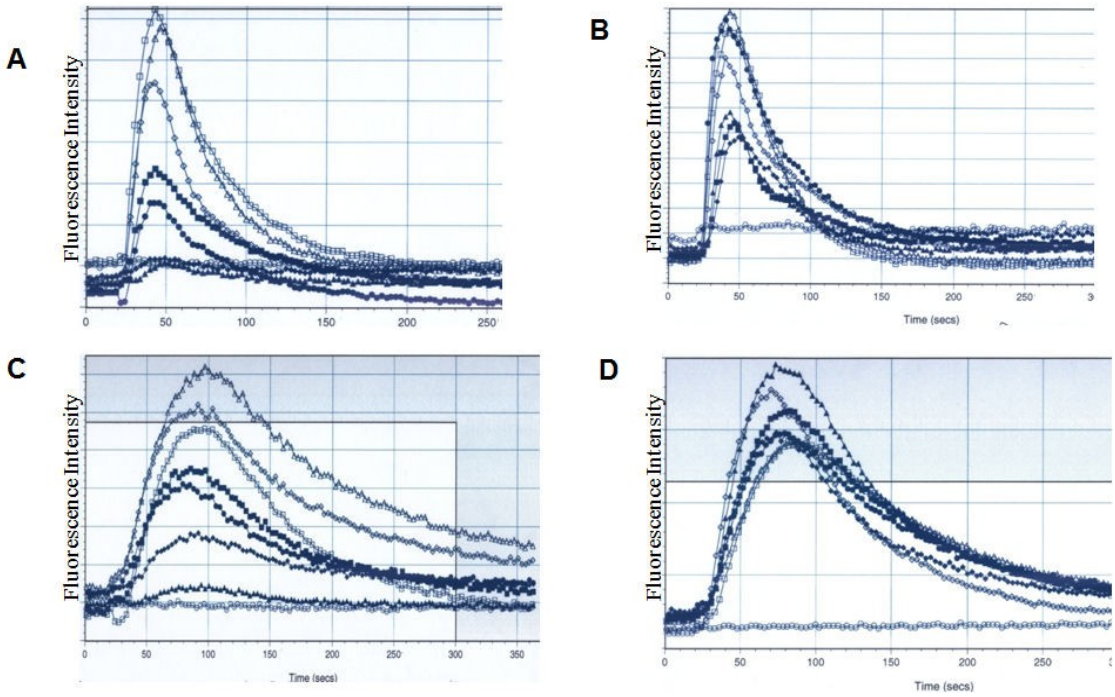


Figure 3.4B. Calcium Flux. A) Calcium graph when stimulated with CXCL12 in the presence of increasing amounts of T140. The symbols in the figure represent: O (negative control, no CXCL12); □ and Δ (positive controls, CXCL12); ◊ (1 μM T140); ● and ■ (10 μM T140); ▲ and ♦ (30 μM T140).
B) Calcium graph when stimulated with CXCL12 in the presence of increasing amounts of spermidine trisphenylguanide. The symbols in the figure represent: O (negative control, no CXCL12); □ and Δ (positive controls, CXCL12); ◊ and ● (20 μM spermidine trisphenylguanide); ■ (50 μM spermidine trisphenylguanide); ▲ and ♦ (100 μM spermidine trisphenylguanide).
C) Calcium graph when stimulated with CXCL12 in the presence of increasing amounts of DNT2300 phenylguanide. The symbols in the figure represent: O (negative control, no CXCL12); □ and Δ (positive controls, CXCL12); ◊ (20 μM DNT2300 phenylguanide); ● and ■ (50 μM DNT2300 phenylguanide); ▲ and ♦ (100 μM DNT2300 phenylguanide).
D) Calcium flux when stimulated with CXCL12 in the presence of increasing amounts of Spermine. This parent compound should not bind to CXCR4. The symbols in the figure represent: O (negative control, no CXCL12); □ and Δ (positive controls, CXCL12); ◊ (10 μM spermine); ● (50 μM spermine); ■ and ▲ (100 μM spermine); ♦ (500 μM spermine). This compound did not result in decreased calcium flux.

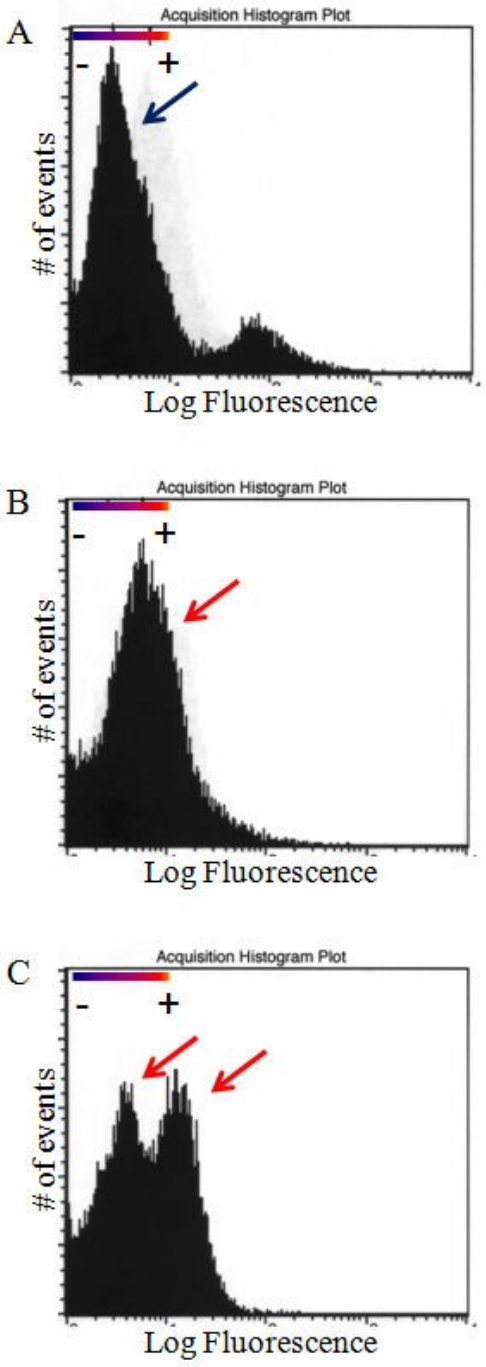


Figure 3.5. Flow cytometry demonstrating CXCR4 expression on CCRf-CEM cells. The purple to red bar indicates negative cell populations (purple) and positive cell populations (red). Purple arrows indicate negative populations and red arrows indicate positive populations. A) Negative Control (Cf2Th-CCR5), B) Positive Control (Cf2Th-CXCR4), C) CCRf-CEM cells. CCRf-CEM cells have a bimodal CXCR4 positive population which has not been explained.

Tissue Culture Wound Assay

In vitro cell migration was tested using the tissue culture wound assay. Each compound that was synthesized was tested in the wound assay to determine if it retarded cell migration into a cleared area (Figure 3.6). While we did not expect any compound that did not bind well to CXCR4 to inhibit cell migration, it was necessary to verify the results using the *in vitro* tissue culture wound assay. This wound assay is a standard and inexpensive way to determine if a compound can retard tumor cell migration. It is not specific and only semi-quantitative, but is effective in identifying compounds of potential interest for further evaluation. All compounds were tested but the only compounds to show repeatable retardation of MDA-MB-231 wound recovery were the four compounds that bound to CXCR4 (Table 3.5).

Many wound assays showed cell death, precipitation of the compound in the culture, or unrepeatable migration inhibition (data not shown). When cell death was readily observed microscopically, the results from those compounds were disregarded as false positive and are not included herein.

Spermidine bisphenylguanide had migration inhibition at the lowest concentration of all of the compounds according to the selectivity index. It displayed migration inhibition at 30 μM levels. Unfortunately, experiments on this compound were limited by the small quantity of material available for testing. Spermidine trisphenylguanide required a higher concentration of compound (100 μM) to get the same distinct migration inhibition. Interestingly enough, it required

100-fold more compound to phenotypically inhibit migration *in vitro* than it did to bind to CXCR4. This emphasizes that having higher SI values are of extreme importance because increasing the dose 100 fold without corresponding toxicity is vital. The assay results also showed migration inhibition for DNT2300 at 86 μM concentration value. This value shows that we may be able to go back and re-evaluate spermidine triphenylguanide and find migration inhibition at less than 100 μM and greater than 30 μM levels.

Spermine tetraphenylguanide showed slowed migration at 30 μM but did not show migration inhibition at the higher concentration of 91 μM. Several repeats with this compound did show repeatable results of the 30 μM slowed wound recovery, but the 91 μM data point could not be repeated due to a shortage of available compound material. The lack of activity at 91 μM could have resulted from precipitation in solution. Due to compound shortage, as well as the questionable variability of this compound, it was not further evaluated *in vivo*.

Compound	Ranking	Concentrations Tested	Results
Spermidine bisphenyguanide	1	10, 30 µM	10=Very slow Recovery, 30=No Recovery
Spermidine trisphenyguanide	2	10, 30, 100 µM	10=Normal Recovery, 30=Slightly slowed Recovery, 100=No Recovery
DNT2300 hexaphenylguanide	3	33, 86 µM	33=Normal Recovery, 86=No Recovery
Spermine tetraphenylguanide	4	1, 10, 30, 91 µM	1 &10=Normal Recovery, 30=Slowed Recovery, 91=Normal Recovery

Table 3.5. Wound assay results for the top four compounds based on the SI. Wound results for the remainder of compounds are not shown due to inactivity.

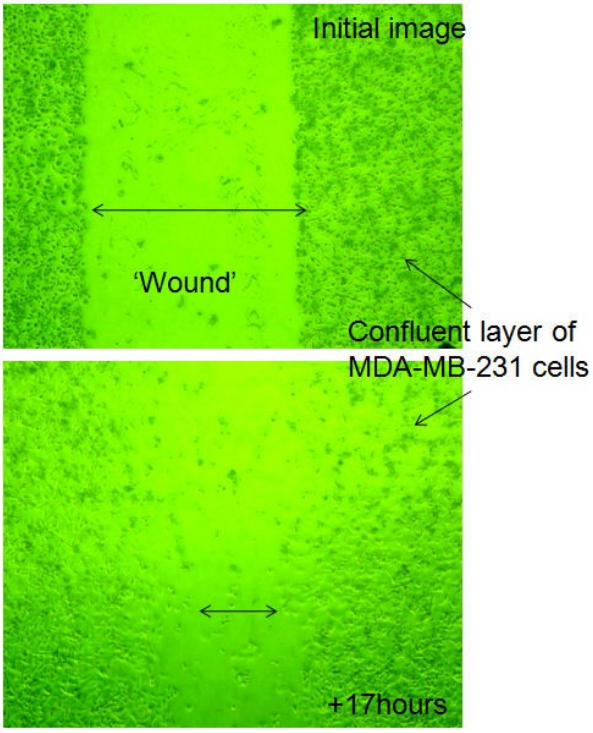


Figure 3.6. Wound assay images. These images were taken of a wound after the wound was initially carved (top) and after 17 hours of growth (bottom) where you can see the cells migrating into the wound and 'healing' it.

Matrigel Assay

The results from the wound assays were encouraging enough to merit further evaluation of those compounds that inhibited wound recovery. Accordingly, the BD Matrigel Assay was performed a total of three times with four replicates of each sample. For the first experiment, MDA-MB-231 cells in DME:F12 plus 10% FCS were used on positive and negative control samples. A negative control sample did not have any CXCL12 to induce migration of the MDA-MB-231 cells through the permeable membrane. The positive control had CXCL12 to induce cell migration but lacked any of the guanide compounds (Figure 3.7). Follow up repeats were unsuccessful due to low FCS in one assay, while another assay used the 3LL cell line that after follow up flow cytometry showed that it did not express CXCR4 on the cell membrane as previous literature searches had suggested (Figure 3.1).

Further matrigel assays evaluating the migration inhibitory effects of our compounds were not completed prior to running out of funding to purchase enough CXCL12 ($250/10 µg) and BD Matrigel plates ($285/plate) to continue these studies. If funding for these studies came available, these studies could be continued using the MDA-MB-231 cell line with 10% FCS and our compounds. Nevertheless, these experiments show that MDA-MB-231 cells show enhanced invasion in the presence of CXCL12.

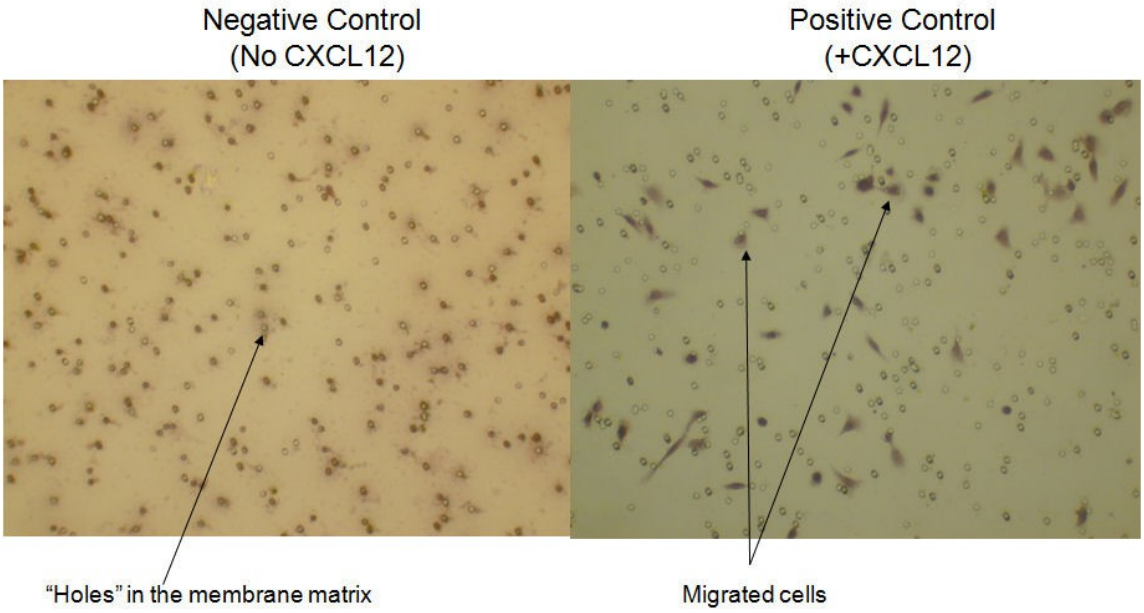


Figure 3.7. Stained Matrigel Membrane. Left: Image from the negative control samples showing the 8 micron "holes" that are in the membrane allowing the cells to migrate through; no cells were observed in this image. Right: Image from the positive control samples showing MDA-MB-231 cells that migrated through the membrane to the other side in response to CXCL12.

In vivo Mouse Lung Metastasis Model

The goal for the *in vivo* study was to examine if blocking the CXCR4 receptor with the guanide compounds would prevent the cells from extravasating and proliferating at sites of high expression of CXCL12, such as the lungs.

While the experiment with the compounds was a priority, the first *in vivo* mouse experiment was completed using alexidine. Alexidine is a biguanide compound that has already been shown to have anti-cancer properties, and thus seemed a logical choice to act as a positive control [26]. The MDA-MB-231 cells that were injected were from primary cancer cultures passaged a maximum of 6 times in tissue culture flasks prior to i.v. injection into mice. The serum from

these mice was submitted for *in vivo* toxicity studies to evaluate for heart (creatinine kinase, CK), kidney (blood urea nitrogen, BUN, serum creatinine CREAT, bilirubin, Bili), and liver toxicity (alkaline phosphatase, ALP, aspartate amino transferase, AST, alanine amino transferase, ALT) (data not shown). None of the tests showed any indication of toxicity *in vivo*. After the mice were euthanized, the lungs were recovered for analysis and the body cavity was checked for the presence of extrapulmonary tumor colonies. No extra-pulmonary tumor colonies were found in any of these experiments. The lungs were stained in Bouin's Fixative in order to clearly differentiate the cell metastases from normal lung tissue [18]. After fixation, the cell metastases were white compared to the yellow lung tissue. Initial results with alexidine using our experimental protocol demonstrated that the experimental procedure yielded similar results to those already reported in the literature, mainly a reduction of tumor metastasis after alexidine treatment (Table 3.6, Figure 3.8) [26]. The final P-value, using the Mann-Whitney U Non-Parametric Two Tailed Test, was 0.076, which confirmed that our mouse model was effective for evaluating the effectiveness of the guanide compounds *in vivo*.

The next *in vivo* experiment was done using 4T1 cells in Balb/c mice where we examined the anti-metastatic effects of spermine bisguanide. The switch in both the cell line and the mouse host was due to a shortage of available SCID mice at the time of this experiment. The results of this experiment failed to show any reduction in the number of lung colonies ($P>0.5$) from treatment with

the spermine bisguanide compound. Further evaluation of the 4T1 cell line showed that there was only a small sub-population of CXCR4 positive cells, and that these could not be selected for through multiple selections for non-adherent cells (Figure 3.2) [9]. Further evaluation of spermine bisguanide was not performed due to the synthesis of four new better compounds (spermidine bis and trisphenylguanide, DNT2300 phenylguanide, and spermine tetraphenylguanide as discussed above). This mouse model and cell line was not used in further *in vivo* studies due to the reasons discussed. Identification of a mouse-origin cell line expressing CXCR4 is required before this model can be further used in these studies.

The top candidate from all of the synthesized guanide derivative compounds as shown in our preliminary data was spermidine trisphenylguanide. It bound with the highest affinity to CXCR4 at 0.2 μM as an inhibitor, was cytotoxic at 500 μM, retarded wound healing at 100 μM, and was available in enough quantity to perform *in vivo* studies. The next step for spermidine trisphenylguanide was to inject it *in vivo* to determine if the migration prevention seen in the wound assay was repeatable in a complex tissue environment. MDA-MB-231 cells were injected into the tail veins of *SCID* mice and the lungs were examined thirty days later for MDA-MB-231 cells that had migrated out of the blood stream (extravasation) and into the lungs. The cells would also have to have the ability to proliferate in this environment in order to be evaluated as tumor lung colonies. Spermidine trisphenylguanide (100 μL of 50 μM or 200 μM)

was injected immediately after the cells were injected with a lag time arising only from switching syringes and finding the vein a second time. The mice were also pretreated with the corresponding amount of compound 24 hours prior to the cell injection and re-treated five days after tumor cell injection. The results showed that spermidine trisphenylguanide did reduce the number of lung metastases (50 μM P-value = 0.34 and 200 μM P-value = 0.3). P values below 0.5 and above 0.05 are considered to be only moderately significant for reduction of lung metastasis.

Following the positive results of the previous experiment, we hypothesized that a higher concentration of spermidine trisphenylguanide would result in a further decrease in the number of lung metastases using the same model. Thus, the concentration was increased to 100 μL of 300 μM spermidine trisphenylguanide. This required additional in vivo toxicity studies at this dose, and these were negative (data not shown). The results from the lung colony assay showed much better reductions in the number of lung colonies (P-value = 0.02). The increase in spermidine trisphenylguanide resulted in fewer lung colonies than the 50 μM or 200 μM. These results demonstrate that spermidine trisphenylguanide administration results in statistical reductions of breast cancer cells capable of metastasizing to the lungs (Figure 3.8 and Table 3.6). Table 3.6 is a chart demonstrating the results from each of these experiments.

Cell Line	Compound	Mouse Strain	U Value	Replicates (Control x Compound)	P Value	Compound Reduce Metastases?
4T1	Spermine Bisguanide (20 μM)	Balb/c	54	9 x 12	>0.5	No
MDAMB231	Alexidine Dihydrochloride (17 μM)	SCID	15	8 x 7	0.076	Yes (Good Confidence Level)
MDAMB231	Spermidine 3ØG (50 μM)	SCID	34	8 x 10	0.34	Yes (Medium Confidence Level)
MDAMB231	Spermidine 3ØG (200 μM)	SCID	32	8 x 9	0.3	Yes (Medium Confidence Level)
MDAMB231	Spermidine 3ØG (300 μM)	SCID	12	7 x 9	0.02	Yes (Good Confidence Level)

Table 3.6. Results from the four *in vivo* experiments. The U-value and P-value calculations are outlined in the next section.

Data Analysis

Statistical data analysis of all the mouse lung colonies were done using the Mann-Whitney U Non-parametric two-tailed test. This rank order statistical analysis is almost exclusively used for these lung colony experiments. The test compares the U-value in each group in order to determine whether the hypothesis given is true or false. The hypothesis for the experiments described herein was simply that following treatment of infected mice with the guanide derivative compounds it is expected that a reduction in the number of lung colonies will be observed by interfering with the CXCR4 pathway. A U value is calculated from each data set and then compared against the number of replicates on the Mann-Whitney Statistical Charts to obtain a P-value. If the P-

value is ≤0.05, then the hypothesis is assumed to be correct, and if the P-value is equal to or greater than 0.5, the results are random and the hypothesis fails. The likelihood of the hypothesis being correct increases with decreasing P-values below P = 0.5. An example calculation is provided below utilizing numbers obtained from the alexidine experiment.

To find the U-value for each data set, the numbers are placed in order of ascending value. In this case, the lung weights (in grams) from the alexidine experiment are demonstrated. Control values are in black and the alexidine treated samples are given in blue text.

Initially, all values per group are placed in ascending order.

Control lung weights: 0.38g 0.49g 0.55g 0.63g 0.70g 0.71g 0.84g 1.01g

Alexidine lung weights: 0.27g 0.43g 0.44g 0.56g 0.57g 0.61g 0.65g

Then all values are placed in increasing order while keeping track of the group it came from.

0.27 0.38 0.43 0.44 0.49 0.55 0.56 0.57 0.61 0.63 0.65 0.70 0.71 0.84 1.01

Next, the values from one of the groups (alexidine for this example) are numbered by the count of the number of weights from the control group ranked below each weight in the alexidine treated sample. For example, there are three control (black) values below the alexidine (blue) value of 0.56. This gives a value of three. There are four control (black) values below the 0.65 alexidine (blue) value. This gives a value of four. The next step is to add up all the numbers as

demonstrated for this example below. The summation of these values yields the U-value.

$$0 + 1 + 1 + 3 + 3 + 3 + 4 = 15 = U$$

The U value is then compared to the Mann-Whitney U 2-Tailed Non-Parametric charts with the number of replicates in each data set to ascertain the P-value of the data set. There are 8 x 7 replicates which give a U value of 15 and a corresponding P-value of 0.076 (Table 3.6).

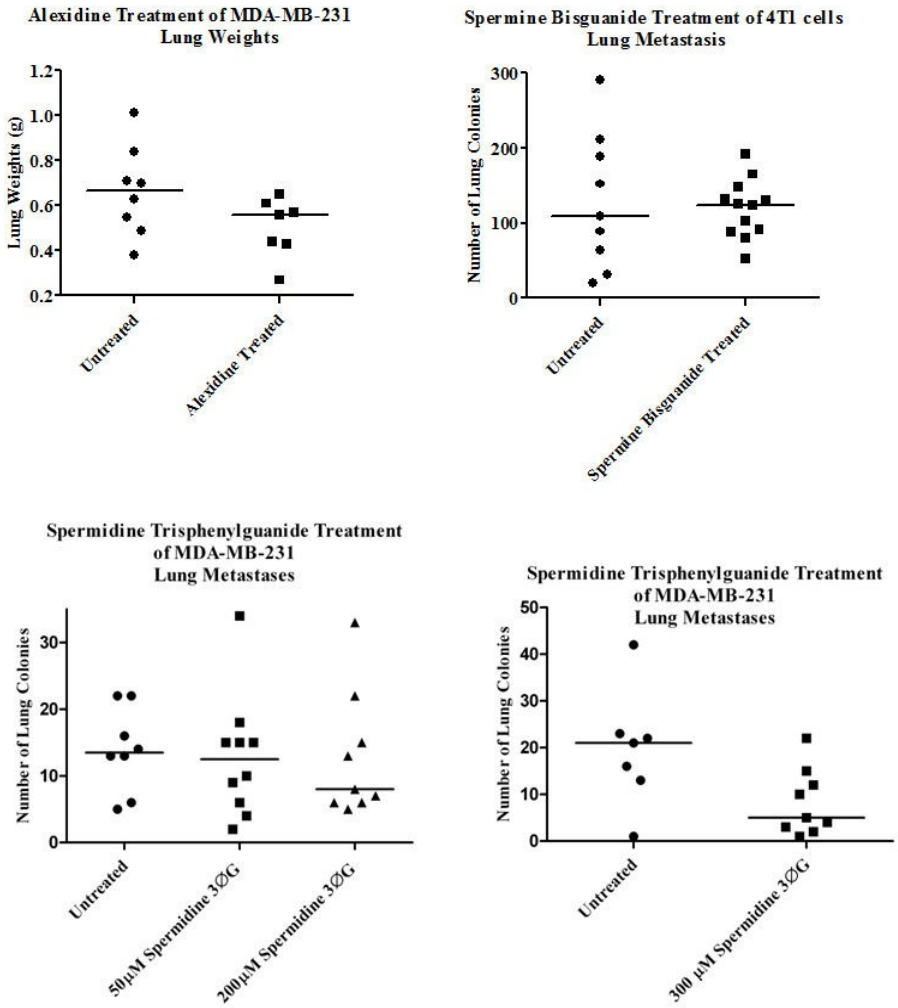


Figure 3.8. Mouse results for *in vivo* assays. Individual dots are individual mice. The lines represent the median value for the group.

