Synthesis of a New Scytovirin Protein Derivative, Sd1-Sd2-Sd2, to Decrease Hiv-1 Binding Affinity

Alexandra M. Wood
University of Mississippi. Sally McDonnell Barksdale Honors College

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SYNTHESIS OF A NEW SCYTOVIRIN PROTEIN DERIVATIVE, SD1-SD2-SD2, TO DECREASE HIV-1 BINDING AFFINITY

By
Alexandra Mae Wood

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2013

Approved by

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Advisor: Dr. John Rimoldi

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Reader: Dr. Lucille McCook

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Reader: Dr. Michael Mossing
SYNOPSIS

Scytovirin is a novel anti-viral protein isolated from the cyanobacterium Scytonema varium. This protein has produced great scientific interest for its anti-human immunodeficiency virus (HIV) activity. Scytovirin disrupts the mechanism of HV infection by binding to HIV-1 proteins glycoprotein 120, glycoprotein 160, and glycoprotein 41. It does not bind to the CD4 receptor on human immune cells. The wild-type protein consists of a single 95 amino acid chain. When folded, Scytovirin’s amino acid sequence is arranged into two distinct domains, each with a high degree of sequence conservation. This implicates the importance of both domains in anti-HIV activity.

Primers were designed and constructed to synthesize three novel, mutant, forms of the Scytovirin protein in an attempt to increase the anti-HIV activity of Scytovirin. Each gene analog featured coding to increase the size of the new mutant proteins by the addition of the second domain onto the nearly wild-type protein. The amino acid sequence connecting this domain was unique to each mutant. Single amino acids were replaced at numerous locations in each domain through site-directed mutagenesis to facilitate proper folding of the proteins. Once the gene constructs were sequenced and purified, all three mutants were expressed in Escheria coli, each with thrombin (THR) fused to its N-terminus. The protein constructs, three versions of SD1-SD2-SD2, were cleaved from THR with restriction enterokinase (rEK). Soluble protein was purified using polyhistidine tag affinity purification through FPLC, and HPLC. NMR determined the structures of the proteins and if proper folding had occurred.
SD1-SD2-SD2 wild-type and short linkers were expressed in Origami cells, purified, and examined by 2D Heteronuclear NOESY NMR. NMR of SD1-SD2-SD2 wild-type linker indicated that the protein had not folded completely. Additional research involving the expression and purification parameters of SD1-SD2-SD2 is required to obtain proper folding before SD1-SD2-SD2 will be screened against HIV-1 to assess anti-viral activity.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>CD4</td>
<td>Receptor HIV-1 binds on human cells</td>
</tr>
<tr>
<td>CB</td>
<td>Carbenicillin</td>
</tr>
<tr>
<td>CM</td>
<td>Chloramphenocol</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine receptor 4</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immune sorbent assay</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope glycoprotein</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography (Nickel Column)</td>
</tr>
<tr>
<td>gp41</td>
<td>Glycoprotein 41</td>
</tr>
<tr>
<td>gp120</td>
<td>Glycoprotein 120</td>
</tr>
<tr>
<td>gp160</td>
<td>Glycoprotein 160</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HR1</td>
<td>heptad repeat 1 region of gp41</td>
</tr>
<tr>
<td>HR2</td>
<td>heptad repeat 2 region of gp41</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthiogalactoside</td>
</tr>
<tr>
<td>JM109</td>
<td><em>E. coli</em> cell line</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out (deleted gene)</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>LL</td>
<td>Long linker</td>
</tr>
<tr>
<td>M9</td>
<td>Minimal Media number 9</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>NaN3</td>
<td>Sodium azide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser Effect Spectroscopy</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>rEK</td>
<td>Restriction enterokinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>SD1</td>
<td>Scytovirin Domain 1</td>
</tr>
<tr>
<td>SD2</td>
<td>Scytovirin Domain 2</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SL</td>
<td>Short linker</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>THR</td>
<td>Thrombin</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

The completion of this thesis was made possible by the advice and support given to me by many people. First and foremost, I would like to thank Dr.’s Robert and Hana McFeeters. I would have never had the opportunity to do so much if not for you extending the invitation to work in your lab. Thank you for seeing the potential in the eager freshman that came to ask you about viruses and your research, and then helping me make my part in your research possible. It was the opportunity of a lifetime, and I am so grateful that I was able to learn so much from such great people.

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I am extremely grateful to my wonderful friends and family, whose continuous support kept me going through good times and bad. Thank you for picking up the phone at 2:00 am when I was lonely and working on experiments, for wisdom and encouragement when things weren’t going the way I had planned, and for encouraging me to explore this opportunity even when it meant summers away from home.
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I. INTRODUCTION

Human immunodeficiency virus type-1 (HIV-1) is a retrovirus which acts by infecting the CD4 (cluster of differentiation 4) positive T-lymphocytes (T-cells) and macrophages of the human immune system. Infection with the HIV-1 virus results in the progressive deterioration and impairment of the host immune system, leading to ‘immune deficiency’. CD4 positive cells form the basis of cell-mediated immunity in humans, comprising T-lymphocytes and macrophages. Viral destruction of these cells leaves the host susceptible to opportunistic infections and cancers, most of which are rarely seen in healthy individuals. Progressive damage to the immune system by HIV-1 and other opportunistic infections eventually leads to acquired immunodeficiency syndrome (AIDS) and death. (Schacker, et al)

