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Synthesis & Evaluation Of Thiophene Derivatives As CXCR4 Antagonists

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SYNTHESIS & EVALUATION OF THIOPHENE DERIVATIVES AS CXCR4 ANTAGONISTS

by

FRANCISCO GARCIA

Under the Direction of SUAZETTE REID MOORING, PhD

ABSTRACT

There is a lack of good CXCR4 inhibitors that treat cancer cell metastasis and autoimmune diseases without serious drawbacks. AMD3100 and WZ811 are the CXCR4 antagonists that were the initial structural basis for our new potential therapeutics. Molecular modeling and the principles of drug discovery and design were used to plan the synthesis of the compounds presented here. These compounds were synthesized via reductive amination, and then purified via flash column chromatography or preparatory thin layer chromatography. The small molecules were then characterized via melting point, high resolution MS, ^{13}C, and ^{1}H NMR spectroscopy. The Matrigel invasion assay and binding affinity assay were used to screen

the potency of the compounds. Compounds that showed high potency in these assays were then subjected to the carrageenan mouse paw edema test. Two lead compounds have emerged as candidates for further testing (**2v**, and **2a**).

INDEX WORDS: CXCR4, CXCL12, GPCR, Chemokine, Chemokine receptor, Cancer metastasis, Immune and inflammatory diseases, Pharmacophore, SAR studies, and AMD3100.

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ANTAGONISTS

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FRANCISCO GARCIA

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

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DEDICATION

I would like to thank Theresa Gaines for being the person I would go to the most when I had questions or problems with my research. Also, Dr. Davita Camp for her assistance in the lab and her instruction. Nikita Burrows for being there to discuss the other things in life apart from chemistry. The undergraduate students: Saniya, Damail, Gregory, Callie, and Chloe for working in the lab to further our group's research goals and to share the joy of learning. I would also like to thank my family. My father for supporting me financially and emotionally throughout my undergraduate and graduate studies. To my mother for always being caring, and someone that believed in me. Finally, my brother who helped me in any way he could. Thank you from the bottom of my heart. Sincerely, Francisco Javier Garcia Jr.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1   INTRODUCTION: THE RATIONALE FOR SYNTHESIZING SMALL MOLECULES AS CHEMOKINE RECEPTOR ANTAGONISTS

1.1   Review of Chemokines

Chemokines (like CXCL12) are small proteins that bind to seven-transmembrane G-protein coupled receptors (GPCRs) like CXCR4, which was previously known as CD184, LESTR[1] and fusin.[2] GPCRs are a large family of chemokine receptors that can be found on the cell surface and are involved in signaling for the immune and inflammatory response throughout the cell.[3] Chemokines are shown to be chemoattractants for various leukocytes, which is the vehicle for immune and inflammatory response.[2, 4] Chemokines are further classified into four subfamilies based on the placement of the conserved cysteine groups on the amino N-terminus: CC, CXC, C, and CX_3C.[5] The name chemokine comes from the word chemotaxis, which refers to compounds that attract other chemicals within the body.[5a] Cytokines are small proteins involved in the immune response, and under this classification, there are subcategories of proteins. These subcategories include lymphokines, interleukins, monokines, and chemokines.[5a] There are chemokines that are promiscuous,[6] viral,[7] specific,[8] and shared.[6] Promiscuous chemokines have multiple binding partners; for example, CXCL12 binds to both CXCR4 and CXCR7.[9] Viral chemokines are chemokines that exist within viral organisms. Specific chemokines are specific to only one binding partner. Shared chemokines may only bind within one family, but can have multiple binding partners within that family. CXCL12 and CXCR4 control various processes including cell migration during the immune response, but such a complex process has also allowed the chemokine/chemokine receptor axis to contribute to the progression of various ailments such as HIV,[2] cancer,[10] and auto-immune diseases (Figure 1).[2, 11]

The large increase in the number of known chemokine receptors and their ligands has allowed for the synthesis of new antagonists to treat the various ailments using this signaling pathway.[12]

In this work, we focus on the interaction between the chemokine receptor CXCR4 and its chemokine CXCL12. The structure of CXCR4 consists of seven transmembrane helixes. Also, there are three extracellular loops (ECL1-3), and two disulfide bridges.[13] Cys274[7.25] found on helix VII forms a disulfide bond with Cys28 in the N-terminal, Cys109[3.25] forms a second disulfide bond with Cys186 on ECL2, which shape the binding pocket and are important in ligand binding.[13-14]

The globular domain of the chemokine CXCL12 is believed to bind to the CXCR4 N-terminus, and the three extracellular loops, which forms an interaction site that gives it affinity and specificity via a conformational change.[13, 15] The N-terminus of the CXCL12 chemokine is then thought to enter the helical bundle of the chemokine receptor shown in green on CXCR4 to activate the majority of receptor signaling.[16] Thus, there is a two site binding model for the chemokine CXCL12 binding to the chemokine receptor CXCR4.[16a] However, blocking the CXCL12 N-terminus from binding to the helical bundle of CXCR4 with a small molecule antagonist does not completely block activation.[15] The CXCL12 globular domain is still able to bind to the CXCR4 N-terminus and three extracellular loops at the top of the protein.[17] Some activity is still required for important biological processes like the inflammatory and immune response. Therefore, an inhibitor that completely blocks the CXCL12 CXCR4 axis is not an acceptable outcome.
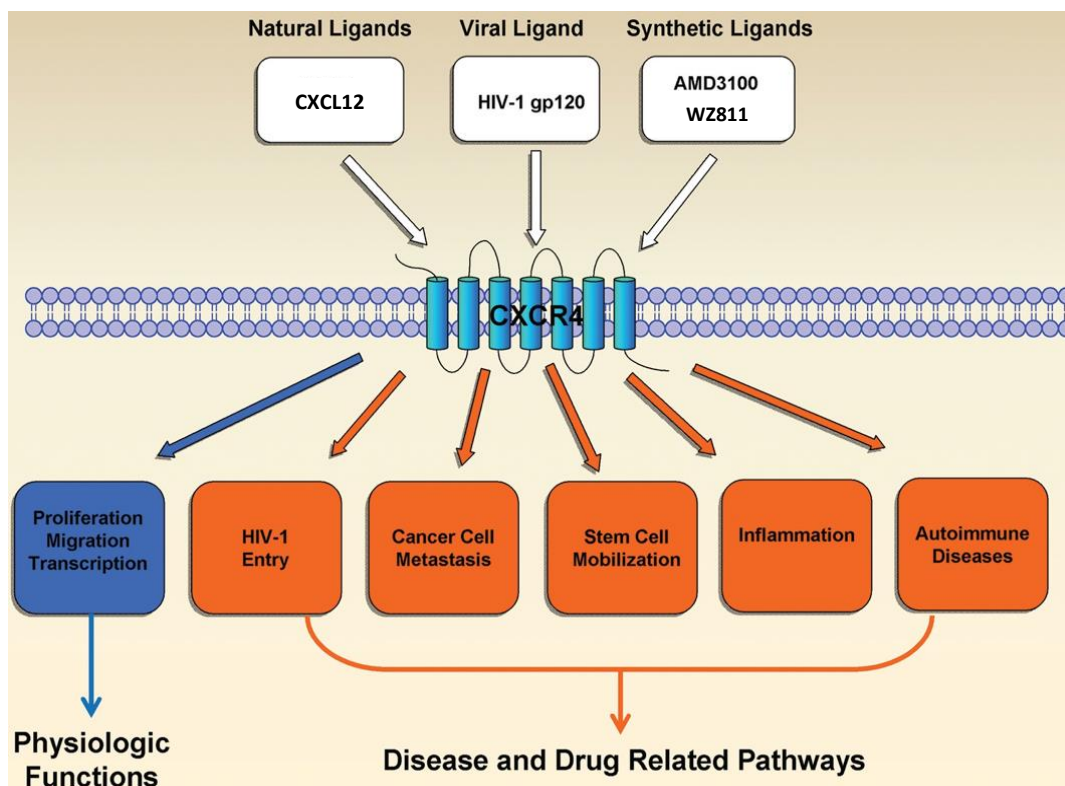
Figure 1: Diagram of various processes regulated by CXCR4. Adapted with permission from the American Chemical Society. Choi, W.-T.; Duggineni, S.; Xu, Y.; Huang, Z.; An, J., Drug Discovery Research Targeting the CXC Chemokine Receptor 4 (CXCR4). *Journal of Medicinal Chemistry* **2011,** *55* (3), 977-994.[18]

CXCR4 is integral to survival. Knockout mice without CXCR4 died *in utero* due to a heart defect, defective vascular development, defective cerebellar neuronal layer formation, and defects in haematopoiesis.[19] The CXCL12-CXCR4 binding axis initiates a cascade of downstream pathways (Figure 2) that include: calcium signaling via the release of intracellular calcium,[20] mobilization of inflammatory and immune cells,[20a] promotion of cell survival,[21] stem cell migration,[22] gene transcription,[11b] and cell adhesion.[11b, 23] Through these pathways, CXCR4 activation is responsible for the processes involved in HIV-1 entry,[24] autoimmune and inflammatory diseases,[25] and tumor cell metastasis.[26] Below is a brief description of the role of the CXCL12-CXCR4 axis in HIV-1, autoimmune and inflammatory diseases, and cancer metastasis.