For our samples, there were two compounds that supported our hypothesis. The alexidine treatment on MDA-MB-231 cells and spermidine trisphenylguanide on MDA-MB-231 cells decreased the number of lung metastases in the mice. We had one experiment that had a null hypothesis (experiment did not show reduction of lung metastases) with a P-value greater than 0.5 and that was the treatment of 4T1 cells with spermine bisguanide.

Naphthylguanide Compounds

Since the phenylguanide compounds were the group of compounds that bound to CXCR4 the best, it was hypothesized that substituting naphthylguanide groups in place of the phenylguanide groups on similar backbone chains might lead to better binding to the CXCR4 receptor (Figure 3.9). Accordingly, two spermidine naphthylguanide derivatives and seven spermine naphthylguanide derivatives were synthesized to test this hypothesis. These compounds were also tested for binding to CXCR4 in the same manner as discussed in Chapter two and demonstrated binding affinities one to five times higher than the previous best compound spermidine trisphenylguanide (Table 3.7). The cellular toxicity of these compounds was also tested and determined to range between 2 μM and 200 μM for CC_{50} values.

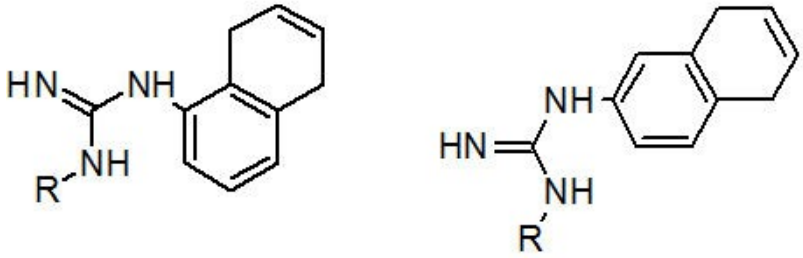


Figure 3.9. The functional groups of the naphthylguanide compounds. On the left is 1-naphthylguanide and on the right is 2-naphthylguanide.

Naphthylguanide Compounds	IC50 (nM)	CC50 (μM)	SI
Spermidine tris-phenylG	280 ± 160	500	1785.7
Spermidine bis-1-naphthylG	220 ±40	118	536.4
Spermidine bis-2-naphthylG	62 ± 23	76.3	1230.6
Spermine bis-1-naphthylG	180 ± 30	297	1650.0
Spermine bis-2-naphthylG	110 ± 20	16.8	152.7
Spermine tris-1-naphthylG	40 ± 8	18	450.0
Spermine tris-2-naphthylG	93 ± 21	15	161.3
Spermine tetra-1-naphthylG	380 ± 40	7.3	19.2
Spermine tetra-2-naphthylG	300 ± 30	20.1	67.0

Table 3.7. Results from Naphthylguanide Compounds. Spermidine trisphenylguanide is given in the first line for comparative purposes.

Since these compounds had much better binding values, we went straight to *in vivo* studies and injected spermidine bis-2-naphthylguanide and spermine tris-2-naphthylguanide into *SCID* mice using the same model as was utilized in the spermidine trisphenylguanide experiments. Initial *in vivo* toxicity studies were done on Balb/c mice prior to lung colony assays with MDA-MB-231 tumor injections in *SCID* mice. Three mice were injected i.v. with 100 μl of 100 μM of each of these compounds on days one, two, and five. Heart punctures performed

under anesthetic conditions were performed to recover serum which was then submitted to the Montana Veterinary Diagnostic Laboratory for evaluation of cardiac, liver, and kidney toxicity. Neither of the compounds showed any heart, liver, or kidney toxicity. Following these findings, the two compounds were then injected into SCID mice with MDA-MB-231 cells to observe lung colony formation. The number of lung colonies was greatly reduced for both the spermidine bis-2-naphthylguanide (P-value = 0.1) and the spermine tris-2-naphthylguanide (P-value = 0.04) (Table 3.8 and Figure 3.10). Both of these P-values indicate that these compounds are effective in reducing MDA-MB-231 lung metastases in our mouse model. They do not show as low of a P-value as 300 μM spermidine trisphenylguanide (P-value = 0.02), but were better than 50 μM and 200 μM spermidine trisphenylguanide (P-values = 0.34 and 0.3 respectively).

Cell Line	Compound	Mouse Strain	U Value	Replicates (Control x Compound)	P Value	Compound Reduce Metastases?
MDAMB231	Spermidine Bis-2-Naphthylguanide (100 μM)	SCID	19	7 x 9	0.1	Yes (Medium Confidence Level)
MDAMB231	Spermine Tris-2-Naphthylguanide (100 μM)	SCID	15	7 x 9	0.04	Yes (Good Confidence Level)

Table 3.8. Mouse results from treatment with the naphthylguanide compounds.

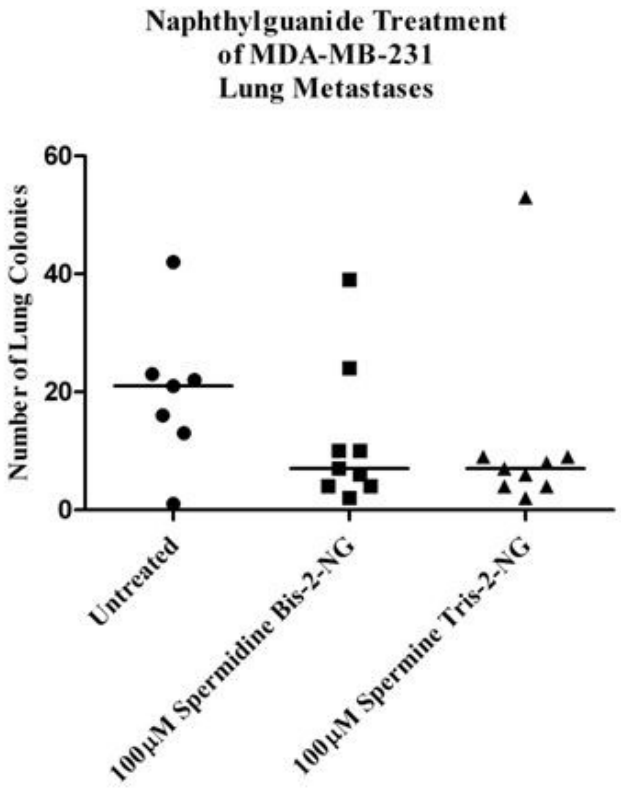


Figure 3.10. Mouse results from *in vivo* assays. Individual dots are individual mice. The line is the median value for the group.

Discussion

The research described herein discusses the evaluation of guanide derivative compounds for their effectiveness in reducing lung colony formation by a breast cancer cell line that overexpresses CXCR4. Successful candidate compounds could ultimately go onto clinical use for breast cancer patients whose

neoplasm overexpresses CXCR4. Neoplasms that do not overexpress CXCR4 would not be affected by these compounds. While there are many types of neoplasms that overexpress the CXCR4 receptor, our project focused on breast cancer metastasis to the lungs.

The most promising compound in our study was spermidine trisphenylguanide. It bound to CXCR4 with an IC_{50} value of 0.2 μM in the initial binding study against T140. This was the highest affinity compound of all compounds that were synthesized for this study until synthesis of the naphthylguanide derivatives. The CC_{50} for spermidine trisphenylguanide was 510 μM when tested on the MDA-MB-231 cell line and 480 μM on the HaCat keratinocyte cell line. These give a selectivity index (SI) value (CC_{50}/IC_{50}) of 2550 and 2400 respectively. High TI values are indicative of a smaller concentration of compound requirement for activity with associated toxicity-caused side effects being less likely due to less toxicity at smaller doses. Values of 2550 and 2400 make spermidine trisphenylguanide an excellent drug lead for developing agents to inhibit breast cancer metastasis.

Preliminary calcium flux data showed that spermidine trisphenylguanide exhibited a dose response of inhibition of intracellular calcium release in response to exposure to CXCL12. It did not show any agonistic activity in the calcium flux assay. The calcium assays were performed with the CCRf-CEM cell line, which naturally expresses CXCR4 on the cell surface. This assay

demonstrated the hypothesized antagonistic activity of the tested guanide derivatives.

Spermidine trisphenylguanide was able to inhibit wound recovery when the compound was added to a confluent growth of MDA-MB-231 cells that had a zone cleared in the monolayer. Such “wound healing” requires cell migration into this zone. Multiple concentrations were tested and showed anti-migration activity for spermidine trisphenylguanide at both 30 and 100 μM concentrations, but not at 10 μM levels. Two other compounds showed promise in the wound assay but were not further evaluated (due to limitations of available material). Many of our compounds caused cell loss in this assay and so could not be evaluated. The ultimate goal of this project was to create a compound that prevents CXCR4-induced metastasis with limited (or no) associated toxicity. Nevertheless, this *in vitro* assay provided a quick way to assess which compounds were potentially the most active.

Ideally, the wound assay results would be followed up by a more robust and specific assay, such as a matrigel assay, where the cells migrate towards a high concentration of CXCL12 through a matrix impregnated filter with 8 micron pores in it. However, due to a shortage of funds to purchase CXCL12, as well as the matrigel inserts, these assays were attempted only three times. One of the three trials was performed with control samples and provided the expected results with the negative control showing no cell chemotaxis and the positive control showing cell chemotaxis (Figure 3.7). The two follow-up matrigel assays

tested our compounds, but the first follow-up matrigel was perfomed without serum in the media, resulting in cell death. The second follow-up matrigel was done using a cell line that did not express CXCR4 on the cell line which was determined after the matrigel failed. Further compound evaluation is merited, but either a new financial source is necessary to buy the CXCL12 or the Teintze lab must synthesize their own CXCL12 (Appendix B) before this experiment can be optimized. Regardless, I am fully confident that using 10% fetal calf serum and a cell line expressing high levels of CXCR4 like the MDA-MB-231 cell line will result in repeatable and reliable results that would confirm the wound assay experimental results.

Despite the inability to run the matrigel assay for confirmation of migration inhibition, we preceded into an *in vivo* lung colony assay in female *SCID* mice using the human cell line MDA-MB-231. The initial *in vivo* experiment was done to verify a proof of principle that the experimental setup would generate results confirming those already in the literature. In the paper by Yip et al., alexidine dihydrochloride was demonstrated to be selectively toxic to cancer cell lines over normal cells [26]. Since the *in vivo* model used in the Yip publication was different than the one described herein (given that our model did not include pretreatment of the cells prior to injection into mice because this led to cell death), it was determined to be worthwhile to retest alexidine dihydrochloride using our model. The results clearly substantiated those in the literature and

provided us with a benchmark P-value of 0.076 for the reduction in the number of lung colonies (Table 3.6).

The next *in vivo* test that was performed switched the model to an all mouse model of the 4T1 mammary carcinoma in immunocompetent Balb/c mice. We chose to use the 4T1 cell line due to information from the literature that it expresses CXCR4 [21]. The compound tested in this system was spermine bisguanide. The results of this assay were negative ($P>0.5$) likely due to the fact that the 4T1 cell line we used did not express CXCR4 on the majority of cells, as was determined through flow cytometry measurements. Due to the very small population of cells that would be affected, any statistical significance of this experiment would have been lost in the noise of the experiment itself. We did attempt to select for the CXCR4 positive population of cells in this cell line using flow cytometry and a non-adherent phenotype, but were unable to obtain a population of 4T1 cells expressing a high level of CXCR4 on the cell surface. These results, along with our control matrigel assay, support the importance of CXCR4 to metastasis inhibition by our compounds. This model was not pursued further and all later lung colony experiments were done using *SCID* mice and MDA-MB-231 cells.

Spermidine trisphenylguanide, being the best compound tested (Table 3.6), was probed for activity in *SCID* mice using the MDA-MB-231 cell line. Two doses of spermidine trisphenylguanide were initially utilized in this experiment (50 μM and 200 μM) and while both were discovered to limit the number of lung

colonies metastasizing to the lungs versus control mice, the higher concentration experiments had a better effect on stopping lung colonization as demonstrated by the P value of 0.3 (200 μM) versus 0.34 (50 μM) (Table 3.6). Given these suggestive results, it was determined to further evaluate spermidine trisphenylguanide at a higher dose of 300 μM. This increase in concentration resulted in an excellent P-value of 0.02 demonstrating a dose respose curve.

Two other recently synthesized compounds were additionally tested. These two compounds, spermidine bis-2-napthylguanide and spermine tris-2-napthylguanide had better binding values to CXCR4 by a factor of 1-5 compared to spermidine trisphenylguanide. Unfortunately, the naphthyl derivatives were more toxic than spermidine trisphenylguanide, running CC_{50} values at 76 μM and 15 μM, respectively, meaning that they are more toxic than spermidine trisphenylguanide (500 μM). Furthermore, while the SI value for spermidine trisphenylguanide was determined to be 1785, the SI for spermidine bis-2-naphthylguanide and spermine tris-2-naphthylguanide were found to be 1230 and 161 respectively. The higher SI value for spermidine trisphenylguanide implies that it is a better compound to work with *in vivo* even though the naphthylguanide compounds bind with higher affinity. While wound assays were not done with the naphthylguanide compounds due to their recent synthesis and evaluation, they were tested in mice after initial *in vivo* toxicity assays. Those initial *in vivo* toxicity tests did not show any elevated serum enzymes in the mice when they were injected at 100 μL of 100 μM levels for both compounds. Lung

colony assays showed moderate and good reductions of lung metastases (P-values of 0.1 and 0.04, respectively). These promising results are not as good as the P-value of 0.02 for the 300 μM spermidine trisphenylguanide, but the compounds are still good candidates for further evaluation at higher concentrations in future work as long as no *in vivo* toxicity is observed at these concentrations.

In conclusion, spermidine trisphenylguanide, spermidine bis-2-naphthylguanide, and spermine tris-2-naphthylguanide all showed activity in reducing CXCR4-mediated lung metastases in a breast cancer (MDA-MB-231) model without demonstrating any correlating toxicity. Further characterization of these compounds is needed but they show excellent promise for development as inhibitors of CXCR4-mediated chemotaxis in CXCR4 positive cancers.

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CHAPTER FOUR

BROAD-SPECTRUM ANTIBACTERIAL ACTIVITY IN NOVEL COMPOUNDS CONTAINING MULTIPLE PHENYLGUANIDE OR BIGUANIDE GROUPS

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

Author: Joyce B. Shepard

Contributions: Tested compounds for antibacterial activity against various pathogens, determined cellular toxicity on two cell lines, evaluated *in vivo* toxicity, performed *in vivo* activity assays, contributed to the manuscript.

Author: Royce A. Wilkinson

Contributions: Synthesized and purified the guanide, biguanide, and phenylguanide derivative compounds, tested compounds for antibacterial activity against various pathogens, contributed to the manuscript.

Co-Author: Catherine Cooper

Contributions: Performed MRSA antibacterial timecourse assay, determined 50% inhibitory concentration of THAM trisphenylguanide on MRSA, assisted with *in vivo* activity assays.

Co-Author: Sarah K. Walton

Contributions: Tested compounds for antibacterial activity against various pathogens.

Co-Author: Amanda R. Radke

Contributions: Tested compounds for antibacterial activity against various pathogens.

Co-Author: Robert L. Watkins

Contributions: Instructed on and assisted with *in vivo* activity assays.

Co-Author: Thomas J. Wright

Contributions: Tested compounds for antibacterial activity against various pathogens.

Co-Author: Elizabeth Erikson

Contributions: Determined cellular toxicity of THAM trisphenylguanide, tested compounds for antibacterial activity against MRSA.

Co-Author: Mohamed Labib

Contributions: Suggested the antibacterial studies; provided NB325 and discussed the results and implications.

Co-Author: Jovanka M. Voyich

Contributions: Designed the MRSA study assays, discussed the results and implications, and contributed to the manuscript.

Co-Author: Martin Teintze

Contributions: Discussed the results and implications and wrote the manuscript.

Manuscript Information Page

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Broad-spectrum antibacterial activity in novel compounds containing multiple phenylguanide or biguanide groups

Joyce B. Shepard[1†], Royce A. Wilkinson[1†], Catherine Cooper[2], Sarah K. Walton[1], Amanda R. Radke[1], Robert L. Watkins[2], Thomas J. Wright[1], Elizabeth Erikson[2], Mohamed E. Labib[3], Jovanka M. Voyich[2] and Martin Teintze[1*]

Department of Chemistry & Biochemistry[1] and Department of Immunology & Infectious Diseases[2], Montana State University, Bozeman, Montana 59717; Advanced BioDevices, Princeton, New Jersey 08540[3]

[†]These two authors contributed equally to this work.

* Corresponding Author. Mailing address: 103 Chemistry & Biochemistry Building, Montana State University, Bozeman, MT 59717. Phone: (406) 994-5390. Fax: (406) 994-5407. E-mail: mteintze@montana.edu.

ABSTRACT

We have synthesized three series of compounds, derived from 12 linear and branched polyamines with 2-8 amino groups, which were substituted to produce the corresponding guanides, biguanides, or phenylguanides. Compounds with multiple biguanide groups, such as chlorhexidine, alexidine, and polyhexamethlyenebiguanide, have broad spectrum antibacterial activity and are widely used as topical microbicides. However, they are too toxic for parenteral use. Preliminary experiments suggested that our compounds might be less cytotoxic, so we hypothesized that some of our new compounds might have antibacterial activity with better selectivity than these other biguanides. We tested the activity of all three series of new compounds against *Acinetobacter baumannii*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Some of the compounds were effective against all of these pathogens *in vitro,* with IC_{50} values in the sub-micromolar range. Other compounds were selectively effective against pathogens *in vitro*, suggesting different mechanisms of action. Using an intraperitoneal mouse model of invasive methicillin-resistant *Staphylococcus aureus* disease, THAM trisphenylguanide, a compound with broad spectrum activity, reduced bacterial burden locally and in deeper tissues. In summary, this study reports the generation of novel guanide compounds with microbicidal activity against a broad range of drug-resistant pathogens, with potential for development as new broad spectrum antibacterials.

INTRODUCTION

Ever since antibiotics were introduced into clinical practice, bacterial pathogens have been developing resistance which reduces or eliminates their effectiveness. In addition, opportunistic pathogens with innate resistance to antibiotics have become emerging problems, particularly in hospital settings. In developed countries, such as the United States, bacterial species that have acquired multiple drug resistance include the ESKAPE pathogens, aptly named for their ability to escape the effects of our current antimicrobial drugs. These include ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa* and ***E****nterobacter* species [4]. As an example, methicillin-resistant *Staphylococcus aureus* (MRSA) was once thought to be problematic only in healthcare settings, but now community-acquired infections are becoming more common. An estimated 12 million outpatient visits occur annually in the US because of *S. aureus* skin and soft-tissue infections [20], and invasive MRSA disease was responsible for nearly 19,000 deaths during 2005 [17]. MRSA are resistant to the entire class of beta-lactam antibiotics including penicillins, cephalosporins, and monobactams. MRSA strains are also becoming resistant to additional classes of antibiotics, including macrolides (such as erythromycin and clindamycin) and quinolones (such as levofloxacin) [18], as well as the glycopeptide vancomycin, the common drug of last resort [21]. Linezolid, quinupristin/dalfopristin (synercid), daptomycin, and tigecycline have been used to treat infections that do not respond to

vancomycin, but the development of resistance to these drugs is also likely. Given how quickly drug-resistance spreads in *S. aureus*, it is apparent we are rapidly exhausting current treatment options.

The same can also be said for the other pathogens used in the current study. *P. aeruginosa* has intrinsically low antibiotic susceptibility [12, 19], attributable to multidrug efflux pumps [24] and low permeability of the cellular envelopes. Chronic pulmonary infections of *P. aeruginosa* are the leading cause of morbidity and mortality in patients with cystic fibrosis. *E. faecalis* is not armed with virulence factors, but is a persistent pathogen in healthcare settings due to its resistance to antibiotics. *E. faecalis* is resistant to aminoglycosides, cephalosporins, clindamycin, penicillins, trimethoprim-sulfamethoxazole, and strains resistant to vancomycin are becoming increasingly common [3]. *A. baumannii* has problematic isolates that are resistant to most antibiotics [11]. It causes opportunistic infections in hospitalized patients, including severe pneumonia and infections of the urinary tract, bloodstream and other parts of the body [14]. Multidrug resistant clinical isolates of *A. baumannii*, *E. faecalis*, and *S. aureus* were included in these studies.

We have synthesized three series of compounds, derived from twelve linear and branched polyamines with two to eight amino groups, which were substituted to produce the corresponding guanides, biguanides, or phenylguanides. The five polyamine precursors that yielded derivatives with antibacterial activity are shown in Figure 4.1, and the derivatization scheme in

Figure 4.2. Some of these compounds were previously found to be effective as antagonists of CXCR4 and inhibited HIV infection by X4 strains [29]. The biguanide series of compounds are chemically related to some commercially important biocidal agents found in common household items such as disinfectants, mouthwashes, contact lens solutions, and cosmetics. Examples include polyhexamethylene biguanide (PHMB), a heterogeneous polymer containing multiple positively charged biguanides, and the small-molecule bis-biguanides chlorhexidine and alexidine. Our new compounds are also polycationic, and preliminary experiments had shown low cytotoxicity *in vitro* [29]; we hypothesized that our compounds might have more selective antibacterial activity than these other biguanides and be candidates for potential therapeutical uses.

MATERIALS AND METHODS

Starting material amine compounds. Spermine tetrahydrochloride was purchased from Sigma (St. Louis, MO). Spermidine was from Acros (Morris Plains, NJ). Priostar™ dendrimers DNT-2200 and DNT-2300 were from Dendritic Nanotechnologies, Inc. (Mt. Pleasant, MI). Trishexylaminomelamine (THAM) was synthesized as described previously [29].

Guanide, biguanide and phenylguanide derivatives. These compounds were synthesized from the starting amines and purified as described previously [29].

Bacterial strains. Bacterial strains used in these experiments were *Escherichia coli* (strain K91), *Pseudomonas aeruginosa* (strain PA01), *Acinetobacter baumannii* (strains ATCC 19606 and ATCC BAA1605), *Enterococcus faecalis* (strains MSU#10 and ATCC V583 [25]), and *Staphylococcus aureus* (strains RN4220 [22] and community-associated MRSA strain LAC, pulsed-field gel-electrophoresis type USA300 [8]). Three of the strains display known drug-resistant phenotypes. Specifically, *A. baumannii* BAA1605 is a multi-drug resistant isolate [6], [26] with resistance to Ceftazidime, Gentamicin, Ticarcillin, Piperacillin, Aztreonam, Cefepime, Ciprofloxacin, Imipenem, and Meropemem. *E. faecalis* V583 is vancomycin and gentamicin resistant [25] and USA300 (strain LAC used in this study) is resistant to penicillin, oxacillin, erythromycin, and shows intermediate resistance to ciprofloxacin [8-9, 17].

Bacterial viability assays. Bacteria were grown at 37°C to mid-log phase (OD_{600} ~0.4). The bacteria were diluted to approximately 10^4 to 10^5 colony forming units (cfu) per milliliter and added to a solution of phosphate buffered saline (PBS, 16 mM phosphate, 0.14 M NaCl, pH 7.2) containing the compound at multiple concentrations. Samples were incubated at 37°C for one hour and then plated on Luria Bertani (LB) agar at appropriate dilutions in PBS. The plates were incubated overnight at 37°C and colonies enumerated the following day. IC_{50}s were calculated from graphs of cfu as a function of compound concentration. Chlorhexidine was used as a positive control for all assays.

Bacterial growth inhibition assays. *S. aureus* strain USA300 was cultured as described previously [28] to mid-exponential (ME) growth phase, washed in PBS, and resuspended at 1×10^6 cfu/ml. The bactericidal activity of THAM trisphenylguanide in culture was then determined by two independent methods. In the first method, 100 µl of *S. aureus* (1×10^5 cfu; ME) was suspended in RPMI plus L-glutamine (Cellgro), at various concentrations of THAM trisphenylguanide, and incubated at 37°C, 5% CO_2. After sixty minutes, *S. aureus* suspensions were diluted in dH_2O and plated on Tryptic Soy Agar (TSA). For the second method, *S. aureus* (1×10^5 cfu, ME) was suspended in Tryptic Soy Broth with various concentrations of THAM trisphenylguanide. *S. aureus* suspensions were incubated at 37°C using a SpectraMax Plus 384 Absorbance Microplate Reader (SoftMax Pro 5.4 software) for six hours with shaking. Every fifteen minutes, suspension turbidity was determined by optical density at 600 nm. After six hours, suspensions were diluted and plated on TSA. Plates were incubated overnight at 37°C, 5% CO_2, and cfu enumerated the following day.

Cell toxicity assays. Compound cytotoxicity was evaluated using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) following the manufacturer's instructions (Promega, Madison, WI) using both the human breast cancer cell line MDA-MB-231 (ATCC HTB-26) and HaCaT human keratinocytes [5]. The HaCaT cell line was grown in Epilife medium (MEPI500Ca) with human keratinocyte growth supplement (Invitrogen, San

Diego, CA). Both types of cells were harvested using Tryspin/EDTA and diluted to 1×10^5 cells per well in a 96-well plate. Ten concentrations covering five orders of magnitude were tested with six replicates per concentration. Cells were incubated for 48-72 hours at 37°C with 5% CO_2. MTS/PMS solution (Promega) was added to the wells and absorbance was measured at 490 nm two to four hours later. Data was analyzed using GraphPad Prism version 5.0 (GraphPad Software) using a variable slope inhibition dose response curve.