HIV is thought to have originated from crossing of simian immunodeficiency virus (SIV) from chimpanzees into humans sometime in the early 20th century in western Africa (Keele, et al). The spread of HIV-1 was initially slow, but spread rapidly worldwide due to rapid urbanization. HIV-1 infection was first recognized by medical publications of advanced immune deficiency, later termed AIDS, in the 1980s. Unusual cases of Kaposi’s sarcoma, a rare form of relatively benign cancer affecting the elderly, among young, previously healthy individuals were reported in large U.S. cities in 1981. The high incidence of this new condition in homosexual men caused the CDC to name the disease gay-related immunodeficiency (GRID). However, cases reported in heterosexuals in December of 1981 caused scientists to rethink the at-risk population. In 1983, cases were reported in Europe, Uganda, Australia, Canada, Latin America and Japan. Later that year, Luc Montangier and Francoise Barre-Sinoussi isolated the suspected virus. In
1984, Robert Gallo confirmed that the causative agent was a retrovirus, which was given the name human immunodeficiency virus. (Barre-Sinoussi, et al)

Transmission of HIV-1 from an infected individual to an uninfected individual occurs by entrance of infected bodily fluids; semen, vaginal fluids, blood or breast milk, entrance into the uninfected individual’s body. The most common modes of infection are unprotected sexual intercourse, injection drug use, and from infected mother to infant during delivery or breast-feeding. Blood transfusions and organ transplants were an initial source of HIV-1 spreading; however, with advanced tests and screenings the risk of infection from these procedures is extremely low. (Kaiser)

Infection with HIV-1 initiates a four-stage, lengthy disease that ultimately results in death by an opportunistic infection taking advantage of the crippled immune system. The first phase of HIV-1 infection is marked by an asymptomatic incubation period, which is the reason that most people do not know that they have become infected. A small number of people develop acute retroviral syndrome at the time of seroconversion, the production of antibodies to HIV-1, 1-6 weeks after the initial infection. (Schacker, et al) Retroviral syndrome is characterized by fever, malaise, pharyngitis, diarrhea, generalized lymphadenopathy, and rash. This complex of symptoms is easily confused with other illnesses such as infectious mononucleosis or influenza; which explains why most people do not learn of their illness until the disease enters the later stages.

During acute primary infection, plasma HIV-1 RNA concentrations can be very high, making the risk of secondary transmission to uninfected individuals extremely high. After the symptoms of primary infection diminish, the infected individual enters the second phase of HIV-1, the asymptomatic latent phase. This stage can persist for several years, averaging 8 to 10, but rapid progression through this stage is usually common. During this stage, HIV is actively replicating
and continuing to weaken the immune system. The symptomatic disease phase, progressing for one to three years, emerges as the peripheral CD4 positive cell count falls below 350 cells per µL of blood. As white blood cell counts continue to fall, many infected individuals become susceptible to infections and exhibit HIV-1 disease-related symptoms. The final stage of infection is marked by the progression of HIV-1 to AIDS, when the immune system’s ability to fight infections unassisted is compromised. A diagnosis of AIDS is given when a patient’s white blood cell count falls below 200 cells per µL of blood and patients experience multiple opportunistic infections. (Schacker, et al)

**Figure 1.1** Diagram depicting the structure and essential proteins of HIV-1. Reprinted with permission from Dr. Robert McFeeters (personal correspondence).

HIV-1 is spherical in shape, has a diameter of 1/10,000 of a millimeter, and is constructed of a protein capsid surrounded by an outer envelope composed of two layers of fatty acids, taken from the membrane of the human cell from which it assembled. Embedded within the viral
envelope are proteins from the host cell membrane as well as approximately 72 copies of the complex protein Envelope Glycoprotein (Env). Env is a conical structure consisting of a cap composed of three molecules of glycoprotein 120 (gp120), and a stem of three molecules of glycoprotein 41 (gp41) that anchors the structure into the viral envelope. (De Clerq) HIV-1 infection of macrophages and CD4 positive T-cells is mediated through the interaction of the viral envelope glycoproteins 120 and 41 (gp120, gp41) with the CD4 molecule on the target cells. (Mondor, et al) CD4 is an integral membrane glycoprotein, belonging to the immunoglobin gene family, which is expressed mainly on the surface of T-cells and the cells of the macrophage/monocyte lineage.

Adsorption begins when Env interacts nonspecifically with heparin sulfate, a sulfated polysaccharide that is involved in adsorption of many enveloped viruses and is widely expressed in animal cells. This initial interaction provides the opportunity for other copies of Env to interact with CD4. Once the virus has attached to the CD4 receptor, the viral Env gp120 interacts with its co-receptor, chemokine receptor 4 (CXCR4). This interaction triggers shape changes in

Figure 1.2: Life cycle of HIV-1. (Reprinted with permission from Nature: open content file)
the gp120-gp41 complex that ultimately changes the secondary structure of gp41. The more stable, double-pronged attachment allows the N-terminal fusion peptide gp41 to penetrate the host cell membrane. (Wyatt, Sodrowski) Interaction of repeat sequences in gp41, HR1 and HR2 causes the collapse of the extracellular portion of gp41 into a hairpin structure. This fuses the viral envelope with the membrane of the target cell, and the viral capsid, containing viral RNA and essential viral protein, is injected into the cell. (De Clerq)