1.2   CXCR4 and HIV-1

CXCR4 is an entry cofactor for T-cell line tropic (TCL) HIV-1 along with CCR5 and CD4.[2] CXCR4 is involved in the later stages of HIV-1 progression as a coreceptor. It has been shown that even at low levels, CXCR4 still allows for significant chemotaxis of naïve T cells like CD4 but levels of CXCR4 rise early on during HIV-1 invasion.[27] The initial invasion of T-tropic HIV-1 called cell fusion was shown to require both CXCR4 and CD4, while M-tropic strains of HIV used a different chemokine receptor.[24b] It was later shown that there exist CD4 independent strains of HIV-1 that also utilize CXCR4.[28]

1.3   CXCR4 and autoimmune diseases

One of the main hallmarks of Rheumatoid arthritis (RA) is activated T cells in the sera and synovial tissue and fluids of the affected region.[29] Mature T cells have been shown to more easily migrate to these regions in RA sufferers.[30] CXCR4 was linked to CD4+ memory T cell accumulation in these regions, and it was shown that IL-15, which was produced as a function of RA, increased CXCR4 expression.[4] Also, CXCL12 stimulates the migration of CD4+ T cells, and inhibits the apoptosis of T cells.[4] Transforming growth factor beta isoforms found within RA synovial fluid amplifies the expression of CXCR4, while CXCL12 allows for the retention of the T cells.[31] Of note, mice given a CXCR4 antagonist showed reduced T cell accumulation in RA areas, which validates our targeting this chemokine receptor in treating inflammatory diseases.[32]

Studies of inflammatory bowel diseases (IBD), which include Crohn's disease (CD), and ulcerative colitis (UC) have reported increased levels of CXCR4 present on cells in the affected region.[33] Lung neutrophils attracted to the lung in the case of lung damage via the immune response have the potential to induce acute lung injury (ALI), and lipopolysaccharide (LPS)-induced lung injury.[34] The problems with the immune response in ALI and LPS is that in the

later stages of these diseases the CXCR4-CXCL12 pathway promotes the migration and

retention of neutrophils, and also protects them from death, which promotes the inflammatory

response.[34-35] There was an increase in the expression of CXCL12 and CXCR4 due to LPS and

ALI.[34-35] The recruitment of various immune cells to these areas of injury or inflammation is

required for host defense, however excess activation and recruitment of these cells leads to these

inflammatory and autoimmune diseases.

1.4   CXCR4 and cancer cell metastasis

CXCR4 is just one of many G protein-coupled receptors found within the body, but

CXCR4 is of note because of its role in cancer cell invasion and metastasis, proliferation, and

angiogenesis.[11b] Cancer cell invasion and metastasis are the processes by which tumor cells

invade surrounding tissues to grow larger, and various regions far from the original tumor

respectively.[36] CXCR4 is highly expressed on many cancer cells including breast(Figure 2)[37],

lung,[37-38] and prostate.[39] In addition, organs and tissues such as the bone marrow,[40] lymph

node,[41] lung,[37] and liver[37] have high amounts of CXCL12 that promote the migration of cancer

cells that express CXCR4 (Figure 2). Research has shown that blocking the CXCL12-CXCR4

axis with anti-CXCR4 antibodies can reduce migration of breast cancer cells to the lymph nodes

and lungs of mice.[37] Also, the use of interfering RNA to silence the CXCR4 gene inhibits breast

cancer metastasis in mice (Figure 3).[42] Further methods of inhibiting metastasis were explored by

using the peptide TN14003 as a CXCR4 antagonist, the results of the inhibition can be seen in

Figure 4.[43]

ErbB2 (tyrosine kinase HER2), which is overexpressed in certain cancers was shown to

increase CXCR4 expression by assisting in synthesizing more CXCR4, and slowing the

degradation of CXCR4.[44] ErbB2 was also shown to be required for invasion, and metastasis in specific types of cancer.[44]
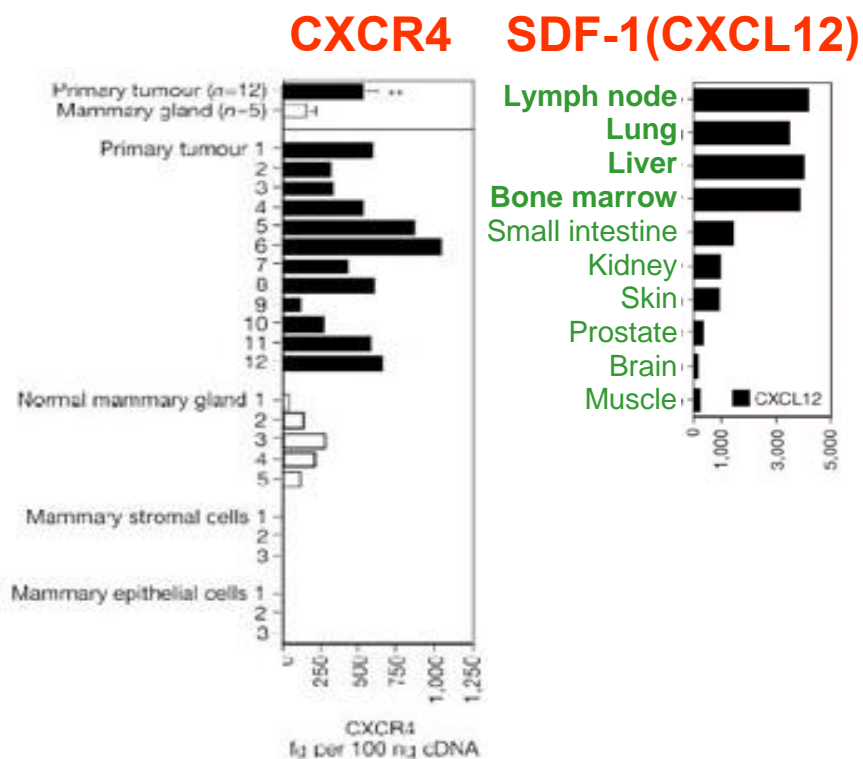
Figure 2: Quantity of CXCR4 present in breast cancer cells, and CXCL12 present in common metastasis sites. Adapted by permission from Macmillan Publishers Ltd. Muller, A.; Homey, B.; Soto, H.; Ge, N.; Catron, D.; Buchanan, M. E.; McClanahan, T.; Murphy, E.; Yuan, W.; Wagner, S. N.; Barrera, J. L.; Mohar, A.; Verastegui, E.; Zlotnik, A., Involvement of chemokine receptors in breast cancer metastasis. Nature **2001,** 410 *(6824), 50-56.[37]*
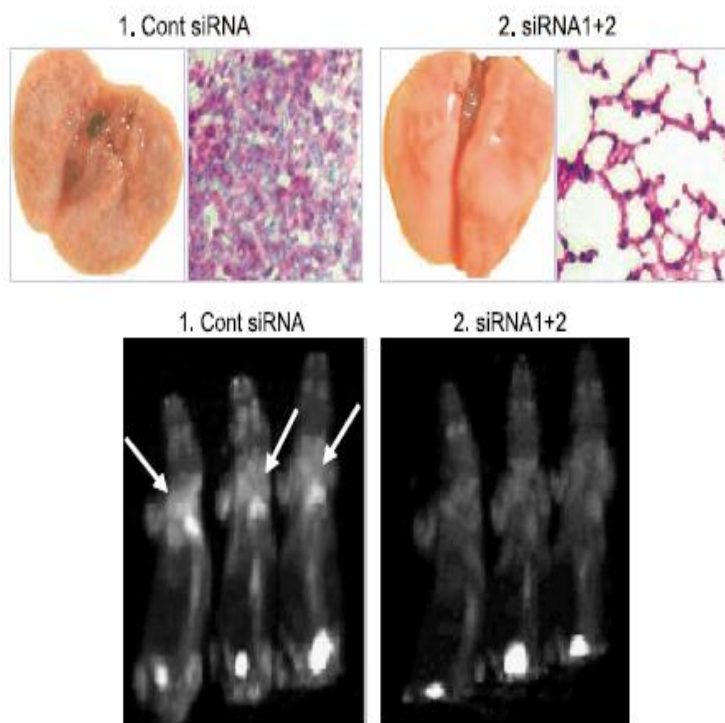
Figure 3: Image of the effect of siRNA blocking CXCR4 function on metastasis, and the control. Adapted by permission from AACR Publications. Liang, Z.; Yoon, Y.; Votaw, J.; Goodman, M. M.; Williams, L.; Shim, H., Silencing of CXCR4 Blocks Breast Cancer Metastasis. Cancer research **2005,** 65 *(3), 967-971.[42]*
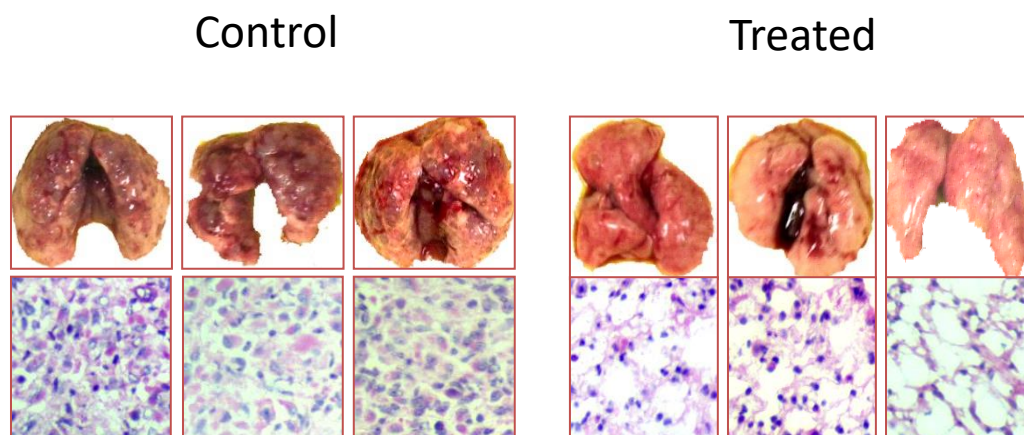
Figure 4: Image of the effect of TN14003 blocking CXCR4 on metastasis, and the control. Adapted by permission from AACR Publications. Liang, Z.; Wu, T.; Lou, H.; Yu, X.; Taichman, R. S.; Lau, S. K.; Nie, S.; Umbreit, J.; Shim, H., Inhibition of Breast Cancer Metastasis by Selective Synthetic Polypeptide against CXCR4. Cancer Research **2004,** 64 *(12), 4302-4308.[43]*