***In vivo* MRSA challenge experiments.** Three groups of Balb/c mice (8 mice per group) were infected by intraperitoneal (*i.p.)* injection with 5×10^7 MRSA in 100 µl sterile PBS. The first treatment group was injected *i.p.* with 100 µl of 100 µM THAM trisphenylguanide in PBS immediately after infection. The second group was injected *i.p.* with 100 µl of 100 µM THAM trisphenylguanide one hour post-infection. The control group was injected with 100 µl PBS. At eight hours post-infection, all mice were euthanized. An intraperitoneal lavage using 10 ml PBS was performed to recover bacteria within the peritoneal cavity and plated at multiple dilutions on TSA plates. The heart and kidney were removed for evaluation of systemic infection. Tissues were weighed, homogenized, and plated at multiple dilutions on TSA plates. TSA plates were incubated overnight at 37°C at 5% CO_2 and cfu enumerated the next day.

RESULTS

Antibacterial activity *in vitro*. We tested the *in vitro* activity of all 48 compounds against gram negative *E. coli* (K91) and *P. aeruginosa* (PAO1) and gram positive *S. aureus* (RN4220) by testing each compound at 30 and 100 μM in a bacterial viability assay. None of the compounds synthesized from 1,4-butanediamine, 1,6-hexanediamine, trisethylaminomelamine, trisbutylaminomelamine, and the dendrimer PAMAM (G0) were active against these bacteria (data not shown), so they were not tested against the remaining bacteria, the gram negative *A. baumannii* and the gram positive *E. faecalis*. For all the guanide, biguanide, and phenylguanide compounds that showed significant inhibition at 30 μM, as well as for the parent amines from which they had been synthesized, we then determined IC_{50} values using the same assay at several different concentrations (Table 4.1). Spermine and spermidine guanides and biguanides are not shown in the table, because they showed no activity in the preliminary screen at 100 μM and were not tested further. Chlorhexidine was used as a positive control with known bactericidal activity [7].

The most active compounds (shaded in Table 4.1) were THAM trisphenylguanide (THAM 3ΦG), DNT2300 hexabiguanide (DNT2300 6BG), DNT2300 hexaphenylguanide (DNT2300 6ΦG) and DNT2200 octaphenylguanide (DNT2200 8ΦG), showing effectiveness against almost all gram negative and positive strains at low micromolar concentrations. THAM trisguanide (THAM-3G) and DNT2300 hexaguanide (DNT2300-6G) were

effective against all the gram negative bacteria tested, while being completely ineffective against the gram positive *E. faecalis* and *S. aureus*. DNT2300 hexabiguanide (DNT2300-6BG) was the only biguanide with strong biocidal activity against both gram positive and gram negative bacteria; DNT2200-8BG was only highly effective against *P. aeruginosa,* and all the other biguanides had no activity. DNT2300 and DNT2200 have closely related structures that vary only in the number of 'arms' extending from the central core of the dendrimer. Thus, it was surprising that the biguanide derivative of DNT2300 (DNT2300-6BG) had much greater and broader antibacterial activity than its DNT2200 counterpart (DNT2200-8BG), even though the parent dendrimer amines, as well as their guanide and phenylguanide derivatives, had similar activities with three or four arms.

In most cases, the drug resistant strains of the bacteria we tested were equally susceptible to our compounds as the related non-resistant strains. There are a few notable exceptions: *A. baumannii* BAA1605 had higher IC_{50} values compared to the non-resistant strain (19606) for THAM-3ΦG (5-fold), DNT2300-6G (6.5-fold), and DNT2200 (8.3-fold); *E. faecalis* V583 had an IC_{50} value approximately 25-fold higher for THAM-3ΦG than the non-resistant strain, and *S. aureus* USA300 demonstrated higher IC_{50} values for DNT2300 (8-fold) and DNT2200 (4-fold).

We tested the ability of THAM-3ΦG to inhibit the growth of *S. aureus* USA300 in a rich medium (TSB) for six hours. Figure 4.3 shows that the active

THAM-3ΦG was able to completely inhibit growth of the bacteria at 5 μM, while the spermidine control did not. This minimum inhibitory concentration (MIC) is similar to the 3.9 μM IC_{50} value determined by counting viable bacteria after treatment in PBS (Table 4.1). We also determined the number of viable bacteria (cfu) following six hours of incubation in the rich medium (Figure 4.3B). The approximately 50% survival for the bacteria treated with 3.75 μM THAM-3ΦG indicates that about half the bacteria contributing to the OD_{600} after six hours were no longer viable, which is also in accordance with the results in Table 4.1.

Cytotoxicity *in vitro*. The toxicity of the compounds to human cells was evaluated using an MTS dye reduction assay in both an epithelial (MDA-MB-231) and a keratinocyte (HaCat) derived cell line (Table 4.2). Many of the compounds were not toxic to the human cells at the highest concentrations tested, and all of them were less toxic than chlorhexidine. THAM-3ΦG was also assessed for plasma membrane damage in human PBMCs and PMNs at 100 μM, by determining percentage of cells staining positive for propidium iodide using flow cytometry, and there was no noticeable effect (data not shown).

MRSA challenge: *In vivo* toxicity studies were carried out prior to the start of experiments in mice challenged with MRSA. Groups of three Balb/c mice each were injected *i.p.* with 0.1 ml of 0, 10, 50, and 100 μM THAM-3ΦG (one of the compounds with the widest spectrum of activity, see Table 4.1) in PBS. The highest *in vivo* dose represented 0.36 mg/kg. Blood was drawn from the mice at two hours post injection and submitted for a standard panel of serum chemistry

and enzyme levels. The chemistry and enzyme levels were not elevated for any of the concentrations tested compared to the mock-treated control group (data not shown).

Since the compound was not overtly toxic, we tested its ability to reduce bacterial burden *in vivo* using a mouse model of invasive staphylococcal disease. Mice were treated with 100 μM THAM-3ΦG either at the same time or one hour after an *i.p*. challenge with a lethal dose of MRSA. The control group received only PBS at the one hour timepoint. The mice treated with THAM-3ΦG immediately after the MRSA challenge demonstrated significantly reduced bacterial burdens in intraperitoneal lavage, kidneys, and heart. They averaged an approximately 3-log reduction in both organs and IP lavage compared to PBS treated mice (Figure 4.4). These reductions were statistically significant with $P < 0.05$. Mice treated an hour after MRSA infection demonstrated an approximately 5 to 10 fold reduction of bacterial cfu (Figure 4.4), although these values did not reach $P < 0.05$ statistical significance.

DISCUSSION

Initial screening of our library of guanide, biguanide, and phenylguanide compounds showed that the higher molecular weight compounds had potent antibacterial activity, whereas derivatives of the smaller amine starting materials (1,4-butanediamine, 1,6-hexanediamine), the short chain melamine derivatives (trisethylaminomelamine and trisbutylaminomelamine), and the small dendrimer

PAMAM G0 were inactive at the highest concentration tested (100 μM). Four derivatives in particular, THAM-3ΦG, DNT2300-6BG and -6ΦG, as well as DNT2200-8ΦG, were effective against all bacteria tested at concentrations comparable to chlorhexidine. At the same time, they were up to 50-fold less toxic to human cells than chlorhexidine (Table 4.2). Therefore, these compounds have potential utility in topical applications replacing chlorhexidine and other biguanides.

A wide variation in activity vs. species of bacteria for each of the compounds was observed. Overall, the compounds ranged from being inactive against all of the bacteria tested, active against only one species (spermine tetraphenylguanide and spermidine trisphenylguanide inhibited only *E. coli* and DNT2200 octaguanide was only active against *P. aeruginosa*), to general antibacterial activity against all of those tested (THAM-3ΦG, DNT2300-6ΦG, DNT2300-6BG and DNT2200-8ΦG). The relatively potent activity of DNT2200 octaguanide only against *P. aeruginosa* (IC_{50} = 5.5 μM) is particularly striking given that it was not active against any of the other gram-negative bacteria and that *P. aeruginosa* is intrinsically resistant to many antibiotics. Therefore, this finding warrants further investigation. We also observed that THAM-3G and DNT2300-6G were selectively active only against the three gram-negative bacteria, but not against the two gram-positive species.

Of the compounds that were notably active against a majority of the bacterial species, most of them were reasonably non-cytotoxic to human cells *in*

vitro (Table 4.2). In particular, the dendrimer phenylguanides DNT2300-6ΦG and DNT2200-8ΦG were 20 to 50-fold less cytotoxic than chlorhexidine, while exhibiting comparable antibacterial activity. Only THAM-3ΦG, DNT2300-6BG, as well as the parent amines for the dendrimers (DNT2200, DNT2300) and spermine had significant cytotoxicity (CC_{50} = 6.8-22 μM) against either of the two human cell lines. Even these compounds were less cytotoxic than the commonly used biguanide antiseptic, chlorhexidine, which had CC_{50} values of 5.1 and 3.8 μM against the human breast cancer cell line and human keratinocytes, respectively. The reduced toxicity toward eukaryotic cells is consistent with the lack of cytotoxicity of THAM-3ΦG in the preliminary screen for membrane damage against PBMCs and PMNs, and in the *in vivo* toxicity experiment in mice. However, given the preliminary nature of these experiments, it is difficult to know how the *in vitro* toxicity really compares to the *in vivo* toxicity.

When all the compounds in Table 4.1 were tested against two eukaryotic pathogens, *Candida albicans* and *Acanthamoeba castellanii,* none of them showed any substantial inhibition at 100 μM against *Candida*, or 200 μM against *Acanthamoeba*, while chlorhexidine and PHMB had IC_{50} values of approximately 10 μM and 10 μg/ml, respectively, against *Acanthamoeba* (data not shown). This was not surprising, given the reduced cytotoxicity for mammalian cells of our compounds compared to chlorhexidine. Others have previously shown that PHMB and chlorhexidine are effective against *Acanthamoeba* [15-16, 27], and

this provides further evidence that they likely act differently on eukaryotic cells than our compounds.

Overall, our compounds were almost equally effective against the drug-resistant isolates of *A. baumannii, E. faecalis,* and *S. aureus* as they were against the non-resistant strains. Among the most broadly active compounds (THAM-3ΦG, DNT2300-6BG, DNT2300-6ΦG, and DNT2200-8ΦG), the only significant resistance was exhibited by *E. faecalis* V583 against THAM-3ΦG; however, this strain was actually more susceptible to DNT2300-6BG and DNT2300-8ΦG. Notably, our most active compounds were effective against both the resistant and non-resistant strains of *S. aureus*, whereas MRSA strain USA300 had 15-fold higher resistance to chlorhexidine.

Apparently, the various mechanisms by which *A. baumannii, E. faecalis,* and *S. aureus* have acquired multi-drug resistance are not generally effective against our new compounds. This lack of resistance to our compounds in *A. baumanni* is particularly noteworthy. Both drug-susceptible (DS) and drug-resistant strains (including BAA1605) of this bacterium have previously been used in a screen of over a thousand compounds, with a high prevalence of known antibiotics that have been assigned US Pharmacopeia status [6]. In this screen, an extremely low hit rate (1.9%, 20 compounds) was found against even a DS *A. baumannii* strain, and there were only four compounds that had equal activity against both the DS and resistant strains [6]. This suggests that even the DS strain of *A. baumannii* is naturally resistant to many old and new classes of

approved antibacterials. Thus the activity of our compounds against this bacterium is of substantial interest.

The mechanism of action for the commonly used biguanide antiseptics, chlorhexidine and PHMB, is not well-defined. Although they are thought to interact with the membrane surfaces [10], resulting in leakage of cellular contents, they do not have long alkyl chains that intercalate into the lipid bilayer like those of the common antibacterial cationic quaternary amine compounds. A PHMB study [2] found induction of the *rhs* genes in *E. coli,* implicating enzymes involved in the repair/binding of nucleic acids, and another study reported cooperative binding of PHMB to nucleic acids [1]. A chlorhexidine study [23] found that the mechanism of action in *P. aeruginosa* may be multifaceted, involving changes in outer membrane permeability, the attenuation of virulence and environmental adaptation processes, and energy deprivation through the repression of genes involved in aerobic respiration. Although the fact that some of our compounds were biguanides led us to infer a similarity to PHMB and chlorhexidine, many of our biguanides had less antibacterial activity than the corresponding guanides or phenylguanides. Therefore, we hypothesize that the active compounds we are pursuing act by different mechanisms than chlorhexidine or PHMB. A compound with four guanide groups, *p*-guanidinoethylcalix[4]arene, was reported to have antibacterial activity, but not the monomeric unit *p*-guanidinoethylphenol [13]. Our data suggest that both the multimeric structure and the functional group are important, with the

phenylguanides being particularly active. Future studies with our compounds are aimed at exploring mechanisms of action.

The results from the initial *in vivo* antibacterial assay performed with THAM-3ΦG in mice infected with lethal doses of MRSA were also promising. Reductions in bacterial load were noted when the drug was given at the same time as the bacterial inoculation as well as when the drug was given one hour post infection. The compound still exhibited antibacterial properties in the complex *in vivo* environment, even though the delivery was likely sub-optimal since the pharmacokinetics are unknown. Optimum dosage levels and dosing regimens for compound use *in vivo* will require additional studies, but nonetheless the current study demonstrates promise for *in vivo* application for this compound and potentially others described in this study.

Our compounds are very different in structure and physical properties from the antibiotics to which the multidrug resistant bacteria have developed resistance, and they have similar antibacterial activity and significantly lower cytotoxicity to mammalian cells compared to the biguanide microbicides. Altogether, the results presented here suggest that some of our compounds, especially DNT2300-6ΦG and DNT2200-8ΦG, may have more desirable properties as topical antiseptics than chlorhexidine, and even have potential utility as systemic antibacterials against difficult to treat infections.

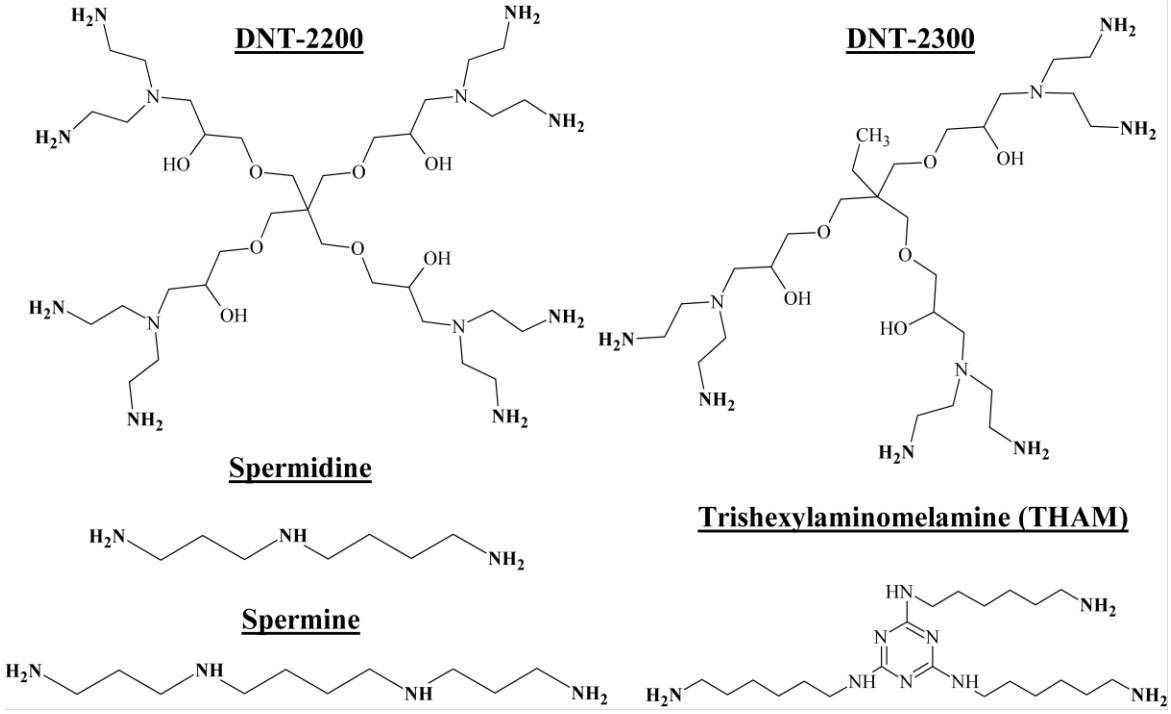


FIG. 4.1. Synthetic starting materials. The structure of starting material polyamines used to synthesize the guanide, biguanide, and phenylguanide derivatives in Table 1. Reactive amines are noted in bold.

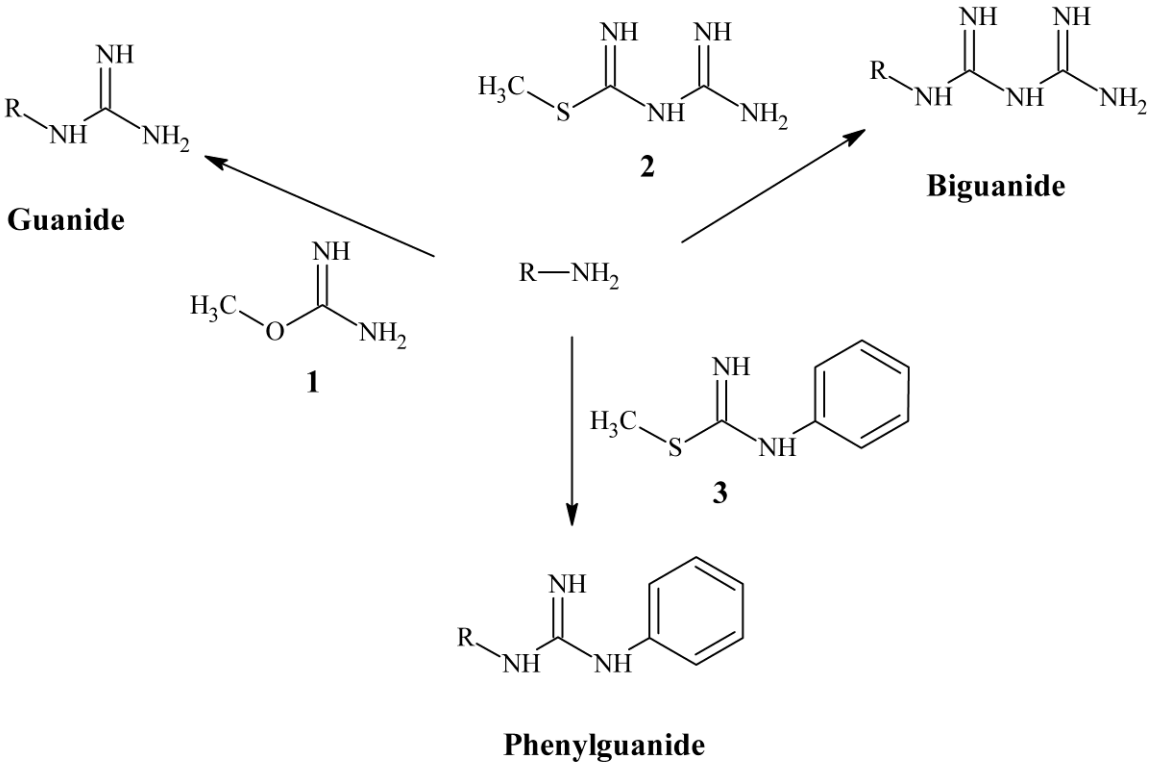


FIG. 4.2. Generic synthetic reaction scheme. Primary and secondary amines treated with the reactive compounds O-methylisourea (1), S-methyl-N-guanylisothiourea (2), or S-methyl-N-phenylisothiourea (3) give the desired guanide, biguanide, or phenylguanide derivatives. (reprinted from [12] Antimicrob Agents Chemother., 2011, 55(1): 255-263)

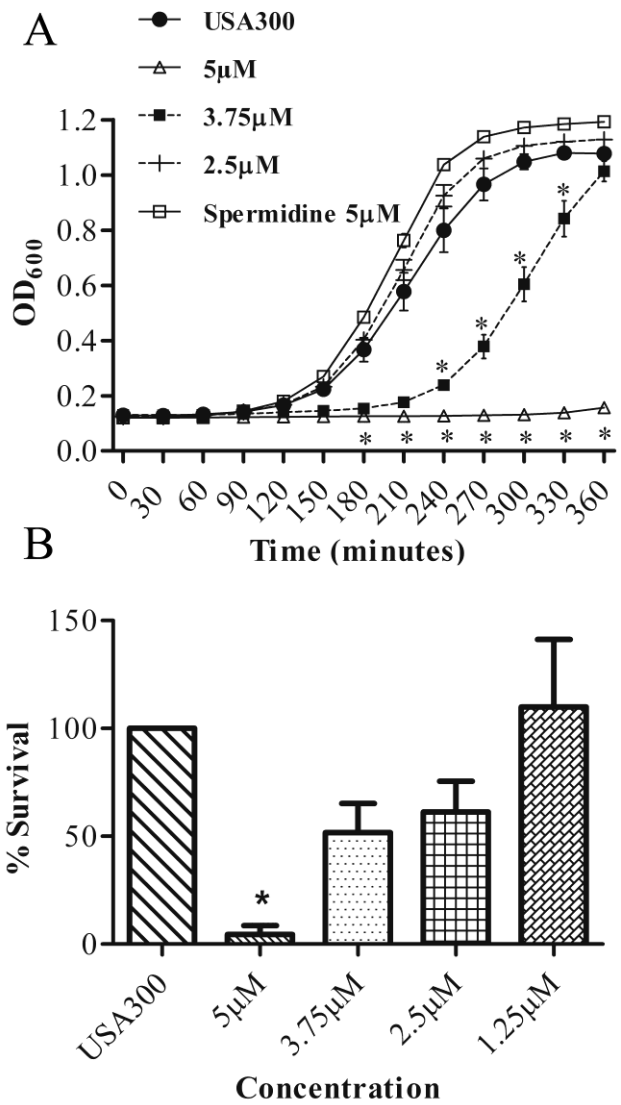


FIG. 4.3. THAM-3ΦG inhibits growth of MRSA for up-to six hours (360 minutes). To assess the ability of THAM-3ΦG to inhibit USA300 growth over time, we incubated *S. aureus* (1×10^5 cfu) in TSB and measured optical density at OD_{600} every 15 minutes for 360 minutes. **A**. OD_{600} measurements at 30 minute intervals up to 360 minutes following treatment with varied concentrations of THAM-3ΦG. * P< 0.001 versus USA300 as measured by two-way ANOVA with Bonferroni post test. **B.** Percent survival following 360 minutes of incubation in TSB with varied doses of THAM-3ΦG. Data were normalized to USA300 without treatment (100% survival). *P<0.05 versus USA300 as analyzed by one-way ANOVA. Data shown is from 3 repetitions. All data sets were analyzed using one-way analysis of variance with Tukey's post-test (GraphPad Prism version 5.0; GraphPad Software). Error bars represent the standard error of the mean.

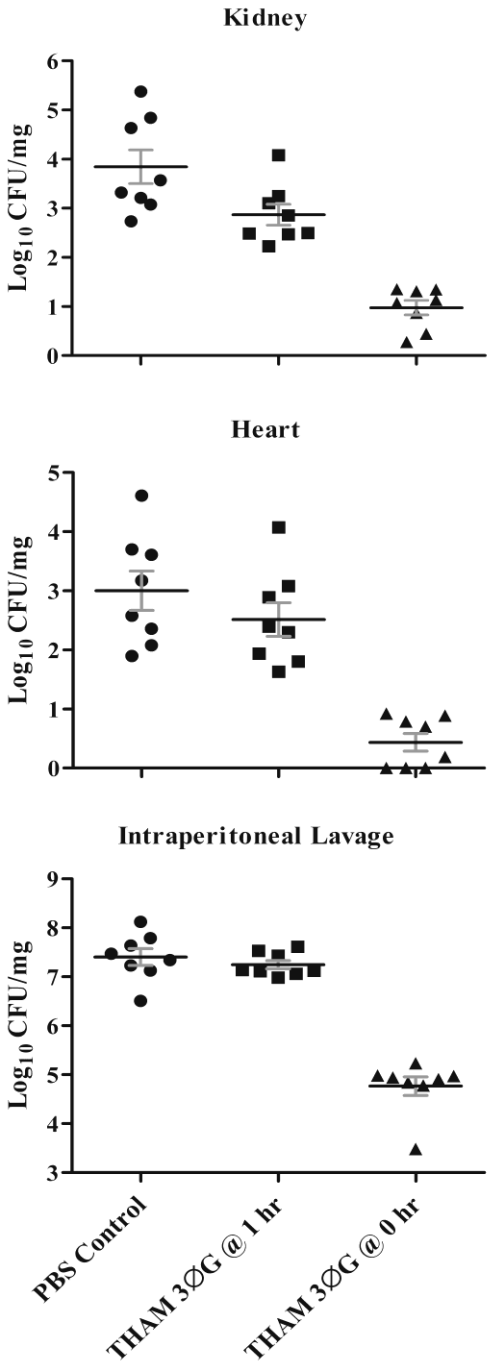


FIG. 4.4. THAM 3ΦG treatment of MRSA infected mice. MRSA infected mice were treated with PBS control or with THAM trisphenylguanide immediately after or at one hour post infection. Bacterial burden at disseminated sites was evaluated in the kidney and heart; localized infection was assessed with the intraperitoneal lavage. Each datapoint corresponds to one mouse. The horizontal bar is the mean of the eight mice per treatment group; error bars represent standard error of the mean.