Once inside the host cell, the viral capsid is uncoated, thought to be accomplished by phosphorylation of the protein matrix surrounding the capsid. Uncoating produces the reverse transcription complex, containing primers and various proteins necessary for transcription. (Cartier, et al) Then, reverse transcriptase transcribes the single-stranded RNA genome of HIV-1 into double-stranded DNA, which is then incorporated into the host’s genome by the viral enzyme integrase. The integrated viral DNA may remain unexpressed for some time, allowing the virus to escape detection by the immune system. Certain transcription factors are necessary for the expression of the viral genes including NF-κB, which is upregulated when the T-cells are currently fighting infection. Therefore, the most active T-cells, the ones fighting the infection, will be the ones to express the viral genes and assemble more virus particles to spread the infection. (Kaiser)

Since its discovery in 1981, HIV-1 has led to the deaths of more than 33 million people worldwide. (UNAIDS) Current therapeutic efforts focus on reverse transcriptase and protease inhibitors. However, these methods do nothing to prevent HIV-1 infection and can only slow the progression of the disease. The chronic nature of HIV-1 implies that therapies are administered for life; therefore, selection of therapeutics is based not only on their efficacy but on their toxicological profile as well. (Blas-Garcia, et al) The emergence of drug-resistant strains of HIV-1 has generated the need for more effective and less toxic anti-HIV agents. In addition, the high mutation rate of HIV-1 prevents the production of an effect vaccine. HIV-1’s high infectivity,
lethality and aggressive rate of mutation have made potential anti-HIV agents a priority research subject. (Schader, Wainberg)

Because of their integral part in the mechanism of HIV-1 infection, gp120 and gp41 are highly conserved in all virulent HIV-1 strains. Consequently, these glycoproteins are of interest in developing anti-HIV agents, particularly those that prevent further or initial infection. In recent years, cyanobacteria have cultivated a high level of interest as a potential new source of bioactive and novel secondary metabolites for use as anti-viral agents.

Scytovirin, a potent anti-HIV-1 protein was originally isolated from aqueous extracts of the cyanobacterium *Scytonema varium*. Scytovirin has a molecular mass of 9713 Da, contains five intrachain disulfide bonds within two domains and one inter-domain disulfide bond. Scytovirin binds to the HIV-1 proteins gp120, gp41, and gp160 but not to the cellular receptor CD4. (Xiong, et al) Low nanomolar concentrations of purified Scytovirin inactivate laboratory strains of HIV-1 by selective interactions between Scytovirin and the high mannose oligosaccharide-bearing glycoproteins gp120, gp41 and gp160.

![Figure 1.3](image1.png)

**Figure 1.3:** Aligned amino acid sequences of the two domains of Scytovirin. Starred amino acids indicate a conserved site. Disulfide bridges are indicated by solid lines above the sequence. Reprinted with permission from Dr. Robert McFeeters (personal correspondence).

The two domain sequences of Scytovirin show strong internal sequence duplication. 36 residues (75%) are directly homologous and 3 residues (6%) represent conservative amino acid changes. (Bokesch, et al) Folding of the amino acid chain clusters aromatic residues in both
binding sites, suggesting an importance to retain aromatic chemistry when substituting amino acids during site-directed mutagenesis. (McFeeters, et al) As these sequences correspond to the binding sites of Scytovirin, it was postulated that both domains were equally important in anti-HIV activity. Research by Liu, et al has indicated this to be true. Because each domain shows approximately equal anti-HIV activity, we decided to attempt to increase the activity of Scytovirin by engineering a Scytovirin protein derivative that contained a third domain. The second domain was selected to be engineered onto the wild-type Scytovirin protein to increase the binding affinity and anti-HIV activity of Scytovirin in the hopes of creating a new, less harmful, anti-HIV drug.
II. DESIGN AND SYNTHETIC APPROACHES TOWARDS THE DEVELOPMENT OF SD1-SD2-SD2 PROTEINS

Scytovirin, a protein isolated from the cyanobacterium Scytonema varium, is a high mannose-binding lectin that shows potent anti-viral activity. Scytovirin inhibits HIV replication with half maximal effective concentrations (EC$_{50}$) in the low nanomolar ranges. (Sato, et al) Scytovirin also acts as a potent HIV-1 entry inhibitor. The mechanism by which Scytovirin inhibits adsorption of HIV-1 is mediated by several carbohydrate moieties on the virus surface, gp120, gp160, and gp41. Scytovirin specifically interacts with oligosaccharides containing α1-2, α1-6 trisaccharide units. Scytovirin is of particular interest as a prophylactic viral inhibitor because it does not bind to the human cellular receptor, CD4 (Xiong, et al)

Due to this potent anti-HIV activity, efforts to increase the binding affinity of Scytovirin have been widespread. Scytovirin is a 95 amino acid, straight chain protein with 10 cysteine residues that form 5 intrachain disulfide bonds. Wild-type Scytovirin has a molecular mass of 9713 Da and folds into a dimeric structure of two domains, which represent its active form. The first comprises amino acids 1-48 and the second stretches from amino acids 49-95. The disulfide bonds are integral in composing these two domains and by extension, the active sites of Scytovirin. Each cysteine residue must find its corresponding partner and create a di-sulfide bond in order to generate properly folded protein. The bonds form by the C20-C26 and C32-C38 disulfides correspond closely to those defined by the C68-C74 and C80-C86 disulfide bonds in the second domain of Scytovirin. (Bokesch, et al) There is also a disulfide bond linkage between the 7th residue in the first domain, and the 55th residue in the second domain, which effectively
links the two domains and contributes to the 3D structure of the protein. The two domains share more in common than just the placement of the disulfide bridges within them.