1.5   Antagonists of CXCR4

There are many routes that chemists can take to synthesize CXCR4-CXCL12 pathway

antagonists. One route is to generate synthetically and modularly modified (SMM) chemokines

from the natural ligands, which in our case would be CXCL12.[45] These unnatural chemokines may inhibit diseases that utilize CXCR4 as a point of entry, but some of these unnatural chemokines would have a lower binding affinity than CXCL12, which would allow one to target only the disease without significant side effects.[45-46] There are also drugs that are made to mimic the structure of proteins found in diseases that bind to an active site (CXCR4) like in the case of the peptide ALX40-4C (Figure 5), which inhibits HIV-1 infection.[46-47] Another route is to take existing, or tentative treatments and modify their structure to obtain better activities like the CXCR4 peptide based antagonist T22 being used as a basis for T140 (Figure 5).[48]

Peptides are an important class of CXCR4 antagonists. One such peptide, T140 (Figure 5) is a 14-residue peptide whose residues Arg2, Nal3, Tyr5, and Arg14 were essential to its activity was elucidated using an alanine scanning study.[49] Structure activity relationship (SAR) studies of a known pharmacophore resulted in the development of cyclic pentapeptides like FC131[50](Figure 5), which led to additional peptide CXCR4 antagonists including T22[49] and TN14003 (Figure 5).[51] From SAR and conformational studies of cyclic pentapeptide based CXCR4 antagonists, indole (Figure 6) derivatives were designed and synthesized.[52] The SAR studies of these cyclic pentapeptide derivatives revealed that a phenyl group, one or two guanidino groups, and a phenol group - if only one guanidino was present – were required for CXCR4 antagonistic activity.[52]

ALX40-4c
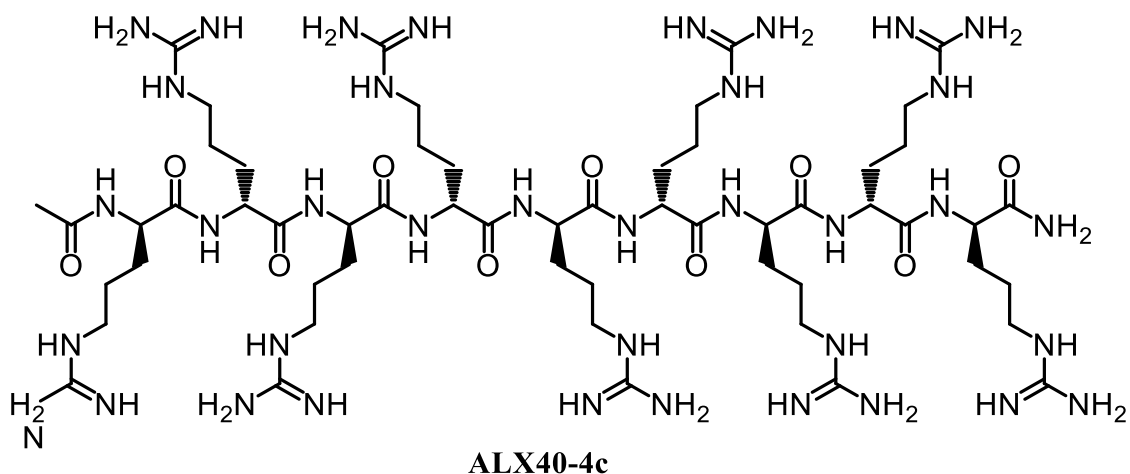
H-Arg-Ala-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH

T140

FC131

TN14003
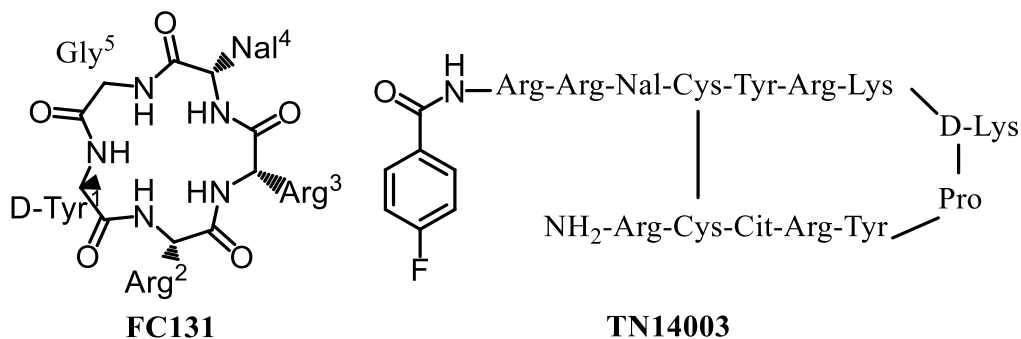
H-Arg-Arg-Trp-Cys-Tyr-Arg-Lys-Cys-Tyr-Lys-Gly-Tyr-Cys-Tyr-Arg-Lys-Cys-Arg-NH$_2$

T22

Figure 5: Structure of various peptide CXCR4 antagonists.

Although there are peptides that are potent CXCR4 antagonists, their major flaw is poor oral bioavailability. Therefore, researchers pursued the synthesis of small molecules that can overcome this challenge. Bicyclams and their derivatives represent the first class of small molecule CXCR4 antagonists. The most widely known bicyclam is AMD3100, (Figure 6) which was found to be effective against HIV-1 by binding to CXCR4.[53] AMD3100 has several drawbacks including poor bioavailability and cardiotoxicity.[54] However, AMD3100 has since

been FDA approved for one time use for stem cell recruitment in cancer patients.[55] Other analogs

of AMD3100 were synthesized and determined that some of the nitrogens on AMD3100 were

redundant, and that the overall charge at physiological pH does not directly affect CXCR4

antagonism.[56] To improve the characteristics of AMD3100, non-cyclam derivatives such as

AMD070 (Figure 6) that is a potent orally available CXCR4 antagonist against HIV were

discovered.[57] This class of drugs was further examined and yielded IT1t (Figure 6) after library

screening.[58]

Tetrahydroquinoline based CXCR4 antagonists were first explored utilizing the lead

AMD070 (Figure 6).[57] One group later modified these tetrahydroquinolines with various rings

that our group is trying to utilize as the core structure[59]. Further modifications involved

constraining the ring[60], and the replacement of benzimidazole to discover alternative ring

systems.[61] WZ811 (Figure 6) is a lead compound that was synthesized from a series of SAR

studies starting with the removal of the bicyclam rings of AMD3100.[62] It was learned that the

central aromatic ring is critical for binding, and the central phenyl ring and the nitrogen of the

linker should only be one carbon apart.[62] From their terminal phenyl rings electron donating

substituents at the para position had lower EC50 values than electron withdrawing substituents

while either electron withdrawing or electron donating substituents had no discernible trend.[62]

The addition of a second nitrogen to the terminal aromatic rings on WZ811 yielded MSX122

(Figure 6). [54b] Many more potential CXCR4 antagonists were synthesized using the structure of

WZ811, and AMD3100 as a basis for modification.[63] An initial study done on guanidine based

CXCR4 antagonists yielded positive results that merited further study, particularly of

phenylguanides (Figure 6).[64] Library screening using structural modeling of CXCR4 indicated

that some quinolines (Figure 6) could be CXCR4 antagonists.[65] A library screening and then

modification of the lead compound yielded KRH1636 [66], further optimization yielded

KRH3955[67] (Figure 6). SAR studies of KRH1636 were done assist in the further development of

this class of compounds.[68] Patents of compounds containing spiro-bonded cyclic groups[69], and