TABLE 4.1. Antibacterial activity of those guanide (G), biguanide (BG), phenylguanide (ΦG), and parent amine compounds that had some antibacterial activity in an initial screen (at 30 and 100 μM) against 5 bacterial pathogens.

	IC_{50} (μM)[a,b,c]							
Bacteria/Strain / **Compound**	***E. coli*** K91	***P. aeruginosa*** PA01	***A. baumannii*** ATCC 19606	***A. baumannii*** ATCC BAA1605	***E. faecalis*** MSU#10	***E. faecalis*** V583	***S. aureus*** RN4220	***MRSA*** USA300
Chlorhexidine	1.7 ± 0.6	2.6 ± 0.5	1.3 ± 0.2	0.78 ± 0.31	14 ± 6	16 ± 6	0.86 ± 0.21	13 ± 0
Spermidine	> 100	> 100	> 100	> 100	> 100	> 100	>100	>100
Spermidine - 3ΦG	3.2 ± 0.9	> 100	> 100	> 100	> 100	> 100	>100	>100
Spermine	> 100	≥ 100	≥ 100	79 ± 24	> 100	> 100	>100	>100
Spermine - 4ΦG	23 ± 16	> 100	≥ 100	≥ 100	> 100	> 100	>500	>100
THAM	> 100	> 100	> 100	> 100	> 100	> 100	>100	>100
THAM - 3G	31 ± 12	14 ± 3	23 ± 14	14 ± 4	> 100	> 100	>100	>100
THAM - BG	> 100	> 100	> 100	> 100	> 100	> 100	>100	>100
THAM - 3ΦG[d]	3.8 ± 1.6	4.5 ± 2.5	0.86 ± 0.25	4.3 ± 1.0	2.8 ± 0.6	65 ± 1	1.7 ± 0.9	3.9 ± 0.5
DNT2300	1.0 ± 0.7	1.4 ± 0.3	1.3 ± 1.1	4.1 ± 2.2	49 ± 22	36 ±12	2.1 ± 1.0	17 ± 3
DNT2300 - 6G	44 ± 1	8.1 ± 5.0	4.9 ± 1.1	32 ± 11	> 100	> 100	>100	>100
DNT2300 - 6BG	1.1 ± 0.9	3.6 ± 1.2	0.59 ± 0.36	1.7 ± 0.9	13 ± 5	2.2 ± 0.7	0.94 ± 0.14	1.3 ± 0.5
DNT2300 - 6ΦG	1.6 ± 0.2	5.7 ± 2.3	3.4 ± 1.0	3.6 ± 0.7	18 ± 15	17 ± 7	5.2 ± 1.7	4.7 ± 2.5
DNT2200	5.9 ± 5.3	2.0 ± 1.7	5.2 ± 4.5	43 ± 17	≥ 100	≥ 100	4.3 ± 1.6	17 ± 2
DNT2200 - 8G	≥ 100	5.5 ± 0.1	≥ 100	> 100	> 100	> 100	>100	>100
DNT2200 - 8BG	21 ± 17	2.3 ± 1.5	69 ± 31	> 100	> 100	> 100	100	100
DNT2200 - 8ΦG	2.7 ± 0.7	7.0 ± 6.5	3.6 ± 1.9	5.4 ± 1.8	11 ± 2	4.0 ± 0.2	5.2 ± 2.0	3.6 ± 0.5

a. >100 means that no activity or toxicity was detected at this concentration, which was the highest tested.

b. Depending on the compound and counterion, 1μM corresponds to 0.5-2.5μg/ml.

c. The average of IC_{50} values from two separate experiments using duplicates of each compound dilution in each test. Results are in μM ± SEM.

d. The most-active, broad-spectrum antibacterial compounds are highlighted with light gray backgrounds.

	CC_{50}[a] (μM)		Relative to chlorhexidine[b]	
Compound	**MDA-231**	**HaCat**	**MDA-231**	**HaCat**
Chlorhexidine	5.1	3.8	-	-
Spermidine	150	>1000	30	>260
Spermidine - 3ΦG	510	480	100	130
Spermine	47	>1000	9.3	>260
Spermine - 4ΦG	>100	96	>20	25
THAM	>1000	260	>200	68
THAM - 3G	83	71	16	18
THAM - BG	>1000	70	>200	18
THAM - 3ΦG	6.8	12	1.3	3.2
DNT2300	47	22	9.3	5.7
DNT2300 - 6G	>50	64	>9.8	17
DNT2300 - 6BG	34	6.3	6.8	1.6
DNT2300 - 6ΦG	140	200	28	52
DNT2200	89	21	18	5.4
DNT2200 - 8G	>50	350	>9.8	91
DNT2200 - 8BG	390	370	77	96
DNT2200 - 8ΦG	>100	79	>20	21

a. CC_{50} is the concentration of compound which killed 50% of the cells in the MTS assay.
b. Factor by which cytotoxicity was reduced relative to chlorhexidine.

TABLE 4.2. Cytotoxicities of the guanide, biguanide, phenylguanide derivatives, and the parent amines, against a human breast cancer cell line (MDA-231) and a human keratinocyte cell line (HaCat).

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CONCLUSIONS AND FUTURE PROSPECTS

We developed novel guanide, biguanide, phenylguanide, and napthylguanide compounds for applications to cancer, HIV, and bacterial infections. The compounds were synthesized as discrete compounds inspired by the structure of the polydisperse mixture NB325, which had previously been demonstrated to bind to CXCR4 and block HIV infectivity [1-5]. The synthesis of these new compounds represented a significant step towards identifying an individual active guanide compounds for use in clinical applications (Chapter 1). Initial applications related to inhibiting X4 HIV infection through the CXCR4 co-receptor. The compounds were also evaluated for use in blocking CXCR4 induced metastasis in a breast cancer model. Lastly, the compounds were probed for effectiveness as broad spectrum antimicrobial agents.

Spermidine bis and trisphenylguanide, DNT2300 phenylguanide, and spermine tetraphenylguanide were all demonstrated to bind to CXCR4 with high affinities, as judged by IC_{50} values that ranged between 0.2 μM and 1.5 μM[6]. Given the ability of these compounds to bind to CXCR4 with relatively good affinity values, the compounds were further evaluated in HIV infection assays [6]. Spermidine trisphenylguanide and spermine tetraphenylguanide displayed the highest inhibition of X4 virus infection, while DNT2300 phenylguanide surprisingly did not show X4 inhibition (Chapter 2). None of these compounds had any activity inhibiting R5 virus [6].

The promising results obtained with spermidine trisphenylguanide suggest that a logical next step for this project is to test this compound for X4 infection inhibition *in vivo* using the cervicovaginal model used by collaborators to test the *in vivo* activity of NB325 [5]. *In vivo* activity would enable possible clinical studies to be started.

Future efforts in this project involve more structure-activity relationship studies involving further probing of the phenylguanide and naphthylguanide binding pockets. Initial studies will be done *in silico* using the crystal structure of CXCR4 to model potential important putative amino acid contacts [7]. Preliminary molecular modeling is already in progress and has given key insights to important amino acids within CXCR4 for phenylguanide binding. The next experiment will be to use that data to enhance the structure of these compounds for better CXCR4 binding affinity. This has also begun with synthesis of naphthylguanide derivatives from the parent spermidine and spermine backbone amines due to the importance of the hydrophobic phenyl groups near hydrophobic tyrosine residues in CXCR4. The naphthylguanide derivatives have demonstrated 1-5 times better binding to CXCR4 than spermidine trisphenylguanide. These derivatives are currently undergoing evaluation for anti-HIV activity.

Ideally, a co-crystal structure of CXCR4 bound with one of the novel phenylguanide or napthylguanide compounds tested herein would allow for optimal structure based design, in the hopes that an extremely tight binding

compound could be synthesized by taking advantage of specific contact regions within the binding pocket on CXCR4. Without a crystal structure, binding analysis with CXCR4 mutants may identify amino acids important for phenylguanide and naphthylguanide binding.

Many types of cancers, including breast cancer, overexpress the CXCR4 receptor [8-19]. The CXCR4 pathway induces cellular chemotaxis, angiogenesis, cell survival and proliferation, and these are all critical pathways for tumor survival [20-24]. Overexpression of CXCR4 may give a competitive edge to the tumor cells, allowing for a selective advantage of these cells.

The results in Chapter two and three show the identification of spermidine trisphenylguanide as one of the high affinity CXCR4 binding compounds that was analyzed. Cellular toxicity results with this compound showed that it had a CC_{50} value of 500 μM, giving a SI value of 2500. Further evaluation of spermidine trisphenylguanide indicated that it inhibited the natural calcium flux induced by the binding of CXCL12 to CXCR4 and prevented *in vitro* cell migration. Finally, and most importantly, it reduced the number of lung metastases in *SCID* mice when tested at three concentrations. The three concentrations showed concentration dependant reductions of lung metastases (50 μM P = 0.34, 200 μM P = 0.3, 300 μM P = 0.02) (Chapter 3). Experiments with spermidine trisphenylguanide at higher concentrations should be performed in future studies. The calculated CC_{50} value for spermidine trisphenylguanide of 500 μM means

that experiments using this compound in the 400 μM to 450 μM range could be performed and might potentially reduce lung metastases even further.

The high CXCR4 affinity naphthylguanides were also examined for anti-metastatic activity. Two naphthylguanide compounds (spermidine bis-2-naphthylguanide and spermine tris-2-naphthylguanide) were tested for *in vivo* reductions of lung metastases. They showed SI values of 1230 and 161, respectively, and caused substantial reductions in the number of lung metastases with P-values of 0.1 and 0.04. Given these promising results, future studies with these compounds are warranted using higher concentration doses to further decrease the number of lung metastases. However, *in vivo* toxicity studies must be performed prior to increasing the concentrations in the lung colony assay to ensure that there are no toxicity issues.

Spermine tris-1-naphthylguanide had the highest affinity for CXCR4 out of all compounds tested in this thesis (IC_{50} = 40 nM) but was not evaluated *in vivo* due to a shortage of available compound. This compound could potentially have better *in vivo* activity than the other two naphthylguanides tested (spermidine bis-2-naphthylguanide (IC_{50} = 62 nM) and spermine tris-2-naphthylguanide (IC_{50} = 93 nM)), and for this reason future experiments using the mouse model optimized in this study with spermine tris-1-naphthylguanide should certainly be performed. The *in vivo* toxicity of spermine tris-1-naphthylguanide would have to be evaluated prior to lung colony assays to ensure that there is not any toxicity

associated side effects, especially since the CC_{50} on MDA-MB-231 cells is 15 μM.

Finally, the calcium flux experiments and matrigel experiments should be completed now that CXCL12 is available in large enough quantities to perform these experiments (Appendix B). Completion of these assays would demonstrate more *in vitro* evidence for the clinical applications of these compounds.

Most published *in vivo* studies on CXCR4-targeted compounds involve pre-treating cancer cells *in vitro* prior to injecting the cells into mice and then monitoring tumor metastasis and growth. It is important to note that in our studies the cancer cells were not pre-treated with compound prior to injection into mice. This is a more realistic model for testing the ability of these compounds to bind to CXCR4 *in vivo* and prevent metastasis from occurring.

The compounds discussed in this thesis have shown the potential to prevent CXCR4 facilitated metastases. There are two main clinical applications for these compounds. One is as a preventative therapeutic given prior to surgery. Surgery is known to result in increased micrometastases released into the vasculature during the trauma, as well as increased cell survival due to increased oxygen availability. Prophylactic therapy to prevent the metastases prior to surgery may be an ideal use for these compounds.

Secondly, mobilization of bone marrow stem cells was a side effect of AMD3100, a CXCR4 antagonist [25, 26] that may help cancer patients with recurrent metastatic disease originating from the bone marrow. Mobilizing the

stem cell population to the circulation with our compounds, followed by chemotherapy, may eliminate latent cancer cells normally protected in the bone marrow, preventing recurrent disease. This application would not include long term use of these compounds, hence eliminating toxicity related side effects.

Long term therapy to prevent metastasis using CXCR4 antagonists in adults has not yet been fully explored. While neonates lacking CXCR4 expression expire *in utero*, blocking the CXCR4 receptor in adults with AMD3100 was well tolerated in healthy volunteers [27]. These observations suggest that our compounds may potentially have applications in long term anti-metastatic treatments in cancer patients.

Unfortunately, the mentor for this breast cancer metastasis project, Dr. Jean Starkey, has retired from MSU. Her expertise in breast cancer and mouse experiments greatly benefited this project and will be very difficult to replace.

Biguanide compounds have been in use as topical antiseptics for over 50 years. Chlorhexidine is one of the oldest and most heavily used biguanide antiseptics. Despite its abundant use, there is minimal microbial resistance to chlorhexidine [28, 29]. However, there is growing resistance to many other oral antibiotics used in bacterial therapy [30]. While we proposed that our biguanide compounds would be active antimicrobial agents due to their similarity to the structures of chlorhexidine and PHMB, we discovered that the phenylguanide compounds were the most active in killing most of the ESKAPE pathogens,

which include *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, and *Enterobacter* bacteria.

The results in Chapter 4 showed that THAM trisphenylguanide, DNT2300 biguanide, DNT2300 phenylguanide, and DNT2200 phenylguanide were the best compounds with the most universal antimicrobial activity. THAM trisphenylguanide was examined further and showed inhibition of MRSA growth for up to six hours, and also demonstrated activity in reducing the bacterial load *in vivo*. One of the *in vivo* models tested herein examined treatment of MRSA immediately after initial MRSA infection. This experiment showed a two to three fold reduction of bacterial burden in the IP cavity, heart, and kidney when mice were sacrificed eight hours post injection. Another *in vivo* experiment examined treatment of MRSA after the MRSA was allowed to disseminate though the mouse for one hour post infection prior to treatment with THAM trisphenylguanide. This did not result in as significant of bacterial reductions as the previous *in vivo* technique, as only 0.5 – 1 log reductions in the IP cavity, heart, and kidney were observed. While this was not statistically as significant as the initial treatment of bacterial burden, it did show that even a disseminated infection could be treated to a moderate extent.

The mechanism that these compounds utilize to cause bacterial death is unknown. There is evidence that chlorhexidine and other biguanide compounds act by binding to the bacterial membrane and disrupting the membrane integrity [28]. While our compounds are similar to chlorhexidine in structure, we have no

evidence that they operate in a similar fashion. We speculate that the mechanism of action is different than the resistance mechanisms known for current antibiotics such as penicillin (β-lactams) and aminoglycosides due to the fact that our compounds were effective against bacteria that are resistant to these groups of antibiotics. Our lab is currently working on identifying the mechanism of action for these compounds by attempting to ascertain the important proteins and metabolites that change in response to MRSA and *Pseudomonas aeruginosa* exposure. Preliminary results from 2-dimension gels stained with differential Z-dyes have shown many different spots that change in response to a non-lethal dose of THAM trisphenylguanide. However, work is ongoing to obtain protein identifications for the spots of interest. Furthermore, initial metabolism studies have been performed on MRSA treated with non-lethal doses of THAM trisphenylguanide and chlorhexidine. Initial reverse phase positive mode data analysis of metabolomic samples revealed a large peak eluting at 50 % acetonitrile (see Appendix C for experimental details). This peak was present in medium or large quantities in all chlorhexidine and THAM trisphenylguanide treated MRSA samples, while it was either absent or present at very low levels in all untreated control samples. Deconvolution of the peak yielded a molecular weight of 3400 ± 250 daltons. Additional evaluation of this peptide is ongoing work, and ultimately positive identification of this peptide may reveal a vital resistance mechanism used by MRSA to grow in the presence of both THAM trisphenylguanide and chlorhexidine that may be more generally applicable to

other phenylguanide compounds. The metabolomics research will be continued in the future in the hopes of identifying mechanism(s) of action for this class of compounds on other bacterial pathogens like *Pseudomonas aeruginosa*.

Further *in vivo* applications of the compounds revealing broad spectrum antimicrobial activity need to be done in topical applications such as in wound models, analyzing for activity against biofilm formation. This topical model may show better results for the compounds that demonstrate high toxicity on mammalian cells. However, further evaluation and optimization of the MRSA sepsis model discussed in chapter 4 is warranted given the promising preliminary data collected on THAM trisphenylguanide.

Overall, the phenylguanide and naphthylguanide compounds turned out to be the most active in all three of the fields we applied them to. High affinity to CXCR4 demonstrated the potential for these compounds to inhibit HIV infection, and reduce breast cancer metastasis. The phenylguanide compounds also had broad spectrum bacteriocidal activity against both drug susceptible and resistant pathogens. These compounds retain a large potential for clinical use in all three of these fields. Further evaluation for inhibition of HIV infection, breast cancer metastasis, and bacterial growth is merited.

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APPENDICES

APPENDIX A

IDENTIFICATION OF CXCR4 AMINO ACIDS IMPORTANT FOR T140 BINDING

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IDENTIFICATION OF CXCR4 AMINO ACIDS IMPORTANT FOR T140 AFFINITY

Joyce B. Shepard[1], Royce A. Wilkinson[1], John Mills[2], Edward Dratz[1], Martin Teintze[1]

[1]Department of Chemistry and Biochemistry, [2]Department of Microbiology, Montana State University, Bozeman, MT 59717 USA

Abstract

Human Immunodeficiency Virus (HIV) uses cellular CCR5 or CXCR4 (G-protein coupled receptors, GPCRs) as co-receptors to enter a host cell. After this was discovered, compounds that bound to CXCR4 and blocked HIV infection began to be identified in research laboratories around the world. T140 is a peptide inhibitor that binds to CXCR4 with high affinity. Without a crystal structure, the CXCR4 binding pocket could only be theorized through homology modeling. However, identifying key CXCR4 amino acids would certainly facilitate drug design by optimizing the drug structure to block HIV infection. We attempted to find the amino acids active in binding ligands within the CXCR4 sequence by crosslinking the T140 peptide to the CXCR4 protein, digesting the complex into small peptides, and identifying the crosslinked peptides through mass spectrometry (MS). An amino acid within CXCR4 where a crosslink was occurring was not identified. Subsequently, a crystal structure of CXCR4 with a T140-like compound was published which identified the T140 peptide binding region.

Introduction

HIV host cells are mostly T-lymphocytes but HIV can also invade B-lymphocytes, monocytes, and neutrophils. HIV gp120 (envelope glycoprotein) initially binds to the CD4 receptor on a host cell. This causes a conformational change in gp120 that exposes a secondary binding site that can then bind to a co-receptor. The co-receptor can either be CCR5 or CXCR4 (Figure 6.1), depending on the gp120 sequence. Once the viral gp120 is bound to both CD4 and a co-receptor, there is another conformational change that exposes the viral protein gp41. Gp41 then inserts a domain into the cellular membrane and

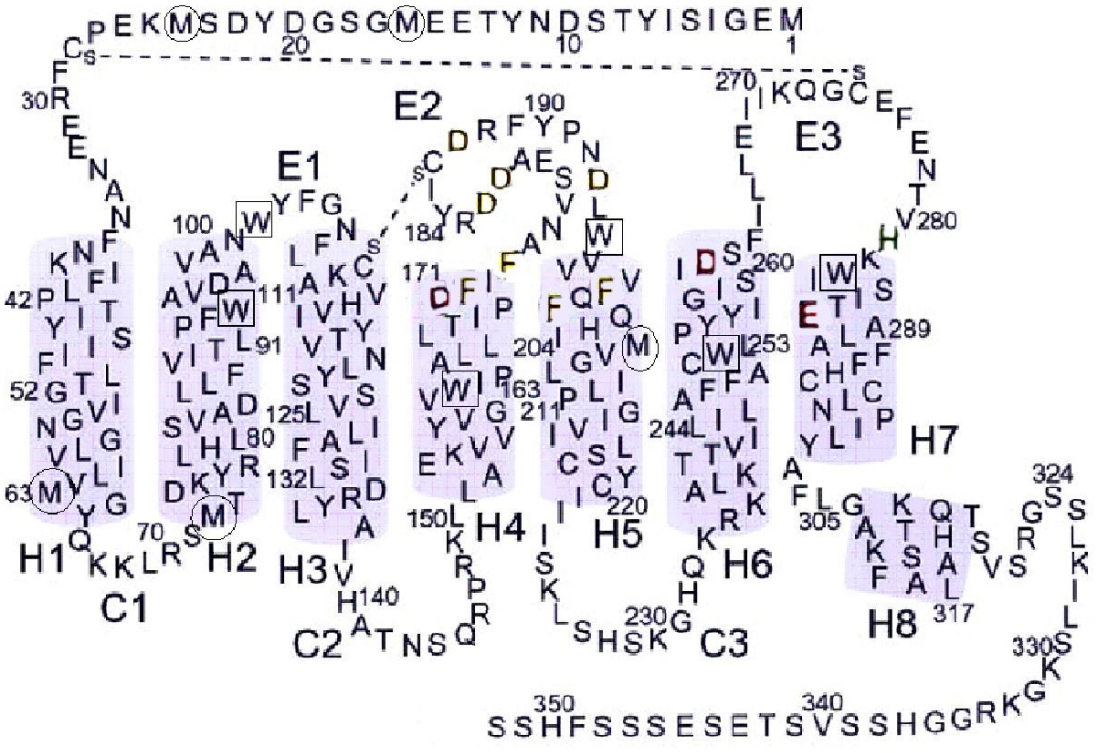


Figure 6.1. CXCR4 sequence and structure based on rhodopsin models. Model adapted from Seibert, et al.[1].

triggers membrane fusion, resulting in viral entry into the host cell.

Maraviroc (Selzentry, Celsentri, UK-427857) was developed as a CCR5 antagonist by Pfizer. It was approved by the FDA in 2007 for therapeutic use in patients infected with R5 (M-tropic) virus. Maraviroc binds to the CCR5 co-receptor and blocks viral entry into the cell [2, 3]. This is different from most other HIV treatments that work to inhibit viral replication once the virus has already infected the host. The drawback to using Maraviroc, though, is that once the CCR5 co-receptor is blocked, any X4 (T-tropic) or dual-tropic viruses latently replicating in the host are selected for and can still replicate within the host. X4 virus typically becomes the predominant viral phenotype at about the time the patient is diagnosed with AIDS. Therefore, it is necessary that a CXCR4 inhibitor also be developed and integrated into the HIV therapeutic regimen.

Many labs have attempted to develop a CXCR4 inhibitor with varying levels of success. The compound with the most success was AMD3100 developed by AnorMed, now Genzyme (Figure 6.2). AMD3100 blocks HIV infection with an EC_{50} value of 3 nM and a CC_{50} (50% cellular cytoxicity) of > 500 μM [4, 5]. This corresponds to a SI (Selectivity Index) value of >100,000. This high of a selectivity index is very promising. AMD3100 went into clinical trials where it failed for use in blocking HIV infection through CXCR4 due to poor bioavailability and cardiotoxicity [4]. However, the side effect of mobilizing stem cells from the bone marrow and into the blood stream was a desired outcome for the entirely different procedure of stem cell donation. Stem cell donation occurs

after ablation of all bone marrow with the replacement of stem cells that were extracted and stored prior to the ablation. AMD3100 (Plerixafor) was approved as a therapeutic one time immunostimulant along with G-CSF for mobilization and collection of stem cells in 2007 [6]. This replaced stem cell mobilization with G-CSF alone, which occasionally failed to mobilize the stem cells from the marrow into the blood [4].

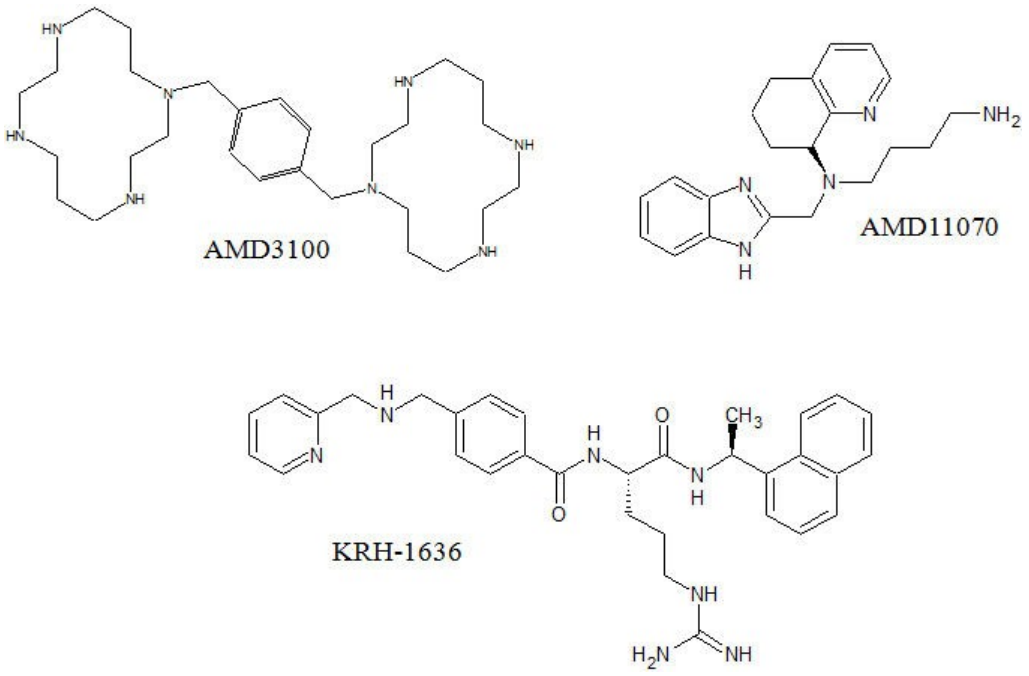


Figure 6.2. Structures of common CXCR4 binding compounds.