Scytovirin has a high level of sequence duplication: when residues 1-48 and 49-95 are aligned, 75% of the residues are identical and 6% make up conservative amino acid changes (Xiong, et al) NMR analysis of Scytovirin reveals that the two domains of the protein have strong symmetry.

The two domains cluster aromatic residues in both sites, suggesting a binding mechanism similar to other known hevein-like carbohydrate-binding proteins. There are three aromatic residues in each domain (SD1, Y6, W8, F37; and SD2, Y54, W56, F85), and with the exception of F85 in SD2, all of the aromatic residues of Scytovirin are clustered near the binding sites. Mutation of these residues to non-aromatic species destroys the antiviral activity of Scytovirin (McFeeters, et al) However, the carbohydrate moieties recognized by Scytovirin are significantly different from hevein-binding carbohydrates. (McFeeters, et al)

Previous experiments by Yinan Liu and affiliates have determined that the activity of Scytovirin and its relatives is significantly affected by the number of carbohydrate binding domains present, rather than their identity. (Liu, et al) However, other experiments by McFeeters and Xiong indicate that this might not be the exact case. ELISA experiments were performed to compare the binding ability to gp120 and gp41 of Scytovirin-derived peptides to Scytovirin.
The results showed that domain 1 of Scytovirin (SD1) appears to have gp120 and gp41 binding ability similar to intact Scytovirin. In contrast, domain 2 (SD2) has 57% reduced gp120 binding ability and approximately 44% reduced affinity to gp41. However, intact, wild-type Scytovirin demonstrates a several fold increase in binding affinity compared separately to either one of its domains. (Xiong, et al) Working with this knowledge in mind, we sought to increase the binding affinity and consequently the anti-HIV activity of Scytovirin by engineering a Scytovirin derivative with an extra carbohydrate-binding domain. As the second domain of wild-type Scytovirin was already equipped to be attached to a previous domain, duplication of this domain and subsequent incorporation onto the end of native Scytovirin protein was undertaken to potentially increase its anti-HIV activity.

![Surface representation of Scytovirin](image)

**Figure 2.2** Surface representation of Scytovirin (SD1 pink, SD2 light blue) colored to show residues having largest change in chemical shift upon tetrasaccharide binding (SD1 red, SD2 blue). Reprinted with permission from Dr. Robert McFeeters (personal correspondence).

The project utilized a pET-32a vector to develop the mutant Scytovirin derivative sequences of SD2C55S. Synthetic sequences for wild-type Scytovirin, SC2, and the first domain,
SD1, were assembled using a pET42b vector. Sequences for both domains were synthesized independently in pET32a vectors and linked together again via gene splicing. Recognizing the difficulty in generating a properly folded protein due to the high-interaction of multiple cysteines, the 55th residue, a cysteine, was replaced by serine using site-directed mutagenesis in the second domain of Scytovirin. This change was introduced to insure that the chemistry between the neighboring residues was retained as serine has the same relative size, shape, and chemical properties as cysteine without the possibility of generating errant disulfide bonds within the mutant protein. Following this change, the mutant protein was designated as SD1-SD2-SD2C55S to denote this mutation.

Inclusion of a BamHI site to the N-terminus of SD2C55S was accomplished by site-directed mutagenesis. A thioredoxine sequence was fused to the N-terminus of SD2C55S to facilitate disulfide bond formation. (Stewart, et al) Additionally, the KpnI digestion site was removed from the pET32a vector to facilitate separation of the construct from its fusion protein during purification. The C-terminal of SD2C55S included an XhoI restriction enzyme digestion site. Digestion of this vector by BamHI and XhoI produced the SD2C55S sequence, which was ligated to the synthetic gene construct for wild-type Scytovirin.

Three different linkers were designed to attach the synthetic, second domain of Scytovirin onto the wild-type, double-domained protein during PCR. Each linker derivative was synthesized and separated from incomplete or mutated sequences by electrophoresis. Bands of 200bp were excised and purified by DNA extraction. Sequencing was used to determine the integrity of the DNA construct. Once extracted, each linker was digested using BamHI and XhoI restriction enzymes. Following digestion, the wild-type, long and short linkers were separately ligated to the BamHI and XhoI digested SD2C55S sequence to create three versions of SD2C55S. Each SD2C55S + linker sequence was separated from partially digested pieces of mutated sequences by electrophoresis, then extracted and purified.
Wild Type linker Sequence

T GCG ↓GAT CCG GGT TCT CGT AAA CCG GAC CCG GGG CCC AAA GGT CCG ACC

A D P G S R K P D P G P K G P T

TAC TGC TG

Y C

Short Linker Sequence

T GCG ↓GAT CCG GGC GGT TCT TCT GGT CCC AAA GGT CCG ACC TAC TGC T

A D P G G S S G P K G P T Y C

Long Linker Sequence

T GCG ↓GAT CCG GGT TCT AGC GGT GGC TCT AGC GGC TCT TCT GGT CCC AAA

A D P G S S G G S S G S S G P K

GGT CCG ACC TAC TGC T

G P T Y C

Figure 2.3 Nucleotide sequences for the synthetic linkers and the encoded amino acid sequences. Reprinted with permission from Dr. Robert McFeeters (personal correspondence).