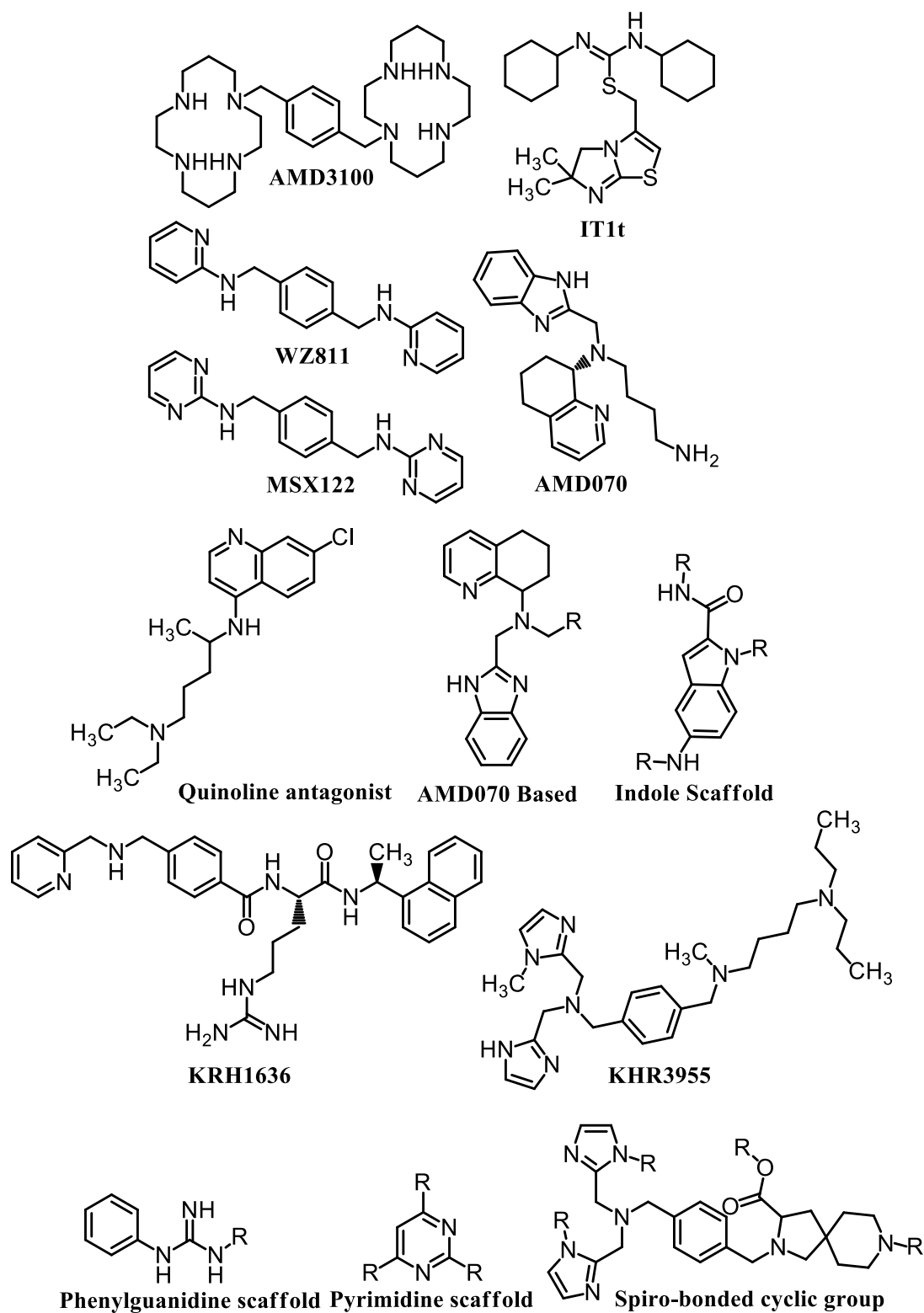pyrimidines[70] (Figure 6) were filed as CXCR4 antagonists as well.

Figure 6: Small molecule CXCR4 antagonists.

1.6   Design and Methodology of Drug Discovery

Drug modification is a field of medicinal chemistry that alters the chemical makeup of known and potential therapeutics to make it a more effective treatment. Homologation is a modification where carbon chains can be lengthened to one, five, or nine to allow the compound entry into the cell membrane.[71] The downside to homologation is that the increase in lipophilicity may prevent it from being water soluble[71]. Chain branching is another modification that adds branching to carbon chains.[71] The branching can potentially prevent it from binding to the target receptor or allow it to bind better.[71] Ring chain transformations allow for more stability, increase lipophilicity, and lock the structure in place.[71] Bioisosterism is the replacement of various substituents with groups that have been found to have similar physicochemical properties.[72] Modifications may create changes in conformation, size, shape, and hydrogen bonding that are all structural effects.[71] Absorption, transport, excretion of the compound, lipophilicity, hydrophilicity, pKa, and hydrogen bonding are a part of pharmacokinetic effects.[71] Blocking, aiding metabolism, chemical reactivity, and toxicity are metabolism effects.[71] Finally, changes to receptor binding interactions are the result of various modifications.[71]

The cytoplasm is an aqueous environment, but the cell membrane is a lipophilic environment. When pH and the pKa are the same 50 % of the drug is ionized, and 50 % is neutral. Therefore, when the compound enters the cell only the neutral compound can enter, and the compound in the cell becomes 50 % ionized and 50 % neutral.[71] The pH inside and outside the membrane will affect the desired pKa of the compound.[71] As for modifications, electron withdrawing groups will lower the pKa, and electron donating groups will raise the pKa. This means that electron withdrawing groups will make acids more ionizable and bases less ionizable. Electron donating groups will make bases more ionizable and acids less ionizable.

The rule of five is a list of five properties that give a greater than 90 % chance of poor oral absorption or bioavailability if two or more of these properties are present.[71, 73] These properties include a compound with a molecular weight no more than 500 g/mol, a log P no greater than five, no more than five hydrogen bond donors expressed as the sum of -OH and -NH groups, and no more than 10 hydrogen bond acceptors expressed as the sum of N and O atoms[73]. Silverman expresses that three hydrogen bond donors and six hydrogen bond acceptors should be the upper limit.[71] Veber and coworkers found that reduced flexibility (10 or fewer rotatable bonds), and a low polar surface area (less than or equal to 140 angstroms squared), a total hydrogen bond count less than or equal to a total of 12 donors and acceptors are important predictors of good oral bioavailability independent of molecular weight.[74] Ajay and coworkers developed screening criteria that found that if the molecular weight, degree of branching, number of rotatable bonds, or the number of hydrogen bond acceptors were increased the compound will be less likely to be central nervous system active.[75] In contrast, if the aromatic density, number of hydrogen bond donors, or log P were increased it would be more likely to be active in the central nervous system.[75]

In this thesis, we detail the methods, and design of potential CXCR4 antagonists. The design of these antagonists was based on AMD3100 and WZ811, which are potent CXCR4 antagonists.[63a] AMD3100 has also shown that inhibition of CXCR4 can help restore the immune system after chemotherapy by dispersing hematopoietic stem cells from the bone marrow.[18, 76] AMD3100 has been shown to be useful for one-time use, but clinical trials resulted in patients exhibiting cardiotoxic side effects.[77] AMD3100 and WZ811 also have poor bioavailability, which drives the need for better CXCR4 antagonists.[18, 63a] Attempts have been made at altering the substituents around the central phenyl ring of WZ811. Additional SAR studies also show that

an aromatic central ring is required for its activity. Our group has been focused on substituting

the central phenyl ring with other heterocyclic aromatic rings such as pyridine rings[78], furans,

and pyrazine. This thesis work will focus on using a Thiophene ring (Figure 7) as the core while

altering the substituents.[62] The synthesized compounds were subjected to a Matrigel invasion

assay and a binding affinity assay. These two biological assays quantified how well the

antagonist blocks the receptor, and the quantity of the antagonist required to effectively bind to

the receptor, respectively.[79] Carrageenan paw edema studies were then conducted with the most

potent compounds according to the assay results. The lab of Dr. Hyunsuk Shim at Emory

University School of Medicine conducted the biological work.
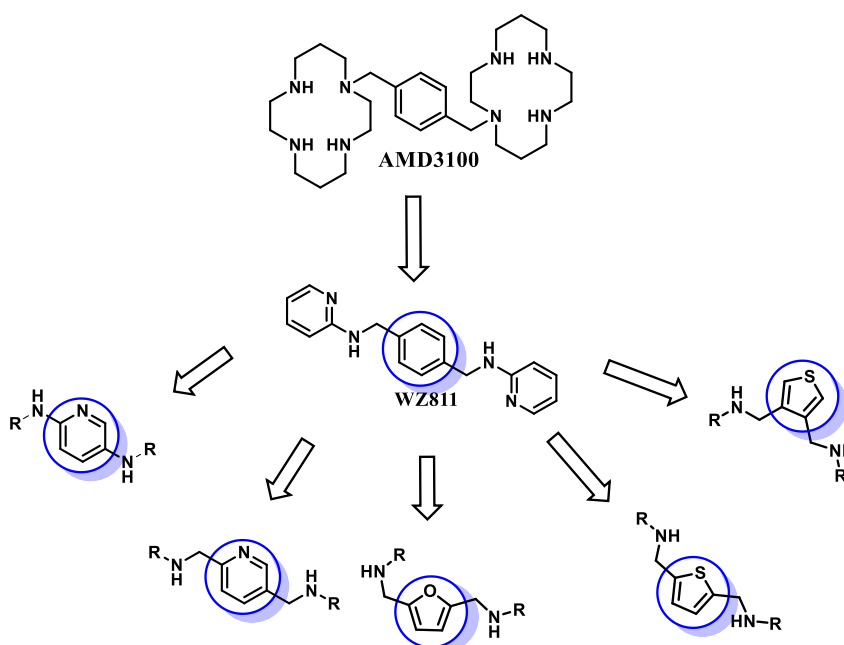