Other compounds that have gone through clinical trials are AMD11070 (Genzyme) (Figure 6.2) and KRH-1636 (Kureha Chemical Industry) (Figure 6.2). AMD11070 (AMD070) was the first orally available HIV inhibitor to enter the clinic

[7]. It inhibits X4 viral infection and not viral entry of R5 virus with a 50% inhibitory concentration (IC_{50}) of 10 nM and a corresponding 50% cytotoxic concentration (CC_{50}) of 30 μM giving a selectivity index (CC_{50}/IC_{50}; SI) of 3000 [7]. The SI values indicate whether a compound has both potency and selectivity. It also inhibits CXCL12 binding and subsequent downstream signaling from receptor activation. There are indications that it interacts with the extracellular loop two, and the transmembrane helicies one, two, and five [7].

KRH-1636 also inhibits X4 viral infection also displays bioavailability through duodenal absorption [8]. The IC_{50} of KRH-1636 is 19 nM with a CC_{50} of 406 μM. This correlates to a SI of greater than 20,000. This compound is also capable of inhibiting CXCL12 binding and downstream signaling cascades from receptor activation[8]. Oral bioavailability is extremely important for any HIV medication due to the chronic lifetime requirement for any HIV regiment.

T140 is a 14 amino acid peptide with the sequence: N-R R Nal C Y R K $_D$-K P Y R Cit C R-COOH (Nal = Napthylalanine, Cit = Citrulline) that was developed from a horseshoe crab 22 amino acid polyphemusin peptide [9-11]. This peptide binds to CXCR4 at 0.43 nM in an HIV infectivity assay [11]. The conformation of the peptide is an antiparallel β-sheet held together by an intramolecular disulfide bond formed between the two cysteine residues [11]. T140 has five amino acids that are critical for CXCR4 binding. They are located on the N- and C- termini of the peptide sequence. Replacing or removing Arg1, Arg2, Nal3, Tyr5, Arg14, or the disulfide bridge results in a reduction in CXCR4

affinity [12, 13]. The middle of the peptide sequence is the least important for binding activity, and this is demonstrated by the ability to replace each amino acid with other amino acids, with the resulting mutant peptides displaying no corresponding loss in activity [14, 15]. However, there are two non-essential lysines located in the center of the peptide and these act to help provide T140 with a high net +7 positive charge [11]. Importantly, of the five total arginine residues present in the sequence, three of them are vital amino acids located at or near the N- and C- termini. The other two arginines in the sequence are not vital amino acids for binding. Lastly, the other active amino acids in the sequence are the hydrophobic Nal3 and Tyr5, and these residues are also vital for binding to CXCR4 with high affinity [11].

In vitro studies on this peptide showed excellent potential for inhibition of X4 HIV infectivity [11, 16]. However, after structure function activity studies were performed that optimized the peptide for the best amino acid sequence for binding activity [12, 14, 15], it was discovered that the peptide underwent rapid degradation in serum [12]. It was revealed that in serum the C-terminal Arg14, which is essential for CXCR4 binding, is readily cleaved. Amidating the C-terminus did improve peptide stability in serum, but the modification added an extra positive net charge to the molecule, making it more toxic to cells and reducing the therapeutic index [12]. Further research on this peptide attempting to balance toxicity and activity led to many T140 derivatives, but ultimately failed to find a single peptide that had serum stability, acceptable toxicity, and excellent

equivalent binding [17-19]. For the studies carried out in the Teintze laboratory, two amino acids (Lys7 and $_{D}$-Lys8) were modified to include a fluorescent tag for easy identification and a crosslinking group to facilitate covalent bond formation between the T140 peptide and CXCR4.

Knowing the high affinity of T140 for CXCR4, our lab wanted to identify the T140 binding pocket within CXCR4 using crosslinking studies. The goal was to initially identify key amino acids in CXCR4 for binding ligands, and then use this information to design a CXCR4-specific ligand without cross reactivity to any of the other eighteen chemokine receptors in the same GPCR family or other receptors in the greater GPCR classification.

Materials and Methods

Cell Lines

The two cell lines used in this study were the canine thymocyte cell line Cf2Th and a canine thymocyte cell line overexpressing CXCR4, Cf2Th-CXCR4 (syn CXCR4) [20, 21]. The syn CXCR4 was a human codon-optimized CXCR4 gene (with C9 1D4 epitope added) cloned into the PACH vector obtained from the NIH AIDS Reagent program and stably integrated into the cellular genome.

Preparation of Sepharose Antibody Beads

Cyanogen bromide (CNBr-) activated sepharose™ 4B beads were suspended (Amersham Pharmacia Biotech AB) in ice cold 1 mM HCl. The beads were washed with 200 mL of 1 mM HCl and the supernatant discarded. Antibody

was added to the beads and incubated overnight at 4 °C. Samples were briefly spun and the supernatant removed and the beads were washed with coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl). Samples were respun, supernatant was then removed, and the beads were washed with 1 M glycine, pH 8.0. Supernatant was again spun, removed, and additional 1 M glycine, pH 8 was added. Beads were incubated with 1 M glycine, pH 8 overnight at 4 °C. Samples were spun briefly, the supernatant was removed, and the beads washed with cymal-5 wash buffer (1% Cymal-5 (Anatrace), 10% glycerol, 100 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl pH 7.5). The cymal-5 wash buffer was spun out and the beads were resuspended in a small amount of washing buffer for storage in the refrigerator until needed.

Rho 1D4 (anti-C9; 1D4) purified monoclonal antibody was purchased from the University of British Columbia. This commercially available antibody binds to the C-terminal nine amino acid sequence N-TETSQVAPA-COOH on rhodopsin. This C9 peptide was added onto the C-terminal of the CXCR4 sequence which allowed use of the 1D4 antibody for purification of the CXCR4/C9 protein referred to in this paper as CXCR4. 4-4-20 (anti-fluorescein) monoclonal antibody was purchased from the National Cell Culture Center.

Protein Purification (1D4)

The cells were grown to confluency in Dulbecco's Modified Eagle Media (DMEM) plus 10% fetal calf serum (FCS) (pH 7.2) with 100 mg/L penicillin and 100 mg/L streptomycin at 37 °C plus 5% CO_2. Cells were harvested using 0.5

mM EDTA in PBS (phosphate buffer system, 0.137 M NaCl, 0.016 M phosphate, pH 7.2). The cells were sonicated for four, thirty-second pulses and the membranes pelleted through ultracentrifugation. The membranes were resuspended in Cymal-5 wash buffer. The suspension was incubated with Rho 1D4 beads for 24 hours at 4 °C. Antibody plus protein was washed with Cymal-5 wash buffer, spun and supernatant was discarded. CXCR4 was competed off the 1D4 antibody with the addition of a large excess of the C9 peptide (sequence: TETSQVAPA, made in the Dratz lab by Dr. Royce Wilkinson) over five elutions.

Western Blot

Samples were run on either 10% SDS PAGE gels or 15% Tris-Tricine gels. Protein was transferred to PVDF (polyvinylidene fluoride) membrane overnight at 25 milliamps. The PVDF was blocked using blotto (5% powdered milk, 0.5% Tween-20). 1D4 was the primary antibody that was incubated on the PVDF for 1 hr. The membrane was then washed with ELISA wash buffer (0.1% Tween-20 in phosphate buffer system; PBS). Secondary antibody (goat anti mouse conjugated to alkaline phosphatase, Pierce) in blotto was added to the membrane. It was washed with ELISA wash buffer followed by one wash with Western Substrate Buffer (WBS, 0.1M Tris pH 9.5, 0.1M NaCl, 1mM $MgCl_2$). Development with NBT (nitro-blue tetrazolium chloride (Sigma)) and BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma)) in WBS was done until optimal color development.

Binding Experiments

Cells were suspended in PBS and were incubated for 30 minutes with the T140 mutant containing a benzophenone (Bpa) group at position seven and SFX (Invitrogen 6-(fluorescein-5-carboxamido) hexanoic acid, succinimidyl ester (Invitrogen)) on the D-8 lysine (made in the Dratz lab by MSU's Dr. Royce Wilkinson). The mixture was exposed to 366 nm ultraviolet light for four rounds of five minute exposures with five minute incubations on ice in between the ultraviolet light exposures.

Proteolytic Digestion

Purified CXCR4 was digested using one or multiple of the following enzymes: Papain Digest (Boehringer Mannheim): 100 ng papain with 0.3% β-mercaptoethanol was added to purified CXCR4/T140 crosslink and digest was incubated at 37 °C for 4 hrs. Proteinase K (Boehringer Mannheim) digest: 100 ng proteinase K was added to purified CXCR4/T140 crosslink and incubated at 37 °C for 4 hrs. Pronase E (Sigma) Digest: 100 ng pronase E was added to purified CXCR4/T140 crosslink and incubated at 37 °C for 4 hrs. Trypsin (Sigma and Promega) Digest: 100 ng trypsin was added to purified CXCR4/T140 crosslink and pH adjusted to ~8. The digest was incubated at 37 °C for 4 hrs. Trypsin/thermolysin (Sigma) Digest: 10 ng trypsin was added to purified CXCR4/T140 crosslink and incubated at 37 °C for 4 hrs. 100 ng thermolysin was then added and incubated at 37 °C for 4 hrs. Thermolysin/trypsin Digest: 10 ng thermolysin was added to purified CXCR4/T140 crosslink and incubated at 37 °C

for 4 hrs. 100 ng trypsin was then added and pH adjusted to ~8. The digest was further incubated at 37 °C for 4 hrs.

CNBr (cyanogen bromide; Acros) Digest: Purified CXCR4/T140 crosslink underwent reduction/alkylation using dithiothreitol (DTT) and iodoacetamide. Protein was precipitated using 10 volumes of acetone and then centrifuged. The pellet was redissolved in trifluoroacetic acid (TFA). CNBr (2.5 mg) was dissolved in TFA and water was added to the protein and incubated overnight at room temperature in the dark. Water was then added (7x volume), the solution was frozen at -80 °C, and subsequently lyophilized. The pellet was redissolved in PAGE sample buffer (1 part 0.5 M Tris pH 6.8, 1 part 10% SDS, 1 part glycerol, bromophenol blue) and run on a 15% tris/tricine gel.

HPLC

High performance liquid chromatography (HPLC) was done on an Agilent 1100 Series LC System. A Phenomenex Jupiter 4u Proteo 90A, 150 x 2 mm, 4 micron column was utilized. Solvent A comprised 100% H_2O plus 0.1% TFA, while Solvent B comprised 95% acetonitrile (ACN), 5% H_20, 0.1% TFA on a gradient flow starting from 5% solvent B and ending at 95% solvent B. The eluted product was monitored via fluorescence with the detector set to an excitation wavelength of 501 nm and emission wavelength of 523 nm.

Solid Phase Extraction

Solid phase extraction was done on Phenomenex Strata-X 33u Polymeric Sorbent Reverse Phase columns. Samples were loaded onto the column in the presence of low concentrations of ACN (<5%) and eluted off with increasing concentrations of ACN up to 100 percent. Samples were acidified with TFA prior to loading and were neutralized with pH 9.5, 1 M Tris buffer after loading and prior to ACN elutions.

Affinity Purification

Anti-fluorescein 4-4-20 antibodies were bound to sepharose beads as described in the Preparation of Sepharose Antibody Beads section [22, 23]. Samples were loaded onto antibody beads overnight at 4° C. They were then spun to remove unbound supernatant. Beads were then washed with PBS five separate times with centrifugation steps in between washes and respective supernatant following individual steps then discarded. Bound protein was eluted with either 0.1 M glycine at pH 2.5 or with 2.5% triethylamine (TEA) in 40% ACN. Improved results in terms of peptide recovery were obtained with the latter elution protocol.

In Gel Extraction

Samples were analyzed by 10% SDS-PAGE. The portion of the gel with fluorescence was cut out and dried. Gel pieces then underwent reduction with 1.5 mg/mL DTT and alkylation with 10 mg/mL iodoacetamide. Samples were

then dried and rehydrated with a 10 mM $(NH_4)HCO_3$ in 10% ACN solution with or without 0.25 µg/µL trypsin solution and allowed to incubate for 16 hours. Following a final centrifugation step, the supernatant was then analyzed for the presence of peptides.

Mass Spectrometry:

Digested protein samples were run on multiple instruments including the Bruker MALDI-TOF (Matrix Associated Laser Desorption Ionization Time of Flight) Biflex II, the Bruker microTOF with the Agilent 1100 HPLC, and the Agilent 6340 XCT Ion Trap with 1100 nanoflow-chip cube HPLC. Experimental masses were compared to those predicted from theoretical digests. Data analysis was performed using Matrix Science MASCOT software.

Results

The main research goal was to identify key amino acids in the CXCR4 sequence responsible for T140 binding. To accomplish this goal, we crosslinked a Bpa amino acid on T140 to an amino acid within the CXCR4 sequence that was in close proximity to the Bpa residue. In order to identify the crosslinked location, a systematic approach was employed that included harvesting Cf2Th-CXCR4 cells expressing CXCR4 on the cell membrane, crosslinking the T140 peptide to CXCR4, purifying the CXCR4/T140 crosslink from the remaining cellular proteins, digesting the crosslink to small peptides that were amenable to purification and finally MS/MS sequence identification (Figure 6.3).

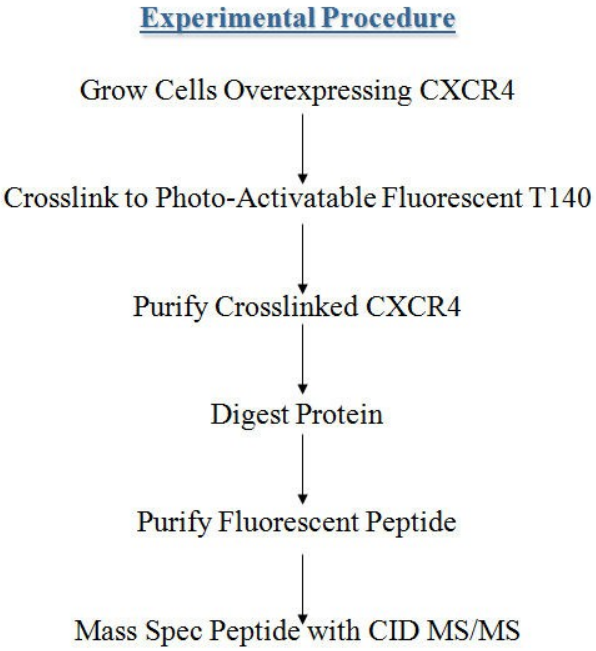


Figure 6.3. Outline of procedural steps taken to identify the crosslinked amino acid in CXCR4.

Expression and Purification of CXCR4

The canine thymocyte cell line Cf2Th-CXCR4 overexpresses the CXCR4 above the normal physiological expression level (1-2 µg from 10^7 cells) [21]. This cell line exhibited the highest expression levels of CXCR4 at the time the work on this project was performed. Since that time, CXCR4 has also been expressed in HEK293 [24], CHO cells [25], and Sf9 insect cells [26]. In order to readily purify CXCR4, an epitope tag was added to the C-terminus of CXCR4 that bound to the monoclonal antibody 1D4. This antibody is specific for the nine residue (C9) C-terminal peptide epitope sequence (TETSQVAPA) on the bovine rhodopsin receptor. The C9 peptide was added to the sequence immediately 5' of the

natural stop codon of the *cxcr4* gene in the PACH vector which was then transfected into Cf2Th cells [20, 21]. These additional amino acids did not alter CXCR4 activity and allowed for highly specific antibody purification of the overexpressed CXCR4 protein. An antibody specific to the C9 peptide had already been developed and proven to be effective [27]. The 1D4 antibody was bound to Sepharose beads to allow for separation using centrifugation. To purify the protein, the Cf2Th-CXCR4 cells were sonicated, the membranes pelleted by ultracentrifugation, and the resuspended solution incubated with the 1D4 sepharose antibody beads. The beads were washed to remove any unbound proteins and the bound CXCR4 was eluted off the antibody by adding excess C9 peptide. We detected the purified CXCR4 using the same 1D4 antibody on a western blot followed by a secondary goat anti mouse antibody that had alkaline phosphatase as the detection method. Addition of NBT/BCIP yielded a purple color as seen in Figure 6.4 after reaction with alkaline phosphatase.

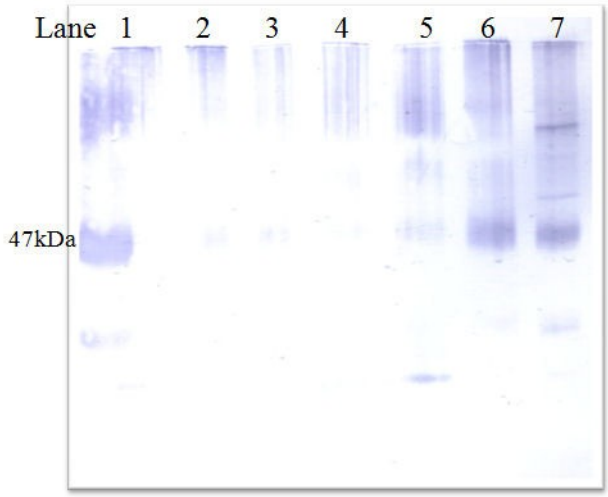


Figure 6.4 Western blot of CXCR4 purification. Lane 1 is unbound flowthrough. Lane 2-4 are washes 1-3. Lane 5-7 are elutions 1-3.

Crosslinking T140 to CXCR4

At the time this work was performed no X-ray crystal structure of CXCR4 was available, thereby prompting the need to (1) identify the ligand binding architecture of CXCR4 and (2) to use this architecture to examine the CXCR4-T140 interface. T140 was an ideal candidate for these studies given the high affinity binding of T140 to CXCR4. To identify the amino acids on CXCR4 important for binding, we crosslinked the T140 peptide and CXCR4 protein together to determine which amino acids were in close proximity. To initiate these studies, two modifications were incorporated on the T140 peptide (Figure 6.6).

The first modification was to replace the seventh lysine amino acid residue with a benzophenylalanine residue (Bpa), which has a photoactive benzophenone group. Bpa has been used by others to map amino acid locations

when the protein and peptide cannot be co-crystallized [28, 29]. We selected Bpa due to the fact that it is more stable than other photoactivateable groups, it can be manipulated in ambient light, and it preferentially reacts with typically unreactive carbon-hydrogen bonds. Further, Bpa reacts with any carbon atom in close proximity unless there is an additional, adjacent methionine residue. The Bpa residue is activated when exposed to controlled 350-360 nm ultraviolet light making it highly desirable to map conformations of protein-peptide interactions [30]. Absorption of a 350-360nm photon results in the promotion of an electron on the oxygen from the nonbonding orbital to an antibonding orbital of the carbonyl group resulting in a diradical state. The electron deficient oxygen abstracts a hydrogen from a nearby carbon atom. The remaining carbon radical readily reacts with the now deficient carbon atom, forming a new carbon-carbon bond (Figure 6.5). If a carbon atom is not located nearby for the oxygen radical to abstract hydrogen from, the Bpa will relax to the ground state and not react. This reaction is highly specific for reacting with carbon atoms in close proximity. In our experiments, the Bpa on the T140 peptide (seventh position) forms a covalent crosslink between the T140 peptide and the CXCR4 protein. The crosslink keeps the T140 and CXCR4 bound together despite further experimental manipulations

(Figures 6.5 and 6.8).

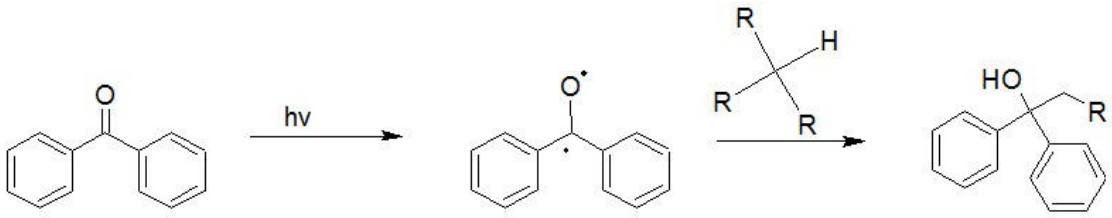


Figure 6.5. Bpa reaction scheme. The R group for our experiment is from the CXCR4 protein.

The second modification to the T140 peptide was the addition of a fluorescent tag on the D8 lysine amino acid located in the center of the peptide. The fluorescent tag utilized in these studies is the NHS-ester tagged SFX (fluorescein-X-succinimidal ester from Invitrogen). The NHS group readily reacted with the primary epsilon amine on lysine 8. The N-hydroxysuccinimide group was the byproduct of this reaction. There is a seven atom aminohexanoyl spacer (6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester) that separates the fluorescent molecule and the lysine side chain. The carbon chain allowed for free movement of the fluorescent group outside of the CXCR4 binding pocket and gave the best fluorescent signal compared to other fluorescein molecules with shorter linkages, such as FITC (fluorescein isothiocyanate) and FAM (5-carboxyfluorescein) (Figure 6.7). The fluorescein labeled lysine enabled us to track the peptide location at all times by merely following fluorescence.

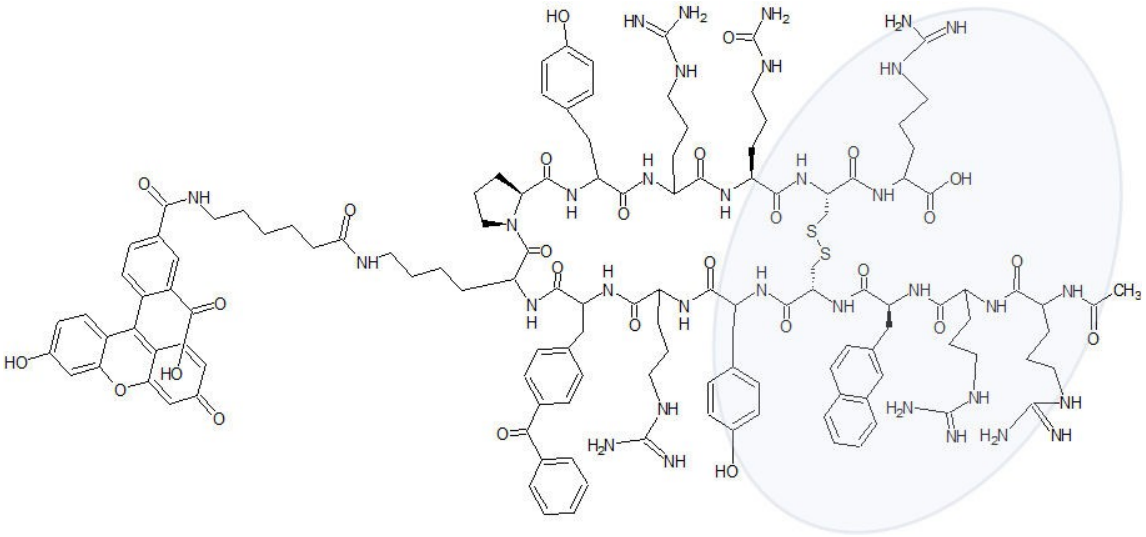


Figure 6.6. Structure of modified T140. Lys8 had a fluorescein fluorescent group added onto the epsilon amine group. Lys7 was replaced with a photoactivatable benzophenone group that forms a covalent bond to the CXCR4 protein. The amino acids circled in blue are the amino acids required for binding to CXCR4.

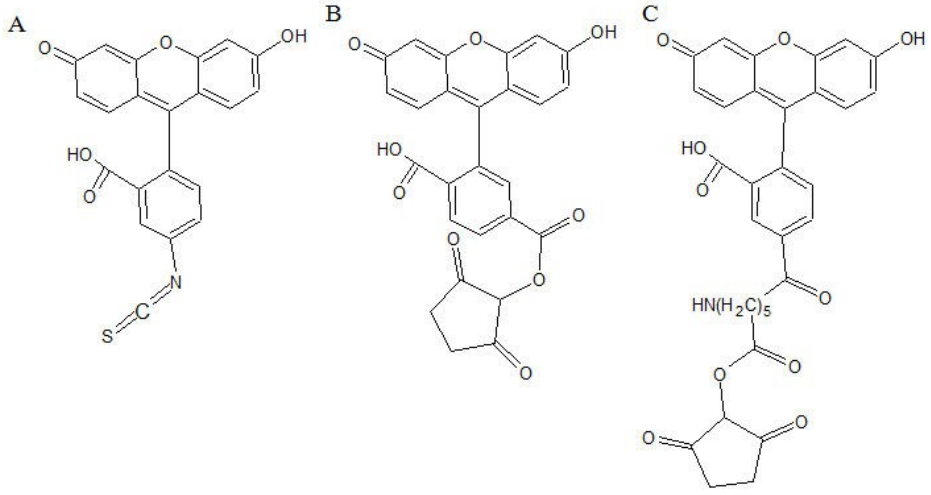


Figure 6.7. Three possible fluorescein compounds. All three have different linkage lengths ranging from shortest (FITC) to longest (SFX). All three of these fluorescent compounds were tested (data not shown). SFX on T140 provided the largest fluorescent signal.

Crosslinking was accomplished by reacting 75 nM T140SFX with Cf2Th-CXCR4 cells and allowing at least 30 minutes for the T140 peptide to locate and bind to the CXCR4 receptor on the cell surface. The cells were then exposed to 366 nm UV light to facilitate the crosslinking reaction between the Bpa on T140 and the CXCR4 protein (Figure 6.8). The crosslinked CXCR4/T140 was then purified using the 1D4 antibody in the same manner as outlined in the previous section for CXCR4 alone. Figure 6.8 shows a fluorescent image of a gel of purified crosslinked CXCR4/T140.