Each of these versions of SD2C55S was then ligated to the end of the second domain of wild-type Scytovirin, SC2, using slow ligation. A histidine tag sequence was introduced onto the ends of all three versions of SD1-SD2-SD2 to aid in later protein purification. The SD1-SD2-SD2C55S constructs were digested with the restriction enzymes BamHI and XhoI, and separated from incomplete sequences using electrophoresis. The constructs were then extracted, purified, and ligated together into the final, previously BamHI and XhoI digested plasmid. The resulting
complete DNA sequences for SD1-SD2-SD2C55S were amplified by PCR, and then purified using the GeneJet PCR Purification Kit.

The purified DNA products for all three versions of SD1-SD2-SD2 were transformed into JM109 competent cells to amplify DNA product and to test the transforming capabilities. SD1-SD2-SD2C55S short linker and SD1-SD2-SD2C55S wild-type linker were transformed successfully. The SD1-SD2-SD2C55S long linker sequence has not yet been successfully transformed. Work on this version of SD1-SD2-SD2 has been halted. Once SD1-SD2-SD2C55S short and wild-type linker sequences were transformed into JM109 cells the transformed cell cultures were miniprepped to extract the recombinant plasmids. Both SD1-SD2-SD2C55S sequences were double digested using BamHI and XhoI and separated using electrophoresis to determine proper ligation. All three versions of SD1-SD2-SD2C55S were sent for sequencing. All three DNA sequences were determined to be as had been designed. It is still not clear why SD1-SD2-SD2C55S long linker will not transform into competent *E. coli* cells.

![SDS-PAGE gel](image)

**Figure 2.4** SDS-PAGE gel depicting expression of SD1-SD2-SD2 WT and SD1-SD2-SD2 SL. 1) Protein ladder 2) SD1-SD2-SD2 WT uninduced 3) SD1-SD2-SD2 WT induced 4) SD1-SD2-SD2 SL uninduced 5) SD1-SD2-SD2 SL induced. Lanes 6-9 represent wild-type Scytovirin protein expression.
Small-scale expressions of both SD1-SD2-SD2 short linker and SD1-SD2-SD2 wild-type linker were carried out in LB, M9, Soc, and Spectra 9 with Origami competent cells at 37 °C. Previous experiments by McFeeters, et al have determined that Origami competent cells express and fold Scytovirin better than other *E. coli* strains.

Using this knowledge, experimentation with expression parameters was carried out to determine the best way to express large amounts of soluble SD1-SD2-SD2. Through these small-scale expressions, it was determined that Origami cells expressed more soluble protein in LB than in any other media. Soluble protein yields are quite high using Origami cells, however properly folded protein yields were not optimal. Steps were taken to determine the optimal parameters to increase proper folding of SD1-SD2-SD2 mutants.

Many large-scale expressions of SD1-SD2-SD2 short and wild-type linkers were performed. Two separate media were utilized, LB and M9. Inoculated cultures of transformed cells were grown at 37 °C until time of induction, at which point they were grown at 18 °C, 30 °C, and 37 °C in both media. Cultures were induced at an OD$_{590}$ of approximately 0.6, and harvested approximately 4 hours after this time. Harvested protein was purified by FPLC, a restriction digest to remove the thioredoxine tag, two buffer exchanges, and reverse phase HPLC.

Protein expressed at 18 °C produced the worst yields; protein that was expressed at 30 °C seemed to demonstrate improved yields of properly folded protein. Purified protein samples were then condensed to a minimal volume using high-speed centrifugation to determine the overall structure using 2D NOESY NMR.
Figure 2.5 2D NOESY NMR of SD1-SD2-SD2 wild-type linker aligned against Scytovirin. Scytovirin is depicted in red, SD1-SD2-SD2 wild-type linker is depicted in blue and green represents the negative spectrum of SD1-SD2-SD2 wild-type linker. Reprinted with permission from Dr. Robert McFeeters (personal correspondence).
III. CONCLUSIONS AND FUTURE PERSPECTIVES

Scytovirin, a novel anti-HIV protein isolated from the cyanobacteria *Scytonema varium*, is a small, 95 amino acid, straight chain protein with a high incidence of internal sequence duplication and 5 intrachain disulfide bonds. Scytovirin demonstrates high anti-viral activity, as well as anti-HIV activity in the nanomolar concentrations. Additionally, Scytovirin binds specifically to gp120, gp41 and gp160 of HIV-1, the proteins essential to viral adsorption but not to the CD4 receptor. This high-mannose oligosaccharide binding specificity offers a new, safe approach to inhibiting HIV-1 infection. As a result, Scytovirin has garnered interest as a possible therapeutic approach to controlling and diminishing HIV levels in affected individuals.