Figure 7: structure of previous CXCR4 antagonists (AMD3100, and WZ811), and the structure of potential CXCR4 antagonists based on altering its central core and substituents.
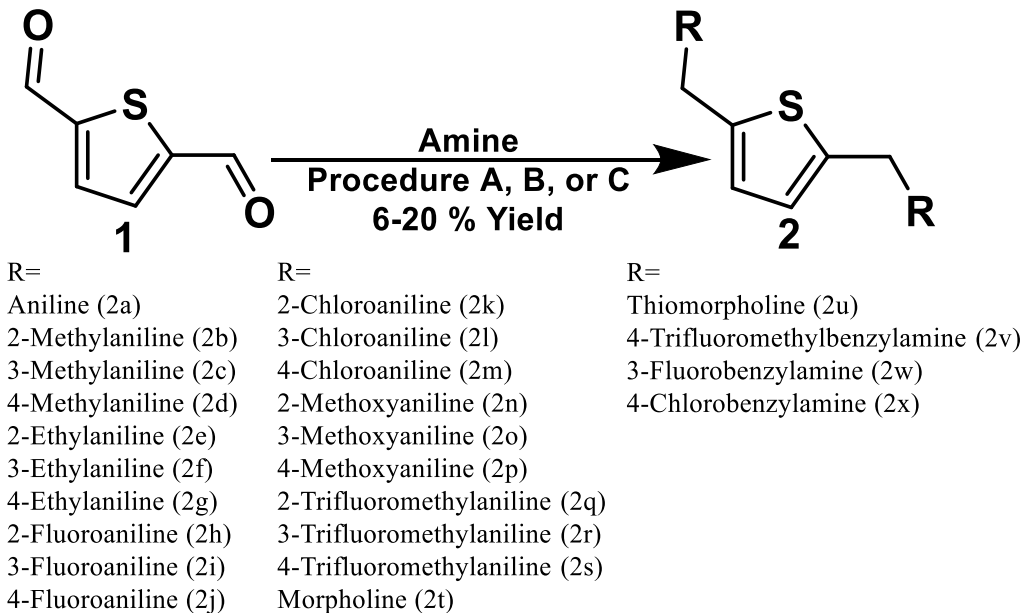
2   DISCUSSION

2.1   Chemistry

The Thiophene analogs were synthesized using reductive amination procedures of

Thiophene-2,5-dicarboxaldehyde with the corresponding primary or secondary amine. Three

different methods for the reductive amination procedure were employed depending on the primary or secondary amine being used.

Method A involved the use of acetic acid as a catalyst and $NaBH(OAc)_3$ as the reducing agent (Yield 3 – 26 %). With Method B, the amine and Thiophene-2,5-dicarboxaldehyde are first reacted at room temperature to form the imine, then $NaBH_4$ is used to reduce the imine to the final amine compound (Yield 6 – 20 %). Method C was the same as Method B, however the dicarboxaldehyde and the amine are heated under reflux conditions to promote a reaction. The reactions that required the use of reflux conditions used secondary amines that were less reactive like Piperidine that did not have an aromatic group. Amines that had a higher pKa and were more sterically hindered than other amines like aniline required reflux conditions to go to completion. However, some reactions did not go to completion even after using reflux conditions. Method B was used to try to get a better yield by forming the imine first and then adding the reducing agent to prevent the reduction of the aldehyde to an alcohol.

R=
Aniline (2a)
2-Methylaniline (2b)
3-Methylaniline (2c)
4-Methylaniline (2d)
2-Ethylaniline (2e)
3-Ethylaniline (2f)
4-Ethylaniline (2g)
2-Fluoroaniline (2h)
3-Fluoroaniline (2i)
4-Fluoroaniline (2j)

R=
2-Chloroaniline (2k)
3-Chloroaniline (2l)
4-Chloroaniline (2m)
2-Methoxyaniline (2n)
3-Methoxyaniline (2o)
4-Methoxyaniline (2p)
2-Trifluoromethylaniline (2q)
3-Trifluoromethylaniline (2r)
4-Trifluoromethylaniline (2s)
Morpholine (2t)

R=
Thiomorpholine (2u)
4-Trifluoromethylbenzylamine (2v)
3-Fluorobenzylamine (2w)
4-Chlorobenzylamine (2x)

*Scheme 1: Synthesis of 2,5-thiophene compounds **2**a-x. These products were synthesized via two different procedures. This list shows the substituents that have been synthesized with this core using aniline (**2a-s**), and cyclic amino (**2t-u**) benzylamine (**2v-x**) derivatives.*

2.2   Biology

2.2.1   *Binding Assay*

The binding affinity assay was used as the initial assay to screen potential CXCR4 antagonists (Figure 8). The MDA-MB-231 breast cancer cells were incubated with the compounds at a concentration of 1, 10, 100, and 1000 nM. The cells were then incubated with a biotinylated peptide TN14003 (a known CXCR4 inhibitor), followed by streptavidin-rhodamine. The fluorescence of the cells was measured to obtain effective concentration (EC), which is the lowest concentration where there is a significant reduction in fluorescence compared to the positive control. The binding affinity assay is the first assay used to screen potential antagonists, and eleven out of the twenty-four compounds synthesized went on to the Matrigel invasion assay by having an EC of 100 nM or less.

Some categories of compounds show promise in the binding assay (Table 1), and are features that will be repeated in other analogs to synthesize better potential antagonists. This includes benzene groups with substituents on the ortho and para position over substituents on the meta position. A phenyl ring with no substituents did well in the binding affinity assay (**2a**). The 2-Chloro, 3-Chloro, and 4-Chloro substituted anilines all did well (**2k**, **2l**, and **2m**). 2-Methyl, 4-Methyl, 2-Methoxy, and 4-Methoxy substituted anilines also did well in the assay (**2b**, **2d, 2n**, and **2p**). Only 3-Trifluoromethylaniline, and 4-Trifluoromethylbenzylamine (**2r,** and **2v**) did well out of all the Fluorine substituted compounds. Thiomorpholine was a compound that while structurally similar to morpholine did well in the binding affinity assay (**2u**).
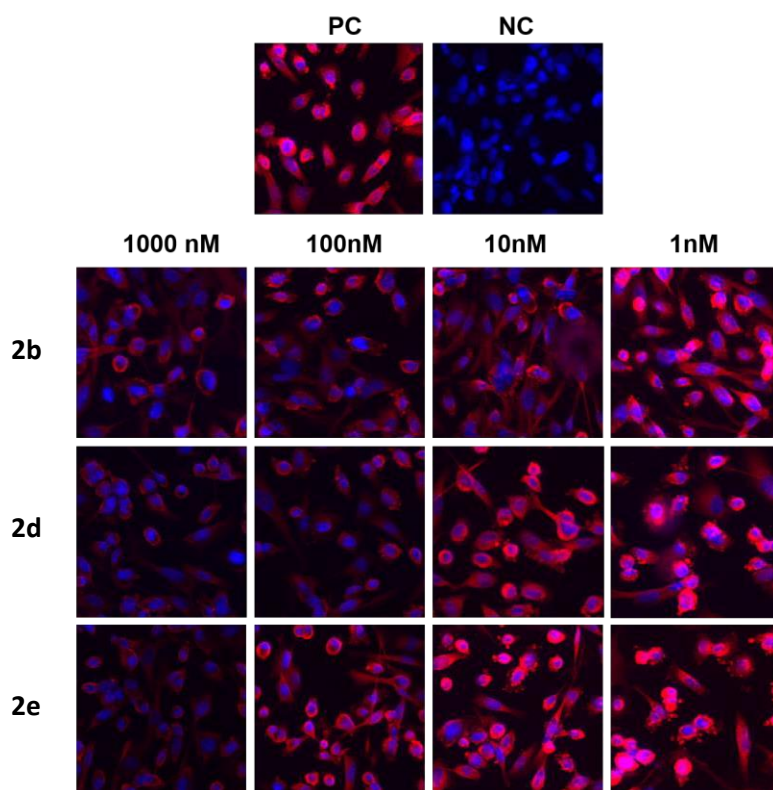

Figure 8: Reduction of inflammation observed for selected compounds. 2i had an EC of 10 nM, 2c had an EC of 100 nM, and 2r had an EC of 1000 nM. Adapted by Permission from Elsevier. Gaines, T.; Camp, D.; Bai, R.; Liang, Z.; Yoon, Y.; Shim, H.; Mooring, S. R., Synthesis and evaluation of 2,5 and 2,6 pyridine-based CXCR4 inhibitors. Bioorganic & Medicinal Chemistry **2016,** *24 (21), 5052-5060.[80]*

2.2.2   Matrigel Invasion Assay

The Matrigel invasion assay was used to indicate if the potential antagonists can block CXCR4/CXCL12 mediated chemotaxis and invasion. Compounds that had an effective concentration of 100 nM or better in the binding assays went on to this Matrigel invasion assay. MDA-MB-231 cells that have been incubated in 100 nM concentrations of the analogs were placed in the top of a special two chambered apparatus, and CXCL12 was placed at the bottom as a chemoattractant. In between the two chambers was a Matrigel matrix that the cancer cells can pass through, and the measurement obtained was the percentage of inhibition of chemotaxis (cells that did not go through the Matrigel matrix compared to the negative control). Once complete the number of cells that migrated was counted. The better inhibitors allowed fewer cells to cross the matrix.

AMD3100 (1000 nM and 62 %)[54b, 81], and WZ811 (10 nM and 90 %)[63a] were used as a benchmark to select the best potential antagonists. Compounds that had an inhibition greater than 50 % were considered favorable and selected for carrageenan paw edema test. Six compounds out of the twenty-four achieve this, and a loose trend appears to favor substituents on the ortho and para position (Table 1). Four compounds, 2-Chloroaniline, 4-Methoxyaniline, Thiomorpholine, and 4-Trifluoromethylbenzylamine, had an inhibition greater than 80 % (**2k, 2p, 2u,** and **2v**).