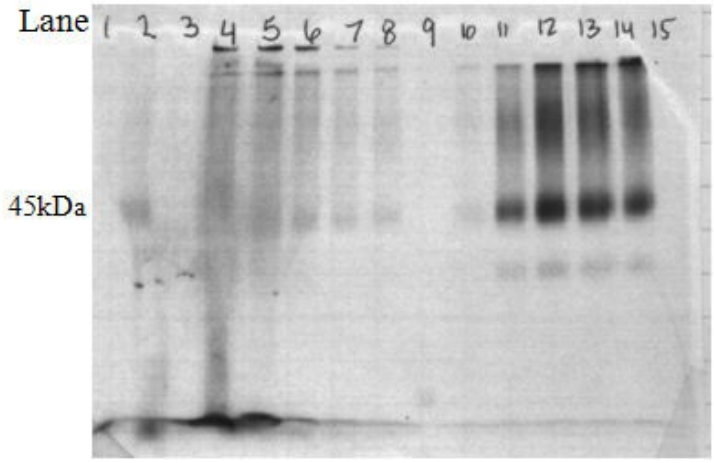


Figure 6.8. Purification of CXCR4/T140 crosslinked sample. Fluorescent scan was done on the BD FX Imager. Lane 2 is unbound flowthrough. Lanes 4-8 are washes 1-5. Lanes 10-14 are elutions 1-5.

Protein Complex Digestion

In order to definitively identify the crosslink, the size of the CXCR4/T140 crosslinked protein needed to be reduced to allow for proper dissociation into

peptide and amino acid components in the mass spectrometer. For this process to occur efficiently, the crosslinked protein needed to be reduced to small peptides that would then undergo peptide bond cleavage when hit with the collisional gas in the mass spectrometer chamber. Collision induced dissociation (CID) is one of the most common methods used to identify amino acid sequences on a mass spectrometer. The mass of the parent ion of interest is selected and all molecules of that mass are isolated. They are then bombarded with neutral atoms from a gas known as the collision gas which is typically argon. The kinetic energy of the collision event is converted to vibrational energy resulting in peptide bond breakage that subsequently allows for peptide sequence identification as each amino acid breaks away. The working hypothesis was that the CXCR4/T140 crosslink could be digested using chemical or enzymatic cleavage protocols, the fluorescent fragment then purified, and analyzed by MS/MS techniques to allow for identification of the crosslinked amino acid moiety.

The first step in implementing this procedure was to digest away most of the protein, leaving the small crosslinked peptide. This peptide would remain fluorescent due to the fluorescein on $_{D}$-Lys8 of the T140, immediately next to the reactive Bpa group. Importantly, following the identification of the amino acid(s) that crosslinked to the T140 peptide, we could then go back to the known amino acid sequence of CXCR4 and hypothesize the binding pocket based on the location of the amino acids.

Several digests of the protein complex with different enzymes were performed in order to identify the best small crosslinked peptide for MS analysis. Our initial tests were done with trypsin since it is the most common protease utilized in protein digestion protocols. However, when the crosslinked product was digested with trypsin, the remaining fluorescent peptide was still larger than the 15 kDa fluorescently labeled lysozyme standard (Figure 6.9). This digested product was too large to obtain adequate CID MS sequence data. The large fragments absorb the fragmentation energy and dissipate it over the entire peptide instead of fragmenting between amino acids, and thus preclude MS analysis. Sequence analysis showed that the CXCR4 sequence has relatively few arginine and lysine residues for trypsin to cleave at. The large peptides seen in the Tris/Tricine gels (Figure 6.9) matched the theoretical large trypsin digest fragments. Thermolysin also did not adequately digest the protein into small enough fragments (Figure 6.9); this protease, which cleaves at hydrophobic residues, was expected to give a small fragment but did not cut the CXCR4/T140 crosslinked protein to any measurable extent.

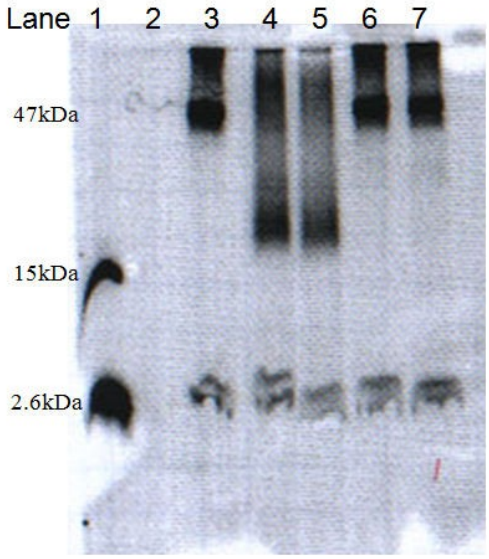


Figure 6.9. Tris/Tricine gel of trypsin and thermolysin digestions of CXCR4/T140 crosslinks. Lane 1 is the lysozyme and fluorescent T140 size standards. Lane 3 is undigested crosslinked CXCR4/T140. Lane 4-5 are trypsin digestion after 4 and 24 hours of digestion. Lane 6-7 are thermolysin digestion after 1 and 4 hours of digestion.

Following this result, other chemical and enzymatic digestions were utilized that should have theoretically yielded small enough protein fragments to obtain CID sequence information. The crosslinked CXCR4/T140 was digested using proteinase K, pronase E, papain, and cyanogen bromide (CNBr). Proteinase K and papain digest nonspecifically at hydrophobic amino acids, while CNBr digests exclusively at methionine residues. Additional trials attempted a double digest of the crosslink for which trypsin and chymotrypsin were utilized (Figure 6.10A).

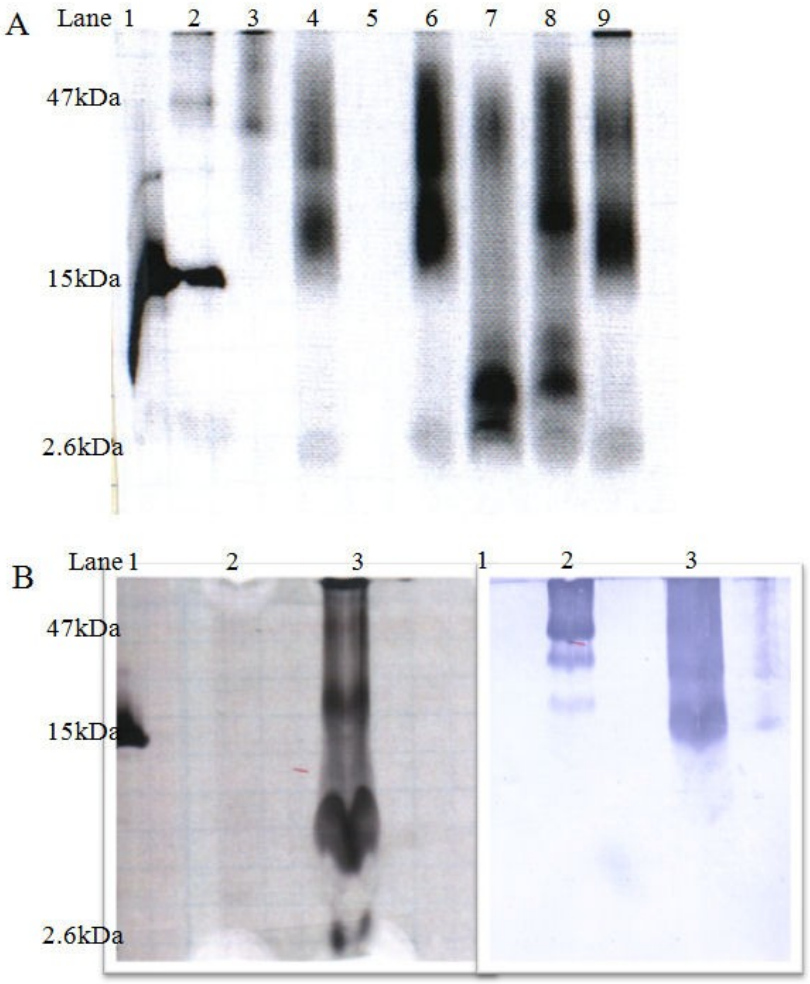


Figure 6.10A. Gel of digested CXCR4/T140 crosslinks. Lane 1 is T140/lysozyme size standard. Lane 2 is undigested CXCR4/T140 crosslink. Lane 3 is CXCR4/T140 digested with Trypsin & Thermolysin in acidic conditions. Lane 4 is digested with trypsin & thermolysin in basic conditions. Lane 6 is trypsin digest alone. Lane 7 is digested with proteinase K. Lane 8 is digested with pronase E. Lane 9 is digested with papain.
Figure 6.10B. Fluorescent image and western blot of CNBr digest of CXCR4/T140 crosslink. Lane 1 is a T140/lysozyme size standard. Lane 2 is an uncrosslinked CXCR4. Lane 3 is the CNBr digest of CXCR4/T140 crosslink. The fluorescent band in the middle of the CNBr digest (larger than the 15kDa lysozyme band) and the large purple band in the western blot CNBr digest are not of equal molecular weight. The band in the fluorescent image is slightly larger than the western blot band proving that the C-terminus is not where the fluorescent crosslink is occurring.

It was discovered that proteinase K (digests at hydrophobic amino acids) gave the smallest peptide fragment following single enzyme digestion. The fluorescent proteinase K peptide is slightly larger than the 2.67 kDa T140 peptide running at the bottom of the gel. The other digests all gave fragments that were larger than the proteinase K peptide (Figure 6.10 A and B). We attempted to further purify the small proteinase K digested fluorescent peptide and get sequence identification on the mass spectrometer.

Purification of Crosslinked Peptides

The purification of the digested fragment had multiple purposes. The ultimate goal was of course to purify the fluorescent crosslinked peptide from the rest of the peptide fragments to allow for identification after mass spectrometry identification, however, the cymal-5 detergent leftover from protein purification needed to be eliminated, since detergents like cymal-5 interfere with peptide ionization on mass spectrometers. Without a charge on the peptide, it would be invisible to the detector and simply not observed in the results. Furthermore, the fluorescent crosslinked peptide needed to be concentrated to detectable levels. To achieve these goals, we tried four separate purification techniques.

The first technique we used was HPLC (high performance liquid chromatography) based. The proteinase K digested sample was run over an HPLC column and elution products were monitored via the fluorescence detector. The fluorescent peptide was discovered to elute from the column with a retention time of 14 minutes (Figure 6.11). Unfortunately, these results were not

repeatable. Later HPLC runs had more than a single fluorescent peak eluting off at various times. When the fractions from the HPLC were run on a gel to verify the elution results, the fluorescent band on the gel did not correlate to the fraction that had the fluorescent signal on the HPLC (Figure 6.11). Eventually, it was determined that air bubbles were getting in the fluorescence detector and causing false positive peaks. For this reason, we desisted using the fluorescence detector to track the fluorescence and instead relied on running individual HPLC fractions on tris/tricine gels. We were able to detect the fluorescent peptide on gels of fractions eluting off in the 100% ACN portion of the chromatogram multiple times. However, silver staining of the same gel as what we fluorescently imaged revealed that the fraction with the most fluorescence did not have any detectable amounts of material in it. Rather, some non-fluorescent material was eluting off with a retention time one minute later than the fluorescent product (Figure 6.11B). This showed that our fluorescent material was not in enough quanitity for the silver stain to detect in the gel. For this reason, we utilized a second technique.

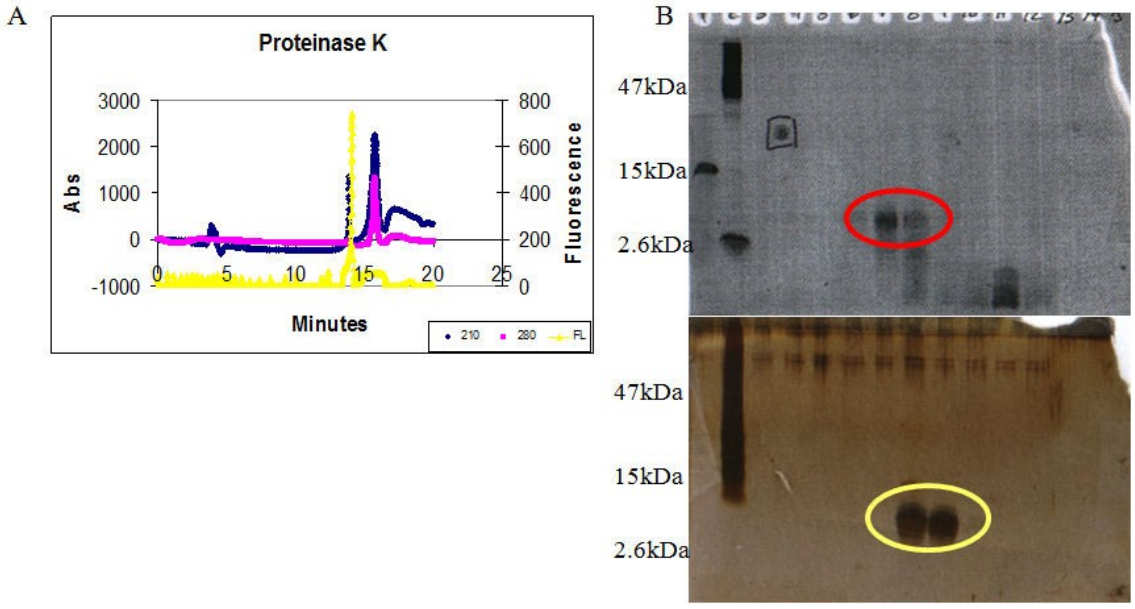


Figure 6.11. Proteinase K digested CXCR4/T140 crosslink. A) The digestion was run on LC to separate out the fluorescent fragment from the remaining fragments and detergent. Fluorescence indicated it was eluting at 14 minutes. B) Corresponding one minute fractions shown on a fluorescent gel (top) and a silver stain of the same gel (bottom). Gels showed elution at minute 15 with a lot of non-fluorescent material coming out at minute 16. The square box is a circular artifact from the imaging apparatus. Lane 1 (left) is a 15kDa lysozyme size standard. Lane 2 is crosslinked 45 kDa CXCR4/T140 and excess 2.6kDa T140.

Due to the problems associated with the above technique, we next attempted to purify the proteinase K fragment by using a Phenomenex Strata-X solid phase extraction column that separates by reverse phase. The fluorescein on the D-lysine of the digested crosslink bound to the column and was the peptide eluted off the column at 60-100% acetonitrile (Figure 6.12). Unfortunately, this result was not consistently repeatable. The fractions with fluorescence from the solid phase extraction column were run on the HPLC as described in the previous section to further purify them. However, these

subsequent HPLC runs showed that no fluorescent peaks were eluted and mass spec analysis on the MALDI Bruker Biflex II did not show any peaks of interest. This purification method was subsequently no longer utilized by itself.

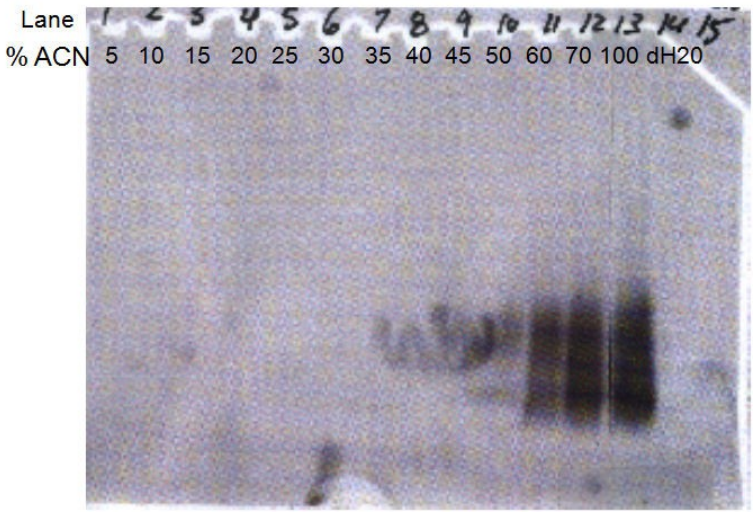


Figure 6.12. Tris-Tricine gel of solid phase extraction fractions. Proteinase K digested fragments elute off at high concentrations of acetonitrile.

The third purification technique that was attempted was to use affinity purification with anti-fluorescein antibodies. However, it was quickly determined that the cymal-5 detergent necessary for purification of the CXCR4/T140 crosslinked protein was incompatible with these antibodies. A ten-fold dilution of the cymal-5 detergent was necessary for the fluorescein tag to bind to the antibodies (Figure 6.13). This dilution effect on the fluorescent peptide was undesired but required for binding. The purification was also performed in the presence of a different detergent (CHAPSO), but this detergent showed similar

loss of retention on the antibodies (data not shown) unless it was also diluted ten-fold. Elution of the fluorescent peptide was difficult, requiring denaturation of the antibody using 5% TEA (triethylamine) and 40% acetonitrile to recover the peptide off the antibodies. The anti-fluorescein antibodies (4-4-20) have such a high affinity for fluorescein that some material was never recovered from the beads. Since our fluorescent peptide was at such low concentrations already, it was not acceptable to lose so much material in a purification step. Samples from the anti-fluorescein antibodies were run on the MALDI Bruker BiflexII and none of the peaks were identified as possible peptide fragments.

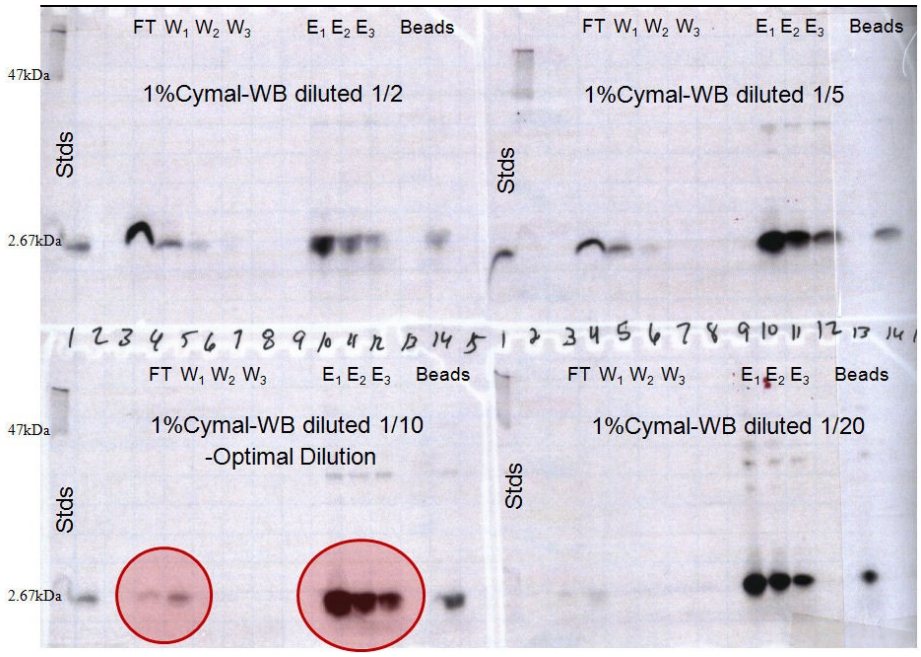


Figure 6.13. Gels of T140SFX elutions off of anti-fluorescein beads. 1% cymal interfered with binding of the fluorescent fragment on the antibodies. These gels show that a 1/10 dilution of the sample allowed for the best binding to the antibody while attempting to minimize sample loss. The standards run in lanes one and two of each gel are CXCR4/T140 crosslinks and the same T140SFX run on the beads to find the best dilution for using the antibodies.

The last purification method that was utilized involved the attempted purification of the proteinase K fragment by gel extraction. This seemed like a logical next step given that we had repeatable fluorescent signals on different gels from multiple purifications. For this procedure, a standard in gel extraction protocol was used to recover the fluorescent peptide. However, when the sample was extracted from the gel and run on the mass spectrometer, we did not identify any peptides from the data (data not shown). The fragments themselves may still have been too large for extraction out of the gel or the fragments may have been

too hydrophobic to be released from the gel in the solvents we were using. Either way, this method was not pursued further.

Each of these purification methods came with its own set of drawbacks and problems that required further optimization or adjustments in order to get the method to work. The biggest drawbacks were usually associated with dilution of the sample; sample loss during purification clearly affected final product concentration which in turn affected the ability to detect the derivatized peptide crosslink with the available techniques. Given these issues, it seemed logical to utilize a new detergent compatible with mass spectrometry when it became available on the market, as this would help limit peptide loss during the purification procedure.

Waters RapiGest™ SF Surfactant is an acid cleavable detergent that enhances enzymatic digestion by increasing protein solubility. Acid exposure degrades it into three separate fragments that are compatible with MS instruments and detection. Moreover, Promega's ProteasMAX™ Surfactant has similar properties as the Waters RapiGest™ SF Surfactant as it is also a protein solubilizing detergent but is designed to degrade over time into products that are compatible with mass spectrometry and liquid chromatography. ProteasMAX™ Surfactant is supposed to enhance trypsin digestion of proteins that are difficult to digest and the digest can be run immediately on the mass spectrometer without any cleanup steps.

We decided to use these detergents to enhance trypsin digestion in a double digest using CNBr and trypsin. Purified and crosslinked CXCR4/T140 went through CNBr digestion as outlined in the materials and methods section. However, instead of solubilizing it in PBS, we solubilized the CNBr digested pellet with either 0.1% ProteasMAX™ in 20 mM NH_4HCO_3 or in 0.2 % RapiGest™ in 20 mM NH_4HCO_3. The pH was adjusted to 8 and trypsin was added to the solution for overnight incubation. Figure 6.14 shows the 15% tris/tricine gel of the final digests. Under these digest conditions, we saw three distinct bands. The smallest band was just larger than our 2.67 kDa T140 peptide. The samples were acidified using formic acid, spun to remove insoluble material, and loaded directly onto the Agilent XCT mass spectrometer.

We also used these latter detergents for second double digest experiments using trypsin and Asp-N (endoproteinase that digests at Asn). The same purification technique was performed including the reduction and alkylation steps for these samples. These samples were only solubilized in 0.2% RapiGest™ in 20 mM NH_4HCO_3. The pH of the solution was adjusted to 8 and trypsin was added to the solution. After the overnight digestion was completed, Asp-N was added to the solution and allowed to digest for four hours. This sample was run on a gel for confirmation of protein digestion and showed the presence of two to three small fluorescent fragments. Formic acid was added to cleave the detergent followed by direct injection onto the Agilent XCT mass spectrometer.

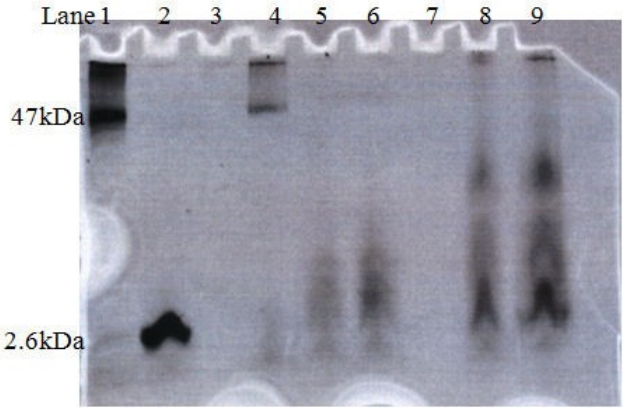


Figure 6.14. Double digests of CXCR4/T140 crosslink. Lane 1) undigested CXCR4/T140 crosslink, lane 2) undigested T140, lane 4) 0.25% of undigested CXCR4/T140, lane 5) 1% of Trypsin/Asp-N digest of CXCR4/T140 crosslink in the presence of Rapigest Detergent Lane, 6) 2% of trypsin/Asp-N digest in the presence of Rapigest detergent, lane 8) 10% of CNBr/trypsin digest in the presence of Rapigest detergent, lane 9) 20% of CNBr/trypsin digest in the presence of ProteasMax detergent.

Mass Spectrometry

Excellent mass spectra were obtained on the T140SFX alone. Using the Agilent 6340 XCT Ion Trap with 1100 nanoflow-chip cube HPLC, the T140 was detected as the +3, +4, +5, and +6 M/Z ion states (Figure 6.15).