Efforts were made to increase the binding affinity, and therefore the anti-HIV activity, of Scytovirin through protein engineering. Because of the high sequence duplication of the two domains of Scytovirin, it was postulated that both have similar binding affinities for glycoproteins on HIV-1. In fact, there is almost no discernible difference in the binding affinities of the domains of Scytovirin (Liu, et al). Increased affinity of Scytovirin is achieved by binding to larger glycoproteins such as gp160, gp41 and gp120 that deliver carbohydrates to both Scytovirin domains. (McFeeters, et al) The high anti-HIV activity of Scytovirin is likely due to this multivalency. Because interaction with both domains increases the activity of Scytovirin, steps were taken to engineer the second domain of Scytovirin onto the end of the wild-type protein in order to attempt to greatly increase its anti-HIV activity.

A pET 32 vector was utilized to clone and express the Scytovirin mutants. Three versions of the DNA sequence coding for the proteins SD1-SD2-SD2 short linker, SD1-SD2-SD2 wild-type linker and SD1-SD2-SD2 long linker were synthesized. A thioredoxin tag was introduced at
an enterokinase restriction site between the Scytovirin peptide sequences and their fusion partner to enhance disulfide bond formation and proper folding during protein expression. (Stewart, et al and Stoll, et al) Several key mutations of cysteine to serine were induced in the recombinant, second domain of SVN to reduce improper disulfide bond formation while maintaining similar amino acid chemistry. DNA sequences for SD1, SD2, and SD2C55S were cloned in JM109 E. coli cells. Extracted DNA was digested, purified, and ligated to create three recombinant plasmids for the three Scytovirin mutants. The integrity of the plasmids was confirmed by DNA sequencing.

Successful expression of SD1-SD2-SD2 wild-type linker and SD1-SD2-SD2 short linker was achieved in Origami cells. Once expression of soluble protein was achieved, efforts were undertaken to increasing the yields of properly folded protein. Multiple parameters were changed to measure the impact on yields of properly folded protein such as temperature and culture medium. It was found that LB media creates a higher yield of protein, as does growth and induction at 37 °C. However, when SD1-SD2-SD2 wild-type linker was compared to Scytovirin using 2D heteronuclear NOESY NMR it was clear that proper folding of the mutant protein was not obtained.

Screening of the SD1-SD2-SD2 mutants has been deferred until proper folding is attained. Many of the folding problems stem from the multiple, sequential disulfide bridges in Scytovirin as well as the interdomain sulfide bridge that is largely responsible for the overall structure of Scytovirin and, consequently, its antiviral activity. Because of this, additional research is required to induce more mutations in the DNA sequence to facilitate proper folding. Additional cell lines will be screened for their expression and possible roles in folding of Scytovirin. Once large quantities of properly folded SD1-SD2-SD2 mutants have been expressed and purified, they will be screened for increased anti-HIV activity to improve the HIV drug cocktail and potentially bring us closer to a cure.
Figure 3.1 2D NOESY NMR of SD1-SD2-SD2 wild-type linker aligned against Scytovirin. Scytovirin is depicted in red, SD1-SD2-SD2 wild-type linker is depicted in blue and green represents the negative spectrum of SD1-SD2-SD2 wild-type linker. Reprinted with permission from Dr. Robert McFeeters (personal correspondence).
IV. MATERIALS AND METHODS

General Methods:

All reactions involving live *Escherichia coli* were carried out in a sterile flame hood using flame-dried glassware. All transformations for the expression of SD1-SD2-SD2 used the Origami cell line unless otherwise noted. All transformations for the purification of DNA used the JM109 cell line unless otherwise noted. All reactions allowed to proceed ‘overnight’ progressed for 12 hours or more unless otherwise noted. All commercially available starting materials and reagents were purchased at the highest commercial quality and used without further purification unless otherwise noted. All PCR procedures were run on the program ‘Mary Sing’ unless otherwise noted. All chemicals were purchased from Sigma, Aldridge, Promega or Fermentas chemicals. All primers used were purchased from ING laboratories in Huntsville, Alabama. The Dr.’s Robert and Hanna McFeeters developed all procedures used unless otherwise noted.

General Procedure for Standard PCR:

A mixture of 5 µL tag10x buffer, 5 µL 2 mM dNTP, 1.25 µL Primer 41, 1.25 µL Primer 49, 4 µL 25 mM MCI₂, 2 µL purified DNA, 1 µL taq polymerase and 30.5 µL dH₂O was mixed on ice before undergoing PCR.

General Procedure for Emerald Amp PCR:

25 µL emerald master mix, 2 µL purified DNA, 1 µL Primer 41, 1 µL Primer 49 and 21 µL dH₂O. Run on program ‘Mary Sing’.
**General PCR Purification Procedure:**

47 µL of binding buffer was added to 47 µL of the PCR mixture and mixed thoroughly in a microcentrifuge tube at 14,000 rpms for 30 seconds. 47 µL of 100% isopropanol was added to the solution per 500 base pairs of DNA. Solution was transferred to the GeneJet purification column. Column was centrifuged for 30-60 seconds at 14,00 rpms. Flow-through was discarded. 700 µL of the wash buffer was added. Mixture was centrifuged for another minute. The flow-through was discarded. The column was then centrifuged for another minute to maximize purification of DNA product. GeneJet column was placed into sterile 1.5 mL microfuge tube. 25 µL of ddH₂O was added to the column. Mixture allowed to incubate at 25 °C for 1 minute. Tube was centrifuged for 1 minute. Another 25 µL of ddH₂O was added to the tube and allowed to incubate for 1 minute at 25 °C. Tube was centrifuged for 1 minute; flow-through was saved.