2.2.3   In vivo carrageenan paw edema test

The mouse paw edema test will be used to determine if these analogs can disrupt the CXCR4-CXCL12 interaction *in vivo* by reducing induced inflammation.[54b, 63a] This test was performed on compounds that did well in the binding affinity, and Matrigel invasion assay. This test may also be used to gain some insight into the toxicity of these compounds based on if a

mouse becomes weak or dies from the potential antagonist. The results of this test can be seen in

Table 2.

3   RESULTS

3.1   Assay Results

Table 1: Results of Matrigel invasion assay, and Binding affinity assay results for all thiophene analogs synthesized. The invasion assay concentration used was 100 nM.

Compound	EC (nM)	Inhibition (%PC)	R=
2a	1	68	Aniline (H)
2b	10	61	2-Methylaniline
2c	>1000	--	3-Methylaniline
2d	100	49	4-Methylaniline
2e	1000	--	2-Ethylaniline
2f	>1000	--	3-Ethylaniline
2g	>1000	--	4-Ethylaniline
2h	1000	--	2-Fluoroaniline
2i	>1000	--	3-Fluoroaniline
2j	>1000	--	4-Fluoroanilne
2k	10	93	2-Chloroaniline
2l	100	51	3-Chloroaniline
2m	1	22	4-Chloroaniline
2n	10	52	2-Methoxyaniline
2o	1000	--	3-Methoxyanline
2p	10	84	4-Methoxyaniline
2q	>1000	--	2-Trifluoromethylaniline
2r	100	77	3-Trifluoromethylaniline
2s	>1000	--	4-Trifluoromethylaniline
2t	>1000	--	Morpholine
2u	1	88	Thiomorpholine
2v	1	97	4-Trifluoromethylbenzylamine
2w	>1000	--	3-Fluorobenzylamine
2x	>1000	--	4-Chlorobenzylamine

Table 2: Result of in vivo carrageenan paw edema test.

Compound	% Inhibition	SEM (Error bar)	R=
2a	30.36	5.56	Aniline
2v	22.62	4.78	4-Trifluoromethylbenzylamine

These three biological evaluations were used to determine the viability of these compounds as small molecule CXCR4 antagonists, and to find potential trends for the design and synthesis of future compounds. The binding affinity assay was the preliminary screen for potent compounds, the Matrigel invasion assay was important to see if our compounds can inhibit chemotaxis, and the carrageenan paw edema test gave us an idea of the activity of our compound *in vivo*. From these data, several hit compounds were discovered and selected for carrageenan induced paw edema test.

Eleven of the compounds had a good binding affinity result, and they are listed here from best to worst: **2u**, **2v**, **2m**, **2a**, **2b**, **2p**, **2k**, **2n**, **2d**, **2l** and finally **2r**. Those eleven compounds then had their Matrigel invasion results obtained, and they are listed here from best to worst: **2v**, **2k, 2u, 2p, 2r**, **2a**, **2b**, **2n**, **2l**, **2d**, and **2m**. Compounds that have a substituent at either the ortho or para position tended to be more active in these assays. The final assay indicates that aniline and 4-Trifluoromethylbenzylamine (**2a,** and **2v)** are currently the best candidates for further analysis. The only exceptions are **2a, 2u, 2l**, and **2r**. The final assay indicates that **2v**, and **2a** would be good candidates for further analysis. 4-Chloroaniline (**2k**) also needs to be submitted for the carrageenan paw edema test.

4   CONCLUSIONS

Aniline was one of the best compounds in the carrageeanan mouse paw edema test and gave rise to some questions. While 4-Trifluoromethylbenzylamine (**2v)** had a higher score on the

percent inhibition of invasion at 97 % than aniline **(2a)** at 68 %, aniline was the best compound in the carrageenan paw edema test out of the aniline based substituents tested. This leads to the idea that the substituents on the aniline yield reduced activity. It is possible that the steric effects of the substituents hinder the compound from binding to the active site of CXCR4. This was supported by the structure of 4-Trifluoromethylbenzylamine **(2v)** when compared to other anilines, the 4-Trifluorobenzylamine **(2v)** added an extra carbon between the aromatic phenyl ring and the amine which gave it more free rotation so it would be less sterically hindered by the phenyl ring. Aniline is less sterically hindered around the phenyl ring when compared to those that have substituents on the phenyl ring. 2-Trifluoromethylanilne, 3-Trifluoromethylaniline, and 4-Trifluoromethylanilne **(2q, 2r,** and **2s)** are only different from the 4-Trifluoromethylbenzylamine (**2v**) by having one extra carbon separating the phenyl ring and the amine. However, 3-Trifluoromethylaniline **(2r)** had an EC of 100 nM and 77 % inhibition of invasion. This gives credit to the idea that the substituents impart some steric hindrance with the binding to the active site, but also potential electronic differences depending on the substituent's position that make the compound better able to bind to the pocket. Molecular docking and more benzylamines are needed to confirm if this greater flexibility assists the compound in its binding to the active site of CXCR4. Nonaromatic cyclic chains in sizes smaller than aniline could help determine if steric hindrance of the amine is a detriment, while chains closer to the size of aniline would determine if a lack of steric hindrance in this region would improve binding. Also, consider that the phenyl ring was said to be vital to binding. The amines that need to be used to further explore the activity of these antagonists include cyclic amines, Trifluoromethylbenzylamines, Methylbenzylamines, and Benzylamine. These compounds would help in determining how steric and electronic differences affect binding for the Thiophene

pharmacophore. The nonaromatic cyclic amines that have already been tried include Morpholine (**2t**), which did not do well, and Thiomorpholine (**2u**), which had an EC of 1 nM and 88 % inhibition of invasion. Thiomorpholine did not have as much steric hindrance as substituted anilines, which supports the idea that reducing steric hindrance around those rings is integral. Sulfur is bigger than oxygen and less electronegative. Since there is no resonance possible in this ring, this one atom difference is what makes this a better compound in the binding affinity assay.

When it comes to the aniline based compounds, it is possible that substituents at the three position structurally hinder binding to the active site of CXCR4. The Methyl and Methoxy substituted compounds, which are electron donating groups bind effectively at the ortho and meta position. However, they do not effectively bind to CXCR4 when they are at the meta position. The Ethyl substituents, which are even more bulky than the Methyl groups do not do well at any position. Likely due to steric effects since methyl has similar electronic effects. Fluoro substituted compounds did not do well, and it can be reasoned that the electronic effects were the biggest problem since Fluorine is not very bulky. The Trifluoromethyl substituted anilines did not do well at the ortho and para position. This is very interesting since at these positions the Trifluoromethyl would be a deactivator that would pull electrons away from the amine. The 3-Trifluoromethyl substituted aniline **(2r)** would not pull electrons from the amine as much, and it is possible that it would then allow the amine to better interact with the active site. The results of the biological evaluations appear to indicate that pulling electrons away from the amine of the potential antagonists hinders binding to CXCR4. This is supported by the 2-Methyl and 4-Methyl substituted anilines that would donate electrons towards the amine, while the 3-Methyl would not. This is also true of the methoxy substituted anilines. The Chlorine substituted anilines all did well, but the 3-Chloroaniline did the worst. This was likely due to its inductive

effects and resonance effects allowing it to do well in all positions. More bulky halogens would possibly introduce detrimental steric effects, but would also reduce the electronegativity, which may have a good outcome.

Synthesis of new potential CXCR4 antagonists will continue to create a sufficient library from this core. Characterization was accomplished via mass spectrometry and NMR. There are binding affinity and Matrigel invasion assays that still need to be completed. The hit compounds (**2a**, and **2v**) will also undergo further analysis to determine their suitability as drug candidates. Also, compounds that had a Matrigel invasion greater than 50 % will be tested via the paw edema test. The trend of the data available indicates that substituents at the ortho and para position on the Thiophene core are the best CXCR4 antagonists barring the exceptions **2a**, **2l**, **2r**, and **2u**.

5   EXPERIMENTAL

Procedure A: To a solution of Thiophene-2,5-dicarboxaldehyde (60 mg, 0.4281 mmol) in DCE (4.3 mL) was added an amine (2.3 equivalents), and acetic acid (0.05 mL, 0.8562 mmol) The solution was stirred for 5 min at room temperature, and then treated with NaBH(OAc)$_3$ (273 mg, 1.2843 mmol). The solution was then stirred from between 5 hrs and overnight. The product was then purified via flash column chromatography, or preparative thin layer chromatography.

Procedure B: To a solution of Thiophene-2,5-dicarboxaldehyde (60 mg, 0.4281 mmol) in Methanol (4.3 mL) was added an amine (2.0-2.3 equivalents). The solution was stirred for 1 h at room temperature, and then treated with NaBH$_4$ (48.6 mg, 1.2843). The solution was then stirred overnight. The product was then purified via flash column chromatography, or preparative thin layer chromatography.

Procedure C: To a solution of Thiophene-2,5-dicarboxaldehyde (60 mg, 0.4281 mmol) in Methanol (4.3 mL) was added an amine (2.0 equivalents). The solution as stirred 5-10 h under reflux conditions. It was then treated with NaBH$_4$ (48.6 mg, 1.2843 mmol). The solution as stirred overnight at room temperature. The product was then purified via flash column chromatography, or preparative thin layer chromatography.