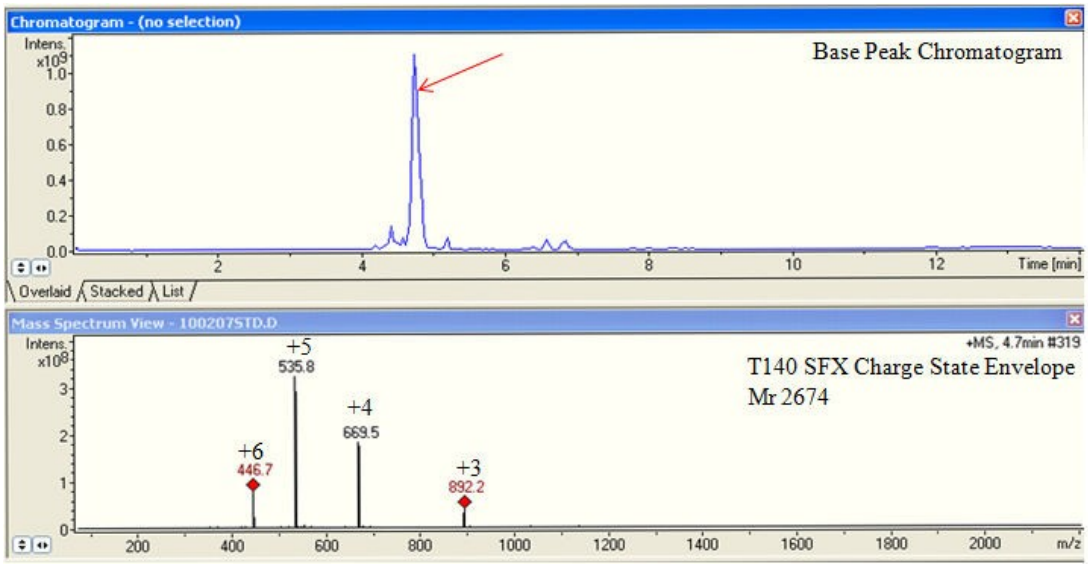


Figure 6.15. Mass Spec of T140 peptide with SFX on it. Top panel is the base peak chromatogram showing the peptide eluting off the C18 column at minute 4.6-4.8. The bottom panel is the charge state envelope of the peptide. It is seen as a +3, +4, +5, and +6 ion giving a total mass of 2674.

A proteinase K digest of T140 by itself without the presence of CXCR4 was performed and excellent CID sequence analysis was obtained on the Agilent 6340 XCT Ion Trap with 1100 nanoflow-chip cube HPLC. Three separate fragments after digestion with proteinase K were detected (Figure 6.16). One was a small fragment that included only the Bpa, lysine with the fluorescein tag, proline, and the tyrosine (Figure 6.17A). The remaining two fragments were larger in size. One of these fragments was only missing the N terminal three amino acids and the Arg6 residue. The other larger fragment was missing those four amino acids (Arg1, Arg2, Nal3, Arg6) as well as the C-terminal Arg14 moiety (Figure 6.17B). Each of these fragments was detected as amino acid sequences from CID. Knowing how the T140 peptide was degraded by proteinase K would

theoretically have allowed us to identify those same fragments in our CXCR4/T140 crosslinked fragment sample.

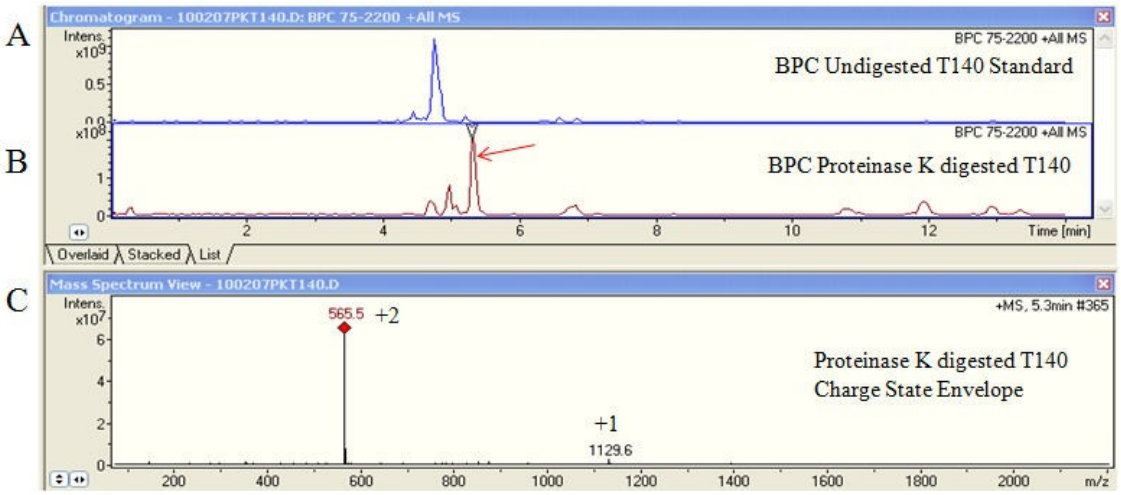


Figure 6.16. Mass Spec of T140 and Proteinase K digested T140. Panel A is the base peak chromatogram of undigested T140. Panel B is the base peak chromatogram of Proteinase K digested T140. Panel C is the +1 and +2 ion m/z of the smallest fragment found.

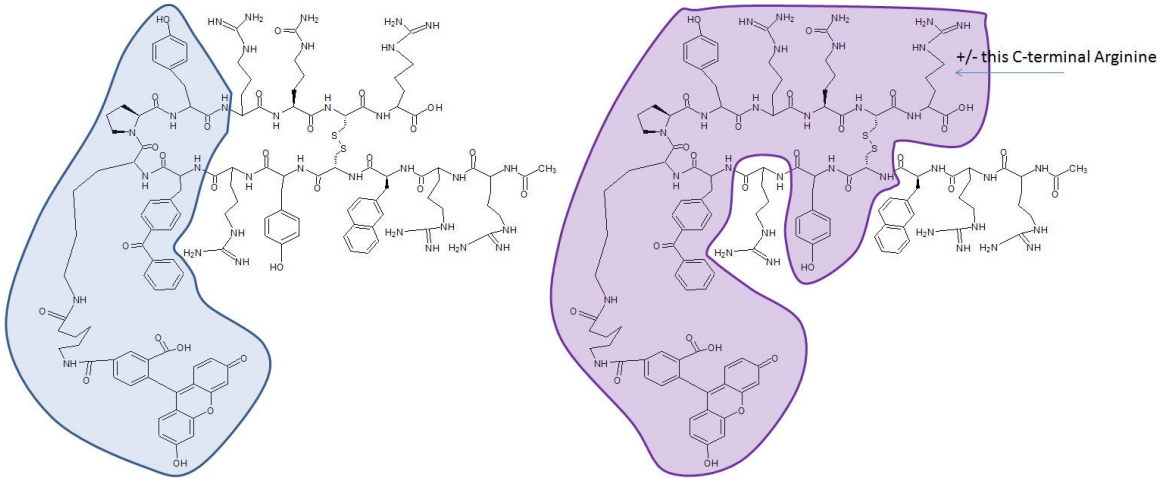


Figure 6.17. T140SFX peptide. Left) The section highlighted in blue is the small fragment found after digestion with proteinase K. Right) The section highlighted in purple is one of the two larger fragments found after digestion with proteinase K. The medium fragment is also missing Arg 14.

Unfortunately, none of our proteinase K digested CXCR4/T140 crosslinks ever gave us an identifiable mass with sequence data from CID despite all the work that went into trying to purify out a small digested fragment. Alongside this work, a new approach was tried using acid-cleavable detergents that are amenable to direct LCMS analysis without further purification.

The double digests performed in the presence of the cleavable detergents ProteasMAX® and RapiGest® showed great promise for identifying the crosslinked fragment. Initial data analysis on the samples after being run on the Agilent XCT identified two peptide fragments. This data analysis was done on Matrix Science search program MASCOT using the mammalian protein database NCBInr (National Center for Biotechnology information). This is a comprehensive non-redundant database. The two peptide fragments from CXCR4 in this database search were a contiguous sequence from helix four and extracellular loop two only seen in the trypsin/asp-N digested crosslinked sample (Figure 6.18). The CNBr/trypsin digested crosslinked sample did not identify any peptide within CXCR4.

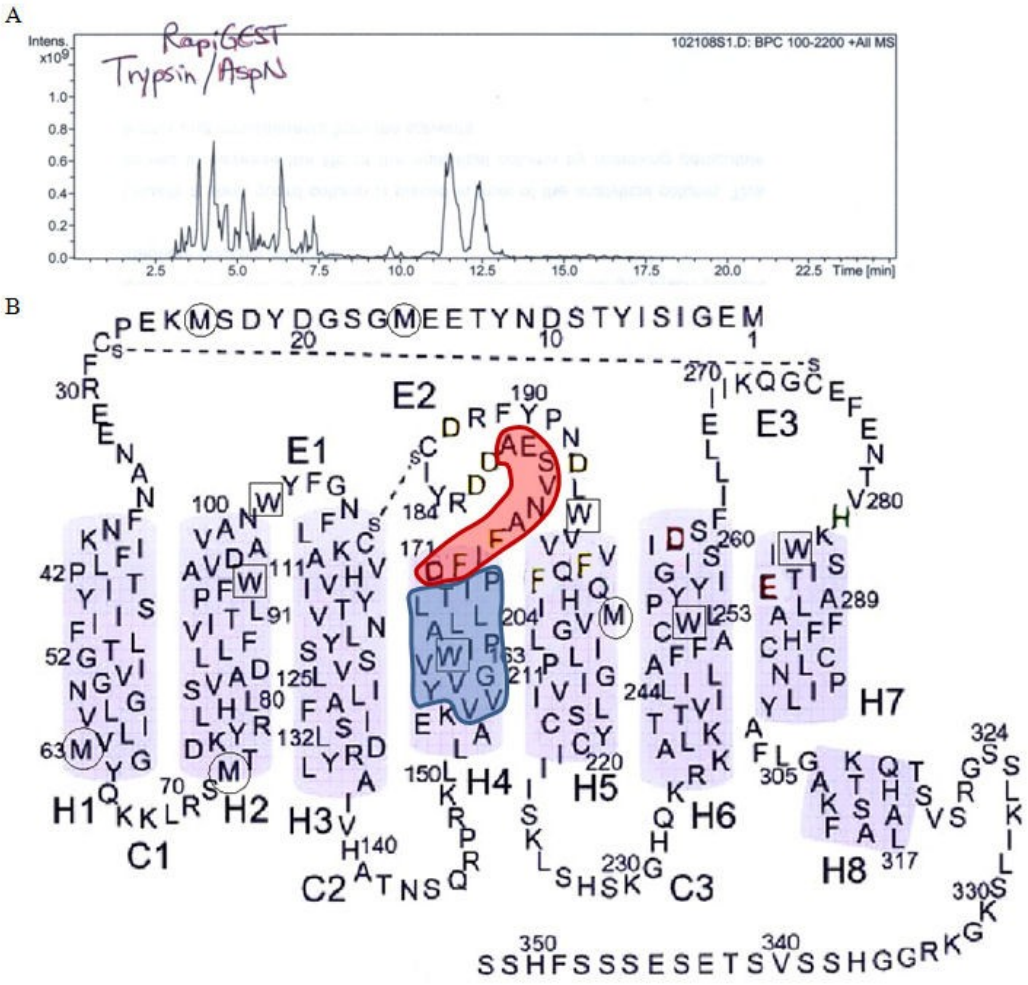


Figure 6.18. Fragments found from the trypsin/Asp-N digest. A) Base peak chromatogram of the digest on the mass spectrometer. B) The two peptides identified in the digested sample highlighted by blue and red.

After these results, we created our own database within the Matrix Science MASCOT program containing only the CCR5 and CXCR4 proteins of interest in our sample. Reducing the number of proteins that the data is searched against would improve identification of low intensity peptides that may be from

our CXCR4 while ignoring protein contaminants. After running the same data through this database, we identified the entire CXCR4 protein sequence between our two digest conditions (CNBr/trypsin and trypsin/asp-N). The CNBr/trypsin digest covered most of the sequence but was missing helicies two and six and the extracellular loops one, part of two, and half of three (Figure 6.19). The trypsin/asp-N digest covered the entire CXCR4 sequence except for helix one (Figure 6.20).

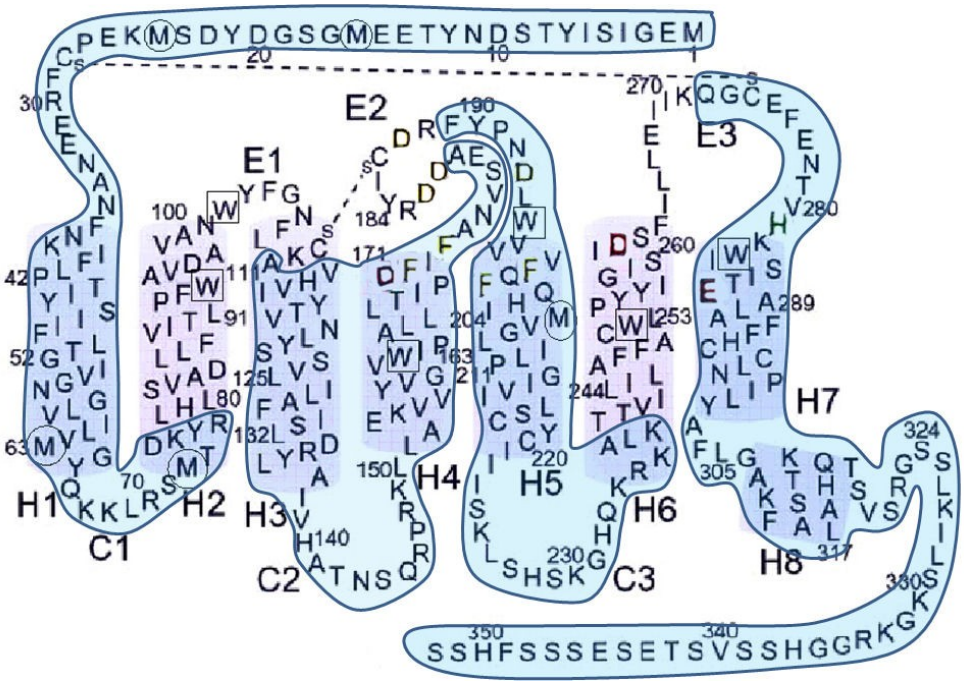


Figure 6.19. Sequence coverage of the CXCR4 receptor from the CNBr/trypsin crosslink digest.

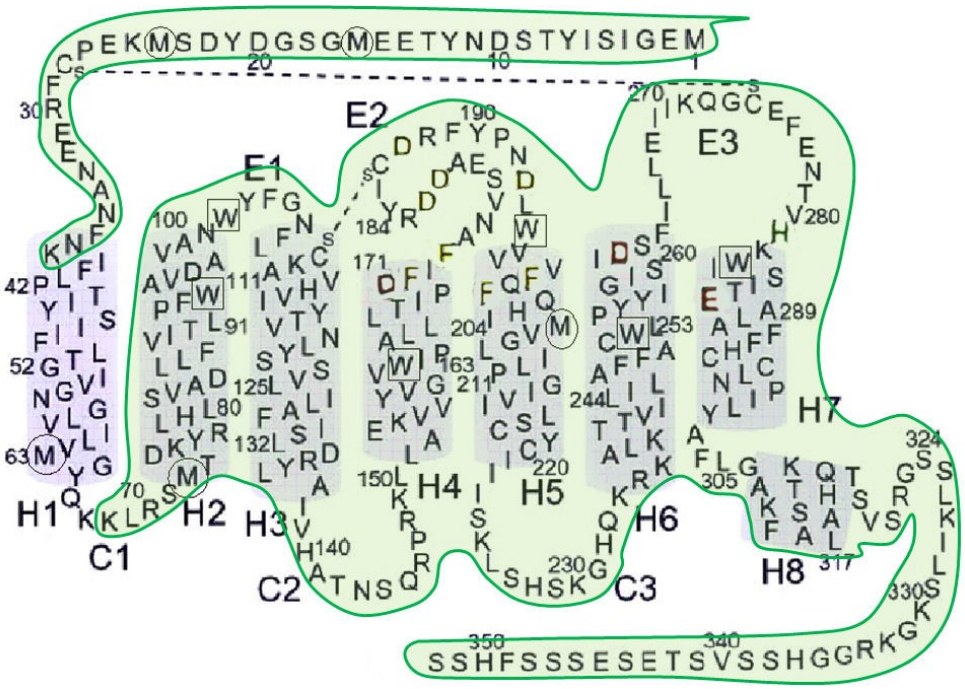


Figure 6.20. Sequence coverage of the CXCR4 receptor from the trypsin/asp-N crosslink digest.

Discussion

The goal of this research was to identify the amino acids in CXCR4 essential for T140 binding. Ultimately, the crosslink digestion LCMS experiments had one usable result for identifying the location of the crosslink. The CNBr digest of the CXCR4/T140 crosslink had one large fluorescent band that did not correlate to the large band that tested positive with the 1D4 antibody. The 1D4 antibody binds the C-terminal C-9 peptide on the CXCR4 protein. This observation meant that the C-terminal fragment that tested positive with the 1D4 antibody was not the fragment with the crosslink. When we evaluated a theoretical digest cleaving the CXCR4 protein at the methionine residues, we

found that there were two large fragments that were close in size to the bands seen on the gels. Either fragment had the potential to be the crosslinked fragment. However, one of them was the C-terminus that would have tested positive for the 1D4 antibody. This left the other fragment as the one with the T140 crosslink by the process of elimination. This fragment included transmembrane helix two through halfway of the fifth transmembrane region (Figure 6.19). However, these conclusions were based entirely on gel chromatography separation. We wanted to narrow down this result to a specific amino acid in the sequence. For that reason, we continued our investigation into the other digested fragments.

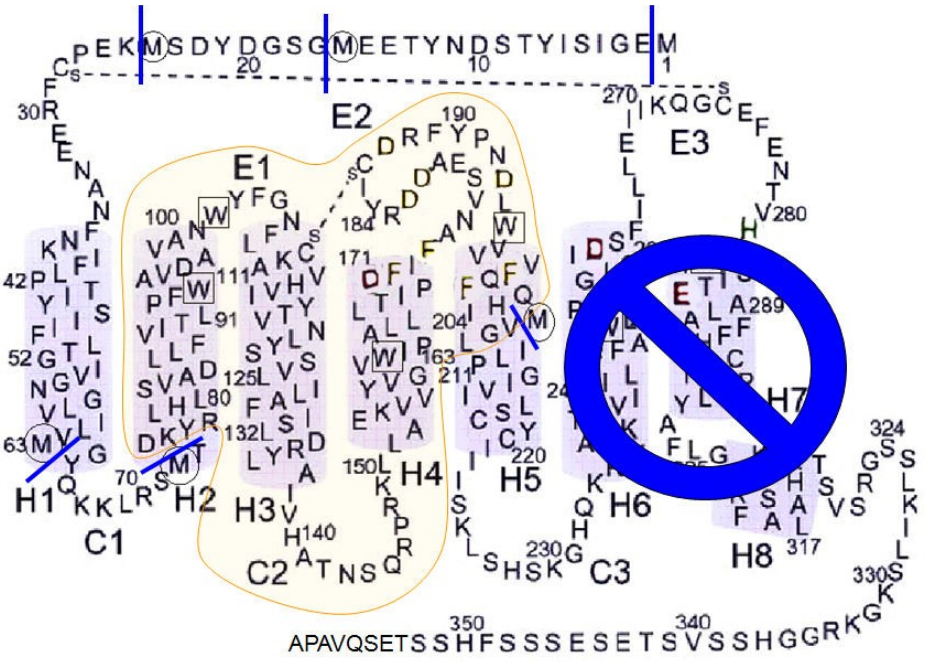


Figure 6.21. Speculated structure of CXCR4. The blue lines indicate the methionine residues where CNBr would theoretically cut. There are two possible large fragments that could have been the one seen on the fluorescent gel. However, one of them was the C-terminus which tested positive in the 1D4 stain as a different molecular weight from the fluorescent band. So this large fragment was ruled out. The fragment circled in orange is the fragment that contains the crosslink on it.

The double digests resulted in multiple fluorescent bands when run on gels (Figure 6.14). Data analysis of mass spectrometry results revealed coverage of the entire sequence between the two different digests (CNBr/trypsin and trypsin/asp-N). Theoretically, the peptide with the crosslinked T140 peptide attached would not be identified from the CXCR4 sequence due to the presence of T140 amino acids on the peptide. However, our data did not reveal a CXCR4 peptide that was missing from sequence identification. We could not identify the crosslink location on CXCR4 based on sequence coverage. This may be

because the crosslinked fragments take up a small fraction of the total CXCR4 protein quantity in the sample. The lack of available data on the efficiency of crosslinking in our sample (unable to quantify the amount crosslinked) combined with the preliminary data demonstrating that the fluorescent peptide is in quantities too low for silver stain to pick up (Figure 6.12), lead us to believe that only a small fraction of CXCR4 are crosslinked to T140. This leads us to believe that we were still able to identify the entire CXCR4 sequence due to the presence of uncrosslinked CXCR4 in the sample. There is the added question of whether the crosslink is occurring to one amino acid within the CXCR4 sequence or whether it is occurring to multiple different amino acids, thereby decreasing the quantity of crosslinked protein even further by the dilution effect of multiple peptides containing the crosslink. Further purification of the crosslink could be done prior to breakdown of the detergents, but these studies were ceased before this work was done. We did not identify a crosslinked amino acid but merely a section of interest in the CXCR4 sequence in the CNBr digest discussed in figure 6.21.

While we were unable to definitively identify the crosslinked peptide within the CXCR4/T140 crosslinked protein, there was another group pursuing almost this exact project. Boulais et al. published a paper entitled “Photolabeling identifies transmembrane domain 4 of CXCR4 as a T140 binding site [24].” They looked for a crosslink between a Bpa labeled on either position 5 or 10 of T140 and the CXCR4. This differs from our experiment in that we put our Bpa on

position 7. They used I-125 (Iodine-125) to track the complex instead of the methodology we employed, which relied on a fluorescent tag.

Once Boulais et al. incorporated I-125 labels on the T140 peptide, the CXCR4/T140 crosslinked protein was digested using three rounds of enzymatic digestion involving the V8 protease, followed by treatment with PNGase F, and finally a second round of V8 protease. The resulting fragment was partially purified through gel electroelution and then subjected to additional digestion with Asp-N. The V8 protease cleaves at the C-terminal side of glutamate residues while PNGase F cleaves between asparagine and N-acetylglucosamines, and Asp-N cleaves on the N-terminal side of aspartate residues.

Based on gel analysis of the molecular weight of labeled peptides remaining after these four digestion steps, they determined that the crosslink was occurring somewhere on the fourth transmembrane helix but could not narrow it down further. This agreed with our results showing that a crosslink between the Bpa on position seven was occurring somewhere between helix two and five, including extracellular loops one and two of CXCR4. Their paper hypothesized that it was the extracellular portion of the fourth transmembrane helix which was also identified in the possible crosslinking area in our results. This paper had results that were no more definitive than those we had obtained previously, but those authors considered their data publishable.

While there was no crystal structure of CXCR4 available when this research was actively being pursued, there have since been five different crystal

structures published. The publication by Wu et al. in 2010 was the first time that CXCR4 was crystallized [26]. Prior to this publication, there were seven other GPCRs that had published crystal structures prior to CXCR4 (rhodopsin, β1 adrenergic receptor, β2-adrenergic receptor, adenosine receptor, dopamine D3 receptor, histamine receptor, and S1P1 sphingosine-1 phosphate receptor). The CXCR4 crystal structures made our research obsolete because they identified the important CXCR4 amino acids involved in binding T140.

Three of the structures published by Wu et al. were co-crystallized with a small molecule antagonist named IT1t, while the remaining two CXCR4 structures were co-crystallized with a T140 peptide derivative called CVX15. All of the structures were published with a T4 lysozyme fusion inserted between the fifth and sixth helices on the cytoplasmic side of the protein. This fusion was inserted in order to stabilize that unstructured loop region within the CXCR4 structure.

The results of Wu et al. demonstrated that the binding pocket in CXCR4 was different than the binding pocket of other published GPCR structures such as rhodopsin. The CXCR4 binding pocket is much closer to the extracellular side of the cell, is much larger, and more open than other crystallized GPCR binding pockets. In fact, the small molecule IT1t is too small to completely fill the binding pocket. However, the fourteen amino acid T140 peptide derivative CVX15 is bulky enough to fill the entire binding pocket. The arginine residues located at positions one, two, and fourteen are all integrally involved in binding to the

CXCR4 receptor (Figure 6.22). Arginine one interacts with asparagine 187, arginine two interacts with threonine 117 and asparagine 171 and may also hydrogen bond with histidine 113. The arginine fourteen forms a salt bridge with asparagine 262. The hydrophobic napthylalanine three is also integral to CXCR4 binding. This residue interacts with a hydrophobic area bordered by helix five. Dispensing of any of these amino acids resulted in a reduction of CXCR4 affinity [13]. Lys7 showing bonding to D193 in the CXCR4 sequence is on the extracellular loop two, the same loop we identified as potentially important from our CNBr digest results. It is possible that our Bpa crosslink was occurring on the extracellular loop two.

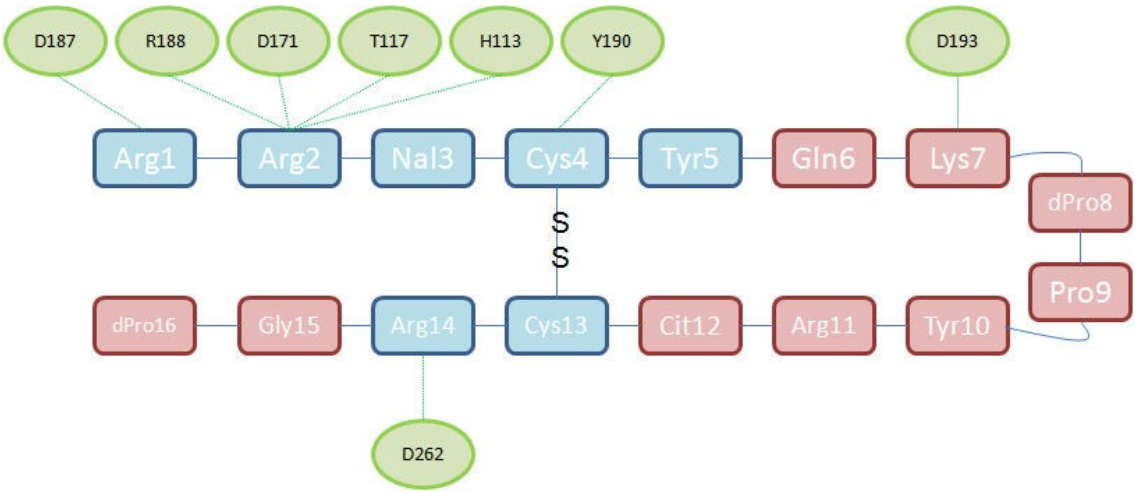


Figure 6.22. Diagram of T140 analog in the CXCR4 crystal structure. Amino acids in blue are the T140 amino acids essential for high affinity binding to CXCR4. Red amino acids are non-essential T140 amino acids. Green amino acids are the CXCR4 amino acids that the T140 interacts with in the crystal structure.