**General Procedure for Transformation of SD1-SD2-SD2 vector for Expression:**

5 µL of desired DNA was added to 50 µL of thawed competent cells in a plastic Falcon tube, then gently swirled. Tubes were allowed to sit on ice for 45 minutes. Tubes were then shocked for 1 minute and 15 seconds in a 42 °C water bath. Tubes were placed back onto ice for an additional 2 minutes. 0.5 L of LB was added to each of the tubes and gently mixed. Tubes were placed in and incubator at 37 °C, 250 rpms for 1 hour. 200 µL of cell/LB mixture were plated to prepped carbenicillin plates in a sterile flame hood. Plates were incubated overnight at 37 °C. 1 mL of LB was added to original tubes along with 1.1 µL of carbenicillin. Tubes were placed in the shaker at 250 rpms and 37 °C overnight.

**Carbenicillin Plate Preparation:**

12.23g of LB agar mixed with 300 mL ddH₂O in a flame-sterilized beaker. Mixture autoclaved on the liquid setting. After autoclaving, 300 µL of carbenicillin was added when mixture reached 25
°C. Plates were poured in sterile flame hood and allowed to harden in the flame hood until transferred to the -20 °C refrigerator for storage.

Before transformed competent cells were spread onto agar plates, all plates were thawed in sterile flame hood and each coated with 100 µL of a 20 µL chloramphenicol to 80 µL LB solution. Plates were allowed to dry fully in sterile flame hood before plating with competent cells.

**Site-directed Mutagenesis Procedure:**

100 µL of ddH₂O was added to designed, dehydrated primers. Primers were vortexed to mix thoroughly. 5 µL of 10x reaction buffer, 5 µL of dsDNA template (SD1 pET42b), 1.25 µL of oligonucleotide control primer #1, 1.25 µL of oligonucleotide control primer # 2, 1 µL of dNTP mix, 1.5 µL of QuikSolution reagent, and 34 µL of ddH₂O were combined in mini PCR tubes on ice, vortexed to mix. Then 1 µL of QuikChange lightning enzyme was added to the reaction mixture, vortexed and gently spun down. Run on program “Morgan” in PCR machine.

**Transformation of Purified PCR Product General Procedure:**

A mixture of 1 µL of p-prime cloning vector, 4 µL of purified PCR product, and 5 µL of ligation master mix in a microcentrifuge tube, thoroughly mixed at 14,00 rpms for 1 minute. Mixture was then incubated at 4 °C for 30 minutes. 7 µL of the mixture was transformed into 70 µL of JM109 E. coli competent cells according to the general transformation procedure. Carbenicillin plates were prepared with 100 µL of 100 mM IPTG plus 20 µL of 50 mg/mL X-Gal. 300 µL of cell and LB broth mixture was plated. Incubated overnight at 37 °C. White colonies were selected for DNA purification.

**Cloned DNA purification general procedure:**

One E. coli colony of medium size and significant distance from neighboring colonies was selected per plate. Colonies were extracted in a sterile flame-hood and transferred to a mixture of 3 mL of LB broth and 3 µL carbenicillin in a 50 mL glass vial. Tubes were placed in the
incubator over night at 37 °C and 250 rpms. 1.5 mL from each tube was transferred to 1.5 ML centrifuge tubes. Mixture was centrifuged at 14,000 rpms for 5 minutes. Supernatant was discarded and procedure was repeated until all of the bacteria from each tube had formed pellets. Pelleted cells were resuspended with 250 µL of Resuspension solution. 250 µL of Lysis solution and 350 µL of Neutralization solution were added to each centrifuge tube and mixed thoroughly by inversion. Tubes were centrifuged for 5 minutes at 14,000 rpms. The supernatant from each tube was loaded into a GeneJet spin column. Supernatant was centrifuged for 1 minute at 14,000 rpms and the flow-through was discarded. 500 µL of Wash solution was added to each GeneJet spin column. The column was centrifuged for 1 minute and the flow-through was discarded. Wash and discarding of the flow-through was repeated. Empty column was centrifuged for one additional minute to maximize separation of DNA product from added solutions. The spin column was then transferred to a new microcentrifuge tube. 30 µL of ddH₂O was added to the column. Column was incubated at 25 °C for 2 minutes. Column was centrifuged inside centrifuge tube at 14,000 rpms for 2 minutes. An additional 30 µL of ddH₂O was added to the column and the incubation and centrifugation was repeated. The flow-through was collected. DNA concentration of the purified product was checked using UV spectroscopy.

**Restriction Digest of synthesized DNA product:**

A mixture of 10 µL of purified PCR product, 2 µ of 10x buffer, 6 µL of ddH₂O, 1 µL BamHI enzyme, and 1 µL of XhoI enzyme was mixed in a centrifuge tube and allowed to incubate at 37 °C for 60 minutes. Digested DNA was then run through a DNA agarose gel to separate DNA sequences by size. Desired DNA pieces were then purified to be used in later procedures.