The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained on a Bruker Ac 400 FT NMR spectrometer in CDCl$_3$, the Mass spectra were obtained on a JEOL spectrometer.

2,5-Bis(anilinomethyl)thiophene (2a) This product was obtained in 26 % yield as an off white solid; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.17 (t, J = 7.83 Hz, 4H), 6.83 (s, 2H), 6.73 (t, J = 7.33 Hz, 2H), 6.65 (d, J = 7.83 Hz, 4H), 4.42 (s, 4H); ^{13}C NMR (100 MHz, Chloroform-d) δ 147.53, 142.19, 129.26, 124.75, 118.06, 113.12, 43.64; Calcd for $C_{18}H_{18}N_2SNa$ ([M + H^{+}]): m/z 317.1087. Found: m/z 317.1088.

2,5-Bis(2-methylanilinomethyl)thiophene (2b) This product was obtained in 15 % yield; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.02 - 7.16 (m, 4H), 6.87 (s, 2H), 6.63 - 6.74 (m, 4H), 4.49 (s, 4H), 2.15 (s, 6H); ^{13}C NMR (100 MHz, Chloroform-d) δ 145.53, 142.26, 130.15, 127.11, 124.80, 122.28, 117.66, 110.14, 43.66, 17.52; Calcd for $C_{20}H_{23}N_2S$ ([M + H^{+}]): m/z 323.1587. Found m/z 323.1576.

2,5-Bis(3-methylanilinomethyl)thiophene (2c) This product was obtained in 14 % yield; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.07 (t, J = 7.58 Hz, 2H), 6.83 (s, 2H), 6.56 (d, J = 7.58 Hz, 2H), 6.39 - 6.52 (m, 4H), 4.42 (s, 4H), 2.27 (s, 6H); ^{13}C NMR (100 MHz, Chloroform-d) δ 147.59, 142.24, 139.05, 129.14, 124.71, 119.01, 113.94, 110.21, 43.67, 21.62; Calcd for $C_{20}H_{18}N_2SNa$ ([M + H^{+}]): m/z 345.1391. Found: m/z 345.1401.

2,5-Bis(4-methylanilinomethyl)thiophene (2d) This product was obtained in 24 % yield; ^{1}H NMR (400 MHz, Chloroform-d) δ 6.98 (d, J = 8.34 Hz, 4H), 6.81 (s, 2H), 6.57 (d, J = 8.34 Hz, 4H), 4.39 (s, 4H), 2.23 (s, 6H); ^{13}C NMR (100 MHz, Chloroform-d) δ 145.28, 142.34, 129.75, 127.28, 124.61, 113.27, 43.98, 20.42; Calcd for $C_{20}H_{23}N_2S$ ([M + H^{+}]): m/z 323.1576. Found: m/z 323.1576.

2,5-Bis(2-ethylanilinomethyl)thiophene (2e) This product was obtained in 14 % yield; ^{1}H NMR (500 MHz, Chloroform-d) δ 7.04 - 7.15 (m, 14H), 6.86 (s, 7H), 6.74 (t, J

= 7.34 Hz, 7H), 6.69 (d, J = 8.02 Hz, 7H), 4.48 (s, 4H), 2.49 (q, J = 7.50 Hz, 4H), 1.24 (t,

J = 7.53 Hz, 16H) ; ^{13}C NMR (100 MHz, Chloroform-d) δ 144.90, 142.33, 127.95,

126.96, 124.75, 117.84, 110.47, 43.68, 23.88, 12.88; Calcd for $C_{22}H_{25}N_2S$ ([M + H$^+$]):

m/z 349.1744. Found: *m/z* 349.1733.

2,5-Bis(3-ethylanilinomethyl)thiophene (2f) This product was obtained in 18 %

yield; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.09 (t, J = 7.45 Hz, 7H), 6.83 (br. s., 7H),

6.60 (d, J = 7.07 Hz, 7H), 6.42 - 6.54 (m, 14H), 4.42 (br. s., 4H), 2.56 (q, J = 7.07 Hz,

14H), 1.12 - 1.27 (m, 21H); ^{13}C NMR (100 MHz, Chloroform-d) δ 147.63, 145.45,

142.22, 129.19, 124.73, 117.79, 112.84, 110.38, 43.69, 28.99, 15.51; Calcd for

$C_{22}H_{27}N_2S$ ([M + H$^+$]): *m/z* 351.1889. Found: *m/z* 351.1889.

2,5-Bis(4-ethylanilinomethyl)thiophene (2g) This product was obtained in 7 %

yield as a yellow colored solid; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.02 (d, J = 8.34

Hz, 4H), 6.83 (s, 2H), 6.61 (d, J = 8.34 Hz, 4H), 4.41 (s, 4H), 2.54 (q, J = 7.58 Hz, 4H),

1.18 (t, J = 7.58 Hz, 6H); ^{13}C NMR (100 MHz, Chloroform-d) δ 145.53, 142.38, 133.93,

128.58, 124.64, 113.26, 44.01, 27.94, 15.92; Calcd for $C_{22}H_{27}N_2S$ ([M + H$^+$]): *m/z*

351.1881. Found: *m/z* 351.1889.

2,5-Bis(2-fluoroanilinomethyl)thiophene (2h) This product was obtained in 6 %

yield as a light yellow oil; ^{1}H NMR (400 MHz, Chloroform-d) δ 6.93 - 7.03 (m, 10H),

6.86 (s, 5H), 6.71 - 6.79 (m, 5H), 6.66 (dd, J = 3.28, 4.55 Hz, 5H), 4.48 (s, 4H); ^{13}C

NMR (100 MHz, Chloroform-d) δ 152.74, 150.36, 141.82, 136.01, 135.90, 124.91,

124.58, 124.54, 117.42, 117.35, 114.61, 114.43, 112.47, 43.23, 29.71;

2,5-Bis(3-fluoroanilinomethyl)thiophene (2i) This product was obtained in 3 % yield as a yellow oil; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.03 - 7.18 (m, 2H), 6.86 (s, 2H), 6.42 (d, J = 7.83 Hz, 4H), 6.28 - 6.38 (m, 2H), 4.43 (s, 4H); ^{13}C NMR (100 MHz, Chloroform-d) δ 141.74, 130.39, 125.01, 108.93, 104.39, 99.94, 99.69, 43.50, 29.71; Calcd for $C_{18}H_{17}N_2F_2S$ ([M + H$^+$]): m/z 331.1070. Found: m/z 331.1075.

2,5-Bis(4-fluoroanilinomethyl)thiophene (2j) This product was obtained in 6 % yield as a white solid; ^{1}H NMR (400 MHz, Chloroform-d) δ 6.76 - 6.93 (m, 6H), 6.49 - 6.63 (m, 4H), 4.36 (s, 4H); ^{13}C NMR (100 MHz, Chloroform-d) δ 157.31, 154.97, 143.85, 142.14, 124.75, 115.80, 115.58, 114.09, 114.02, 44.26;

2,5-Bis(2-chloroanilinomethyl)thiophene (2k) This product was obtained in 5 % yield as a clear oil; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.22 - 7.31 (m, 3H), 7.12 (t, J = 7.45 Hz, 2H), 6.87 (br. s., 2H), 6.60 - 6.77 (m, 4H), 4.51 (br. s., 4H); ^{13}C NMR (100 MHz, Chloroform-d) δ 143.32, 141.63, 129.18, 127.77, 124.90, 119.34, 117.91, 111.59, 43.27.

2,5-Bis(3-chloroanilinomethyl)thiophene (2l) This product was obtained in 3 % yield; ^{1}H NMR (500 MHz, Chloroform-d) δ 7.08 (t, J = 8.12 Hz, 7H), 6.85 (s, 7H), 6.70 (d, J = 7.82 Hz, 6H), 6.63 (s, 7H), 6.52 (dd, J = 1.76, 8.22 Hz, 7H), 4.42 (s, 4H); ^{13}C NMR (100 MHz, Chloroform-d) δ 148.57, 141.68, 135.03, 130.24, 125.05, 117.96, 112.79, 111.39, 43.41; Calcd for $C_{18}H_{17}N_2Cl_2S$ ([M + H$^+$]): m/z 363.0478. Found: m/z 363.0484.

2,5-Bis(4-chloroanilinomethyl)thiophene (2m) This product was obtained in 5 % yield as a white solid; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.07 - 7.13 (m, 4H), 6.81

(s, 2H), 6.51 - 6.57 (m, 4H), 4.38 (s, 4H); ^{13}C NMR (100 MHz, Chloroform-d) δ 145.98, 141.88, 129.07, 124.85, 122.61, 114.19, 43.64;

2,5-Bis(2-methoxyanilinomethyl)thiophene (2n) This product was obtained in 7 % yield; ^{1}H NMR (400 MHz, Chloroform-d) δ 6.82 - 6.90 (m, 4H), 6.75 - 6.81 (m, 2H), 6.63 - 6.74 (m, 4H), 4.46 (s, 4H), 3.83 (s, 6H); ^{13}C NMR (100 MHz, Chloroform-d) δ 146.94, 142.20, 137.53, 124.70, 121.23, 117.20, 110.37, 109.56, 55.44, 43.44; Calcd for $C_{20}H_{22}N_2O_2SNa$ ([M + H^{+}]): m/z 377.1300. Found: m/z 377.1300.