The results summarized within this Chapter highlight the experimental difficulties in crosslinking experiments. Ultimately, without the ability to obtain larger amounts of the crosslinked product for more thorough MS analysis, we could not define the exact amino acid identity of the crosslinked product. Regardless, the data we obtained did narrow the T140 binding pocket to a region of CXCR4, which was confirmed to be correct in the following years by the determination of the CXCR4 structure with a T140 analog bound.

Acknowledgments

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APPENDIX B

EXPRESSION AND PURIFICATION OF CXCL12

Expression and Purification of CXCL12

Performing the matrigel assays and calcium flux assays discussed in detail in chapter three required large quantities of CXCL12 to continue these studies. The cost of CXCL12 was prohibitive to continuing these studies. For this reason, making our own CXCL12 was required to complete the studies. Veldkamp et al. published a solid-phase method for milligram scale production of natively folded CXCL12 which I followed to make CXCL12 [1]. It is important to note that they added an N-terminal hexahistidine tag followed by an eight amino acid sequence (ENLFYQGM) that included a methionine residue. CNBr (cyanogen bromide) cleaved off the hexahistidine and eight amino acid tag at the methionine residue, leaving a native N-terminus on the CXCL12 protein after purification and proper folding of the protein was completed.

Figure 7.1 shows a western blot of CXCL12 before purification and CNBr cleavage (Lane 1) and after purification and CNBr cleavage (Lane 2). Figure 7.2 is the corresponding mass spectrum from lane 2 analyzed on the Bruker MALDI Autoflex III showing the correct mass of the CXCL12 after purification, CNBr cleavage, and proper folding (MW 7.9 kDa).

The CXCL12 was not tested for activity in the calcium flux assays nor the matrigel assays due to a time constraint. However, recent testing in an ERK-phosphorylation assay done by Amanda R. Radke demonstrated CXCL12 induced activity using these samples. It will be used for the continuation of those studies.

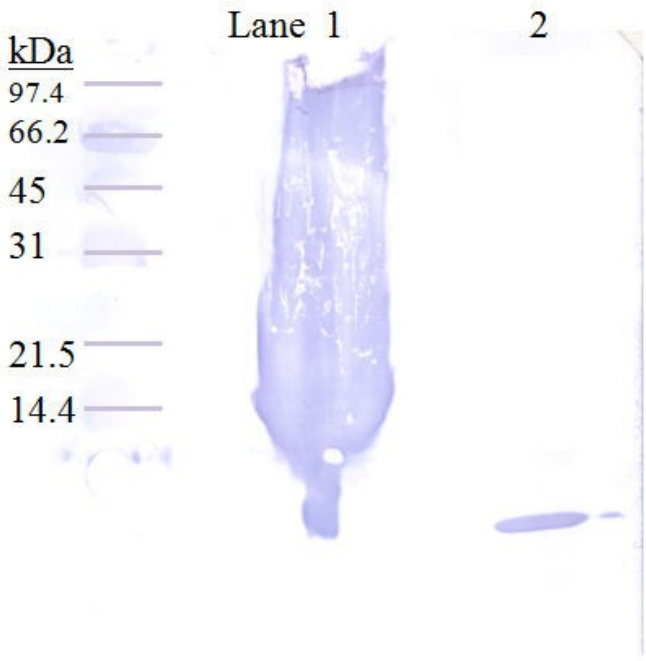


Figure 7.1 CXCL12 expression and purification. Lane 1) CXCL12 from E.coli with a hexahistidine tag (MW 10.3 kDa), Lane 2) CXCL12 after purification, proper folding, and cleavage of the hexahistidine tag (MW = 7.9 kDa).

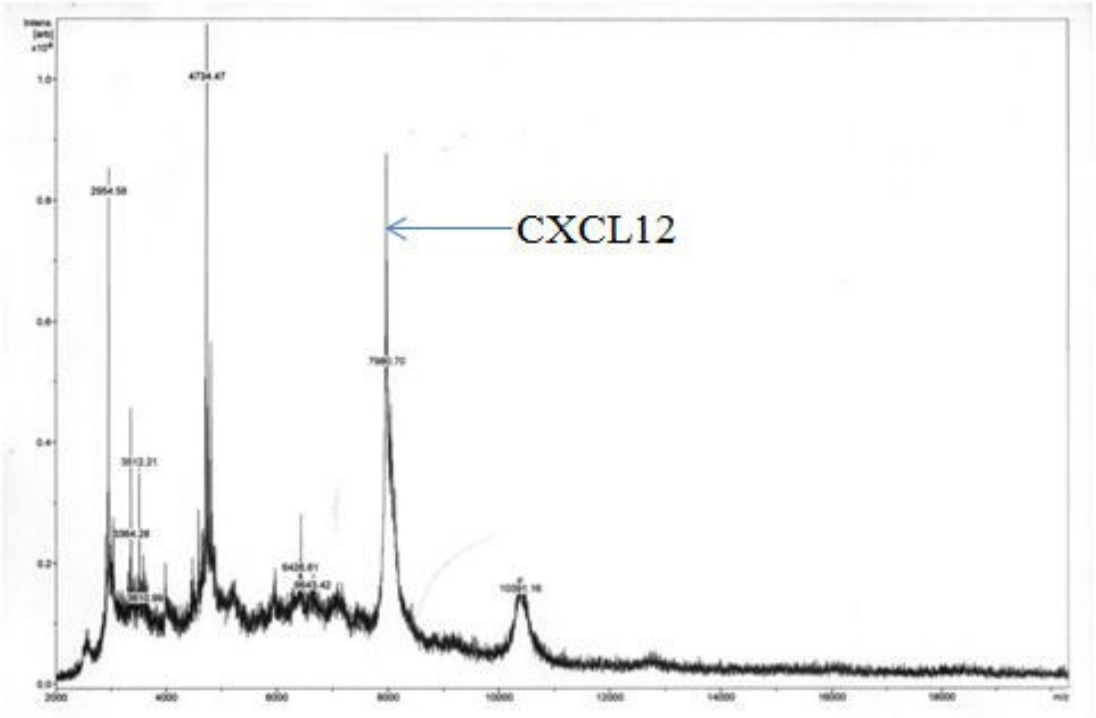


Figure 7.2 Molecular weight from MALDI of the purified and folded CXCL12 (MW = 7.9 kDa).

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APPENDIX C

EXPERIMENTAL PROTOCOL FOR METABOLOMICS STUDIES

Experimental Protocol for Metabolomics Studies

The protocol for metabolite extraction was adapted by Josh Heinemann from the protocol by Canelas et al. [1]. Methicillin-resistant Staphylococcus aureus (community-associated MRSA strain LAC, pulsed-field gel-electrophoresis type USA300 [2]) bacteria were grown to mid-log phase (OD_{600} = 0.4) and 2 μM THAM trisphenylguanide, 5 μM chlorhexidine, or PBS (phosphate buffered saline, 0.137 M NaCl, 0.016 M phosphate, pH 7.2) were added and the cells grown for one additional hour at 37 °C. The TSB (tryptic soy broth) was discarded after spinning the cells for 30 minutes at 5000 x g at 4 °C. The cell pellet was washed with PBS and respun under the same conditions. The PBS was discarded and the bacteria were put in chloroform and methanol and shaken for fourty five minutes at 4 °C. The insoluble pellet was spun down for 30 minutes at 5000 x g at -5 °C. The supernatant was removed and saved as part of the metabolite sample vial. More chloroform and methanol were added to the insoluble pellet and vortexed three times. The samples were incubated on ice when not vortexing. The samples were again spun for 30 minutes at 5000 x g at -5 °C. The supernatant was added to the metabolite sample vial and 50% methanol was added to the insoluble pellet. The samples were vortexed three times, placed on ice in between vortexes and spun for 30 minutes at 5000 x g at -5 °C. The supernatant was added to the metabolite sample vial and the insoluble cell pellet was discarded. Seven volumes of acetone were added to the metabolite sample vial and frozen overnight at -80 °C. The samples were spun

down for 30 minutes at 5000 x g at -10 °C and the insoluble protein pellet was discarded. The supernatant was subsequently dried down under nitrogen and the dried sample placed in the freezer until analysis was done. The dried samples were resuspended in 50% methanol, spun briefly to remove any insoluble material, and run on the Agilent 6538 QTOF with 1200 Agilent HPLC. Samples were run in positive mode on a C18 reverse phase column.

Data analysis revealed one large chromatogram feature eluting out at approximately 50% acetonitrile at medium or large intensities in both of the treated MRSA samples. Untreated control samples showed very low intensity or no intensity correlating to this peak (Figure 8.1 and 8.2). Deconvolution of the mass spectrum revealed a mass of 3500 +/- 250 daltons for that peak (Figure 8.3). Further evaluation of this peak is necessary to identify it. Due to the overwhelming signal seen from this unknown material in a few samples, further data analysis comparing untreated and treated samples cannot be done due to ionization interference from this overwhelming unknown material. Initial experiments done using a molecular weight cutoff filter were not effective in eliminating the unidentified material from the sample to allow comparison analysis of other metabolites in the sample. Future metabolomic studies will require elimination of this material before other metabolites changing in response to chlorhexidine and THAM trisphenylguanide can be identified.

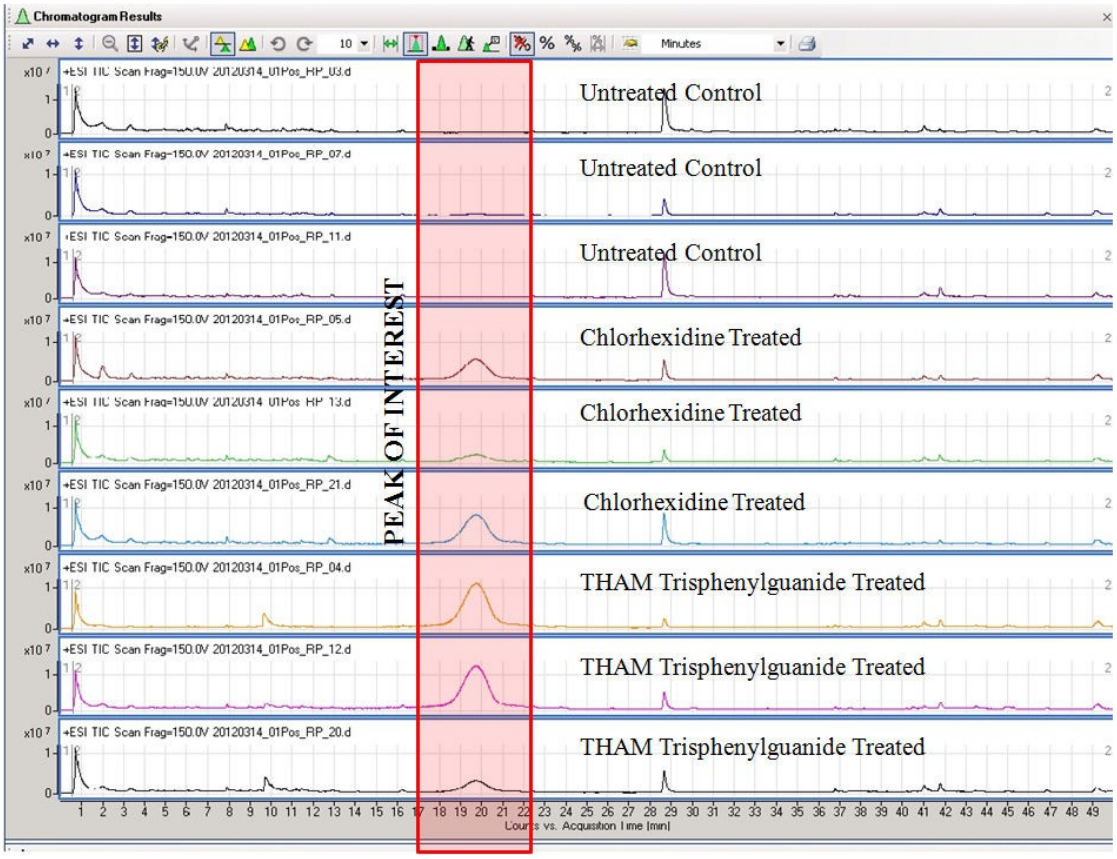


Figure 8.1 Chromatograms of extracted metabolites from three replicates of untreated control, chlorhexidine treated, and THAM trisphenylguanide treated MRSA. The peak of interest is in the red box.

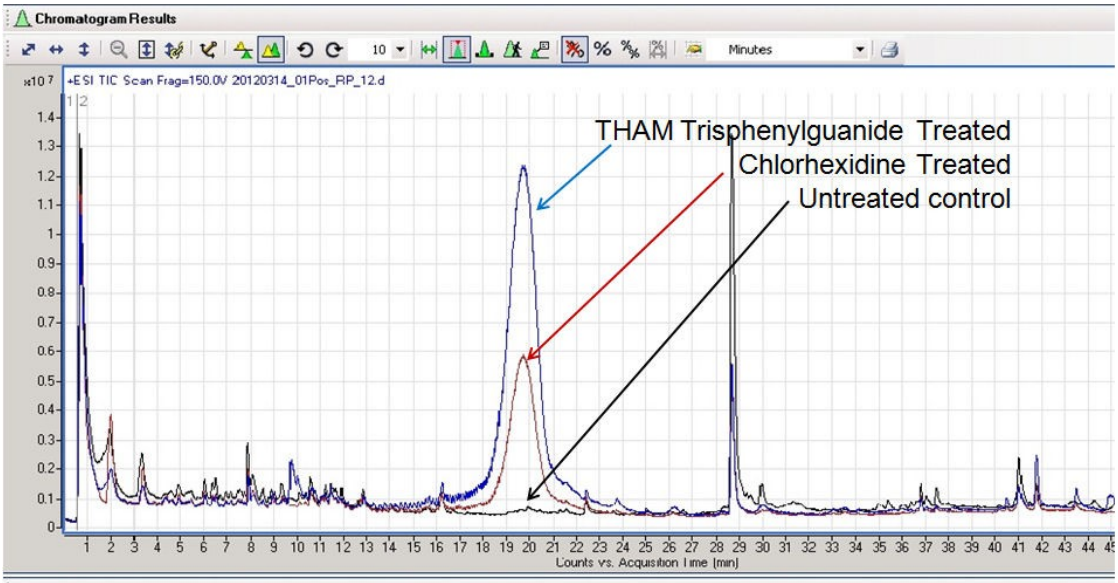


Figure 8.2 Overlaid Chromatograms from one replicate of each sample for comparison.

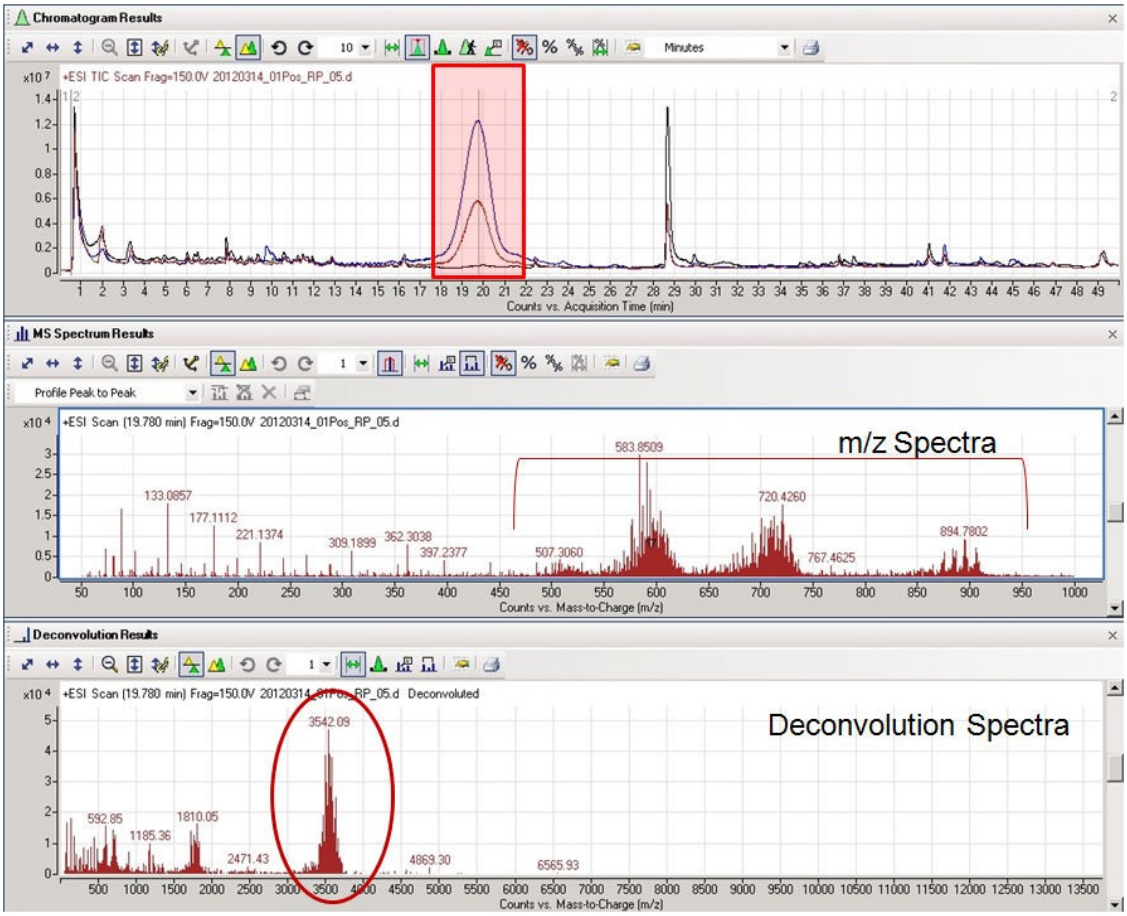


Figure 8.3 Mass Analysis of the peak of interest. Top: overlaid peaks of interest, middle: the m/z masses found in that peak of interest, bottom: deconvolution of the m/z spectrum. Peak of interest has a molecular weight of 3500 +/- 250 Da.

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2. Diep, B.A., et al., *Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus.* Lancet, 2006. **367**(9512): p. 731-9.

APPENDIX D

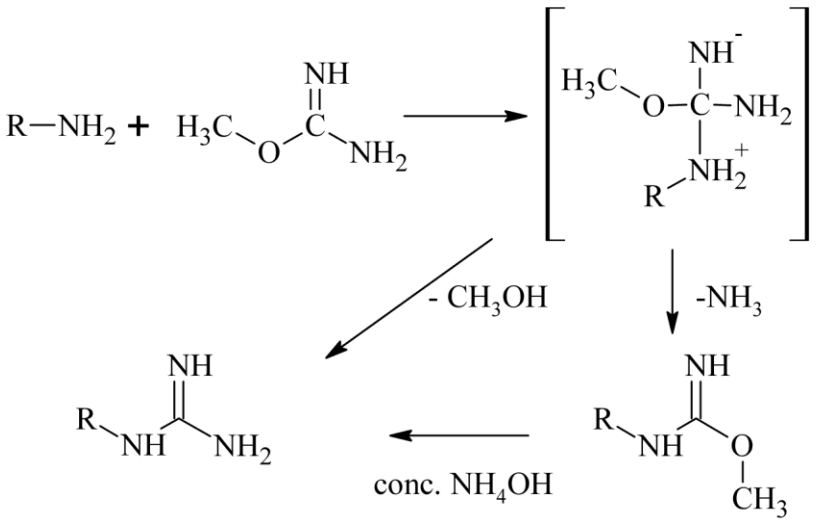
SUPPLEMENTARY MATERIAL FOR CHAPTER 2

Supplemental Table 9.1

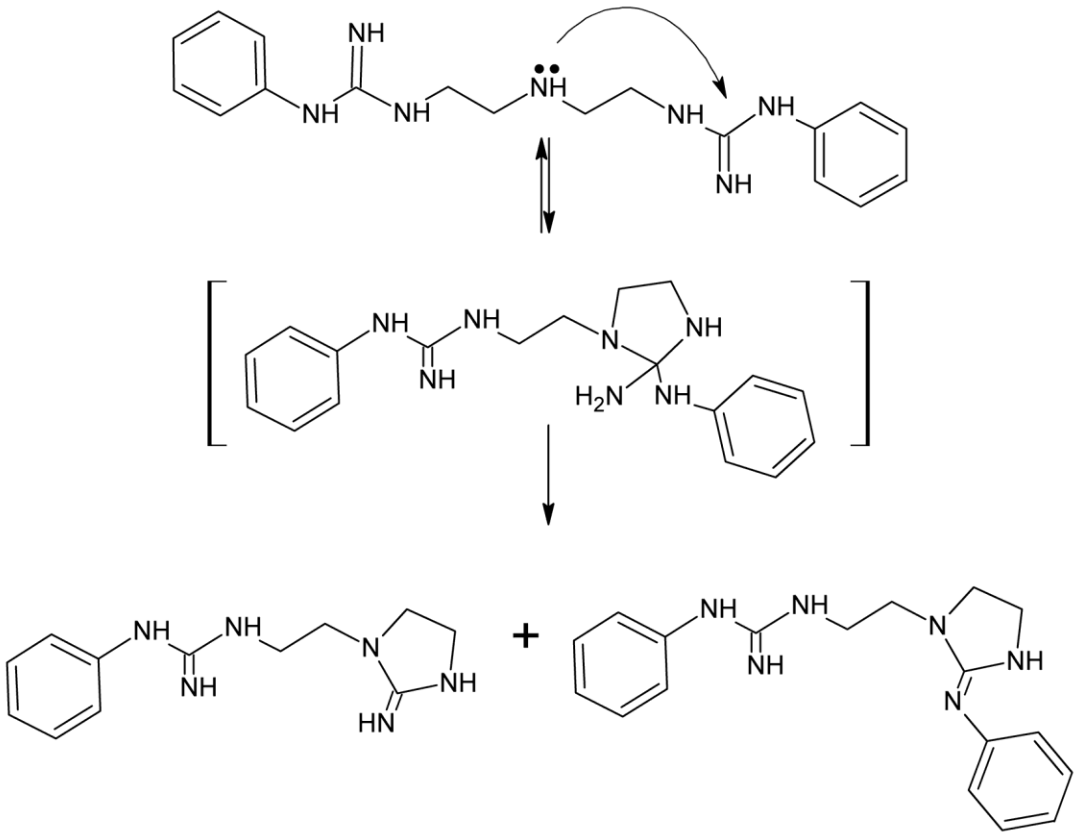
Table 1: Compound cytotoxicity against CXCR4 expressing human MDA-MB-231 cells

Compound	**CC_{50} (μM)**
Spermidine bisguanide	500
Spermidine trisguanide	500
Spermine bisguanide	20
TEAM trisguanide	ND
TBAM trisguanide	1000
THAM trisguanide	75
PAMAM G0 tetraguanide	> 100
DNT2300 hexaguanide	> 50
DNT2200 octaguanide	> 50
Spermidine biguanide	100
Spermine bisbiguanide	> 50
DNT2300 biguanides (3-6)	20
Hexanediamine bisphenylguanide	> 50
DETA monophenylguanide	1000
DETA bisphenylguanide	500
spermidine bisphenylguanide	> 1000
spermidine trisphenylguanide	500
spermine tetraphenylguanide	200
TEAM trisphenylguanide	> 100
TBAM bisphenylguanide	> 1000
TBAM trisphenylguanide	200
BEMA dimer monophenylguanide	400
PAMAM G0 tetraphenylguanide	> 100
DNT2300 hexaphenylguanide	> 100
DNT2200 octaphenylguanide	> 100
NB325**	0.03%

* CC_{50} values >x mean no toxicity was observed at the highest concentration tested (=x μM). ND=not determined. **Assuming an average MW~1kD, 0.03% would be about 300μM



Supplemental Fig. 9.1. Mechanism of formation of O-methylisourea reaction byproduct. Some reactions with O-methylisourea yielded a moderate amount (20-50%) of a +15 amu byproduct. Formation of this product can be rationalized through the zwitterionic tetrahedral SN_2 intermediate eliminating NH_3, instead of the expected CH_3OH, to give the O-methylisourea byproduct. Treatment of this compound with concentrated NH_4OH yields the desired guanide.



Supplemental Fig. 9.2. Byproducts formed during the synthesis of DETA phenylguanide. Mass signals in the MALDI analysis of the DETA phenylguanide synthesis suggest some side reactions occurred. In the proposed mechanism, a nucleophilic amine with the correct spacing to form a five-membered ring intermediate attacks the phenylguanide imine, which can either revert to the bis-phenylguanide or eliminate ammonia or aniline to give both the byproduct masses observed by mass spectrometry. Similar byproducts were not observed with reactions of any of the other starting amine compounds.

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