2,5-Bis(3-methoxyanilinomethyl)thiophene (2o) This product was obtained in 12 % yield; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.08 (t, J = 8.08 Hz, 2H), 6.83 (s, 2H), 6.14 - 6.36 (m, 6H), 4.41 (s, 4H), 3.74 (s, 6H); ^{13}C NMR (100 MHz, Chloroform-d) δ 160.79, 148.90, 142.08, 130.02, 124.83, 106.21, 103.22, 99.21, 55.08, 43.64; Calcd for $C_{20}H_{22}N_2O_2SNa$ ([M + H^{+}]): m/z 377.1300. Found: m/z 377.1300.

2,5-Bis(4-methoxyanilinomethyl)thiophene (2p) This product was obtained in 3 % yield as a light yellow solid; ^{1}H NMR (400 MHz, Chloroform-d) δ 6.75 - 6.85 (m, 6H), 6.63 (d, J = 8.84 Hz, 4H), 4.39 (s, 4H), 3.74 (s, 6H); ^{13}C NMR (100 MHz, Chloroform-d) δ 152.55, 142.41, 141.75, 124.61, 114.86, 114.56, 55.75, 44.66; Calcd for $C_{20}H_{22}N_2O_2SNa$ ([M + H^{+}]): m/z 377.1300. Found: m/z 377.1300.

2,5-Bis(2-trifluoromethylanilinomethyl)thiophene (2q) This product was obtained in 20 % yield as a clear oil; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.45 (d, J = 7.58 Hz, 2H), 7.34 (t, J = 7.71 Hz, 2H), 6.85 (s, 2H), 6.70 - 6.81 (m, 4H), 4.77 (br. s., 2H), 4.51 (d, J = 5.31 Hz, 4H); ^{13}C NMR (100 MHz, Chloroform-d) δ 144.79, 141.32, 133.10, (126.74, 126.69, 126.63, 126.43), 124.97, 123.72, 116.69, 113.99, 112.20, 43.09;

2,5-Bis(3-trifluoromethylanilinomethyl)thiophene (2r) This product was obtained in 7 % yield as a clear oil; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.20 - 7.33 (m, 2H), 6.97 (d, J = 7.83 Hz, 2H), 6.82 - 6.92 (m, 4H), 6.72 - 6.82 (m, 2H), 4.47 (s, 4H), 4.24 (br. s., 2H); ^{13}C NMR (100 MHz, Chloroform-d) δ 147.59, 141.58, (131.75, 131.44), 129.71, 125.62, 125.18, 116.03, (114.53, 114.49), (109.37, 109.33), 43.40;

2,5-Bis(4-trifluoromethylanilinomethyl)thiophene (2s) This product was obtained at 6 % yield as a light yellow solid; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.39 (d, J = 8.59 Hz, 4H), 6.85 (s, 2H), 6.63 (d, J = 8.34 Hz, 4H), 4.25 - 4.61 (m, 6H); ^{13}C NMR (100 MHz, Chloroform-d) δ 149.86, 141.56, (126.71, 126.67, 126.64, 126.59), 126.24, 125.14, 123.55, (120.05, 119.73, 119.41, 119.08), 112.24, 43.09.

2,5-Bis(morpholinomethyl)thiophene (2t) This product was obtained in 10 % yield as an off white/ light yellow solid; ^{1}H NMR (400 MHz, Chloroform-d) δ 6.74 (s, 2H), 3.68 - 3.77 (m, 8H), 3.66 (s, 4H), 2.49 (br. s., 8H); ^{13}C NMR (100 MHz, Chloroform-d) δ 140.76, 125.66, 66.98, 57.80, 53.27; Calcd for $C_{14}H_{23}N_2O_2S$ ([M + H^{+}]): m/z 383.1475. Found: m/z 383.1475.

2,5-Bis(thiomorpholinomethyl)thiophene (2u) This product was obtained in 6 % yield as a white solid; ^{1}H NMR (400 MHz, Chloroform-d) δ 6.71 (s, 2H), 3.67 (s, 15H), 2.61 - 2.84 (m, 59H); ^{13}C NMR (100 MHz, Chloroform-d) δ 141.05, 125.45, 58.18, 54.57, 28.00; Calcd for $C_{14}H_{23}N_2S_3$ ([M + H^{+}]): m/z 315.1018. Found: m/z 315.1023.

2,5-Bis(4-trifluoromethyl-1-phenylmethylaminomethyl)thiophene (2v) This product was obtained in 16 % yield as a light yellow oil; ^{1}H NMR (500 MHz, Chloroform-d) δ 7.58 (d, J = 8.02 Hz, 4H), 7.47 (d, J = 8.02 Hz, 4H), 6.76 (s, 2H), 3.95

(s, 4H), 3.89 (s, 18H); ^{13}C NMR (100 MHz, Chloroform-d) δ 144.13, 143.01, 128.36, 125.36, 125.33, 124.68, 52.16, 47.88; Calcd for $C_{22}H_{21}F_6N_2S$ ([M + H$^+$]): *m/z* 459.1324. Found: *m/z* 459.1324.

2,5-Bis(3-fluoro-1-phenylmethylaminomethyl)thiophene (2w) This product was obtained at 9 % yield as a light yellow oil; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.23 - 7.33 (m, 2H), 7.04 - 7.15 (m, 4H), 6.88 - 6.99 (m, 2H), 6.76 (s, 2H), 3.94 (s, 4H), 3.83 (s, 4H); ^{13}C NMR (100 MHz, Chloroform-d) δ 164.24, 161.80, 143.04, 142.77, 142.70, 129.87, 129.79, 124.62, 123.67, 123.64, 115.04, 114.83, 113.97, 113.76, 52.15, 47.80; Calcd for $C_{20}H_{20}F_2N_2SNa$ ([M + H$^+$]): *m/z* 381.1207. Found: *m/z* 281.1213.

2,5-Bis(4-chloro-1-phenylmethylaminomethyl)thiophene (2x); This product was obtained at 3 % yield as a clear oil; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.21 - 7.38 (m, 8H), 6.75 (s, 2H), 3.93 (s, 4H), 3.80 (s, 4H); ^{13}C NMR (100 MHz, Chloroform-d) δ 143.04, 138.46, 132.71, 129.53, 128.53, 124.61, 51.96, 47.77; Calcd for $C_{20}H_{20}Cl_2N_2SNa$ ([M + H$^+$]): *m/z* 413.0616. Found: *m/z* 413.0622.

5.1.1 *Binding affinity assay*

The binding affinity assay is a competitive assay where twenty thousand MDA-MB-231 breast cancer cells are incubated in an 8-well slide chamber for two days in 300 µL of medium. The compounds were also incubated in separate wells at several concentrations (1, 10, 100, 1000 nM) for ten minutes at room temperature. The cells were then fixed in a chilled solution of 4 % paraformaldehyde. After the cells were rehydrated in phosphate-buffered saline (PBS), the slides were prepared by incubating them with 0.05 µg/mL biotinylated TN14003 for thirty minutes at room temperature. These slides were washed three times with the PBS solution and were then

incubated for thirty minutes at room temperature in streptavidin-rhodamine (1:150 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). The slides were washed again with the PBS solution and were mounted in an antifade mounting solution (Molecular Probes, Eugene, OR). A Nikon Eclipse E800 microscope was used to analyze the samples[63a, 82].

5.1.2   *Matrigel invasion assay*

This assay was performed using a Matrigel invasion chamber (Corning Biocoat;Bedford, MA). In the bottom chamber, a solution of CXCL12 (100 ng/mL; R&D Systems, Minneapolis, MN) was added to the apparatus. 100 nm of the selected compounds (or AMD3100 as a control) were added to the MDA-MB-231. The cells were then placed in the top chamber. The apparatus was then incubated in a humidified incubator for 22 hours. The remaining cells in the top chamber were removed using a cotton swab and the invading cells in the bottom chamber were stained hematoxylin and eosin (H&E) and fixed with methanol. The rate of invasion was calculated by counting the invading (stained) cells[63a, 82].

5.1.3   *Paw inflammation test*

In this test, C57BL/6J does (Jackson Laboratories) are subcutaneously injected with λ-carrageenan (50 μL in 1 % w/v in saline) in the right hind paw to trigger inflammation; the other hind paw is used as the non-inflammation control. The selected analogues were prepared in 10 % DMSO and 90 % of 45% (2-hydroxypropyl-β-cyclodextrin (CD) in PBS. Doses of the analogues were set at 10 mg/kg and the does for TN14003 was set at 300 μg/kg. The TN14003 dose was set lower for this experiment because it was found that 300 μg/kg gave the maximum efficacy at minimum concentration in breast cancer metastasis in an animal model. The mice were dosed 30 minutes after the carrageenan injection and then once a day following the initial dose. The mice were sacrificed 74 hours after inflammation was induced and two hours after the last injection of

the selected analogues. The hind paws of the mice were photographed and calipers were used to measure the thickness of the paw from front to back. To quantify the edema, the volume of the untreated paw was subtracted from the volume of the treated paw. The inflammation suppression percentage was determined by comparing the analogue treated groups to the control group. Each analogue was tested in quintuplicate using the above procedure[54b, 63a]. Paw tissue slices were also collected and stained with H&E. Tissue slices were scanned and digitized by NanoZoomer 2.0 HT. The software NDP.view2 was used to view the slices in detail.

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APPENDICES

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