**DpnI Digest of Site-directed Mutagenesis Amplification Products:**

2 µL of the DpnI restriction were added to each amplification reaction. Reaction was mixed thoroughly and spun down briefly. Mixture was incubated at 37 °C for 30 minutes. Digested DNA was immediately transformed in XL-10 gold E. coli competent cells. 2 µL of DNA, 2 µL of
the β-ME mix and 45 µL of XL-10 gold cells were combined on ice. Mixture was allowed to incubate on ice for 30 minutes. Competent cell mixture was heat shocked for 30 seconds at 42 °C, placed back on ice for another 2 minutes. 0.5 mL of preheated NZY+ broth was added to each transformation tube. Tubes were placed in the incubator for 1 hour at 37 °C and 250 rpms. 200 µL of competent cells were plated on Kanamycin plates and incubated at 37 °C overnight. Colonies containing the desired plasmids appear blue on agar plates containing 80 µg/mL X-Gal and 20 mM IPTG.

**DNA Extraction and Purification Procedure:**

Excise the desired bands of DNA from the stained agarose DNA gel with a razor blade.

Determine the approximate volume of the DNA piece by weighing, then transfer the piece to a sterile 1.5 mL centrifuge tube. If the piece is larger than 0.40g, a larger centrifuge tube is necessary. 3 equivalents of Binding solution was added per 1 equivalent of DNA gel. The tube was then placed in a 55 °C water bath and incubated for 5 to 10 minutes or until the DNA gel had liquefied. The tube was briefly centrifuged every 3 minutes of incubation. Once liquefied, add a volume of TE buffer to bring the total volume to 100 µL. 2 µL of silica powder suspension was added per 1 µL of DNA and mixture was incubated for 5 minutes at 55 °C. Silica powder suspension was resuspended to produce a homogenous suspension by rigorous mixing every 1 to 2 minutes while incubating. The silica powder and DNA mixture was centrifuged for 5 seconds at 14,000 rpms. The supernatant was removed and set to the side. 500 µL of ice cold wash buffer was added to resuspend the pellet. Mixture was then centrifuged for 5 seconds at 14,000 rpms and the supernatant was discarded. Wash procedure was repeated 3 more times. The washed silica and DNA pellet was then resuspended with 500 µL of ddH₂O. Tube was incubated at 55 °C for 5 minutes. Mixture was centrifuged for 30 seconds at 14,000 rpms. Supernatant containing purified DNA was then carefully extracted and placed into sterile centrifuge tube.
General Slow-Ligation Procedure:

3 µL of previously digested SC2 Bam HI kpnKO, 12 µL of previously digested SD2C55S linker, 2 µL of buffer, 3 µL of dH₂O, and 1 µL ligase were combined in a sterile microcentrifuge tube and allowed to incubate at 25 °C for 1 hour. 10 µL of ligation product was transformed into 120 µL of JM109 cells and plated according to the general transformation procedure for JM109 cells.

Procedure for SDS-PAGE 12% Protein Resolving Gel:

2.4 mL of H₂O, 1.1 mL of 2M Tris-HCl, pH 8.8, 37.5 µL of 20% SDS, 2.4 mL of Acrylamide/Bis-acrylamide 30%/0.8% (w/v), 40 µL of 10% ammonium persulfate (w/v), and 14 µL TEMED were combined in a 50 mL glass beaker by gentle pipetting. Solution was poured into protein gel mold, topped with distilled H₂O and allowed to polymerize.

Procedure for SDS-PAGE 4% Stacking Gel:

1.8 mL of H₂O, 0.75 mL of 0.5M Tris-HCl pH 6.8, 15 µL of 20% (w/v) SDS, 0.40 mL of Acrylamide/Bis-acrylamide 30%/0.8% (w/v), 30 µL of 10% (w/v) ammonium persulfate, and 6 µL of TEMED were combined in a sterile, 10 mL glass beaker. Water over polymerized resolving gel was removed, and the stacking gel mixture was pipetted on top of the polymerized resolving gel. Stacking gel was allowed to polymerize.

Protein Gel Silver Stain Procedure:

All steps were performed in a single, clean plastic staining tray with constant, gentle shaking. Protein gel was washed 2 times with ddH₂O for 5 minutes. Protein gel was fixed twice for 15 minutes in 30% etOH: 10% acetic acid solution. Protein gel was washed twice for 5 minutes in 10% etOH, then twice for 5 minutes in ddH₂O. Sensitizer Working solution was prepared by mixing 50 µL sensitizer with 25 mL H₂O. Protein gel was sensitized for 1 minute, then washed twice for 1 minute each in ddH₂O. Protein gel was stained for 30 minutes with silver stain. Gel was washed twice for 20 seconds each with ddH₂O, then gel was developed for 2 to 3 minutes or until bands began to appear. Development was quenched with 5% acetic acid for 10 minutes.
M9 Media:
3.4 g Na₂HPO₄, 1.5 g KH₂PO₄, 0.25 g NaCl, 1.0 g dextrose, 0.5 g NH₄Cl, 5 mL divalent, 1 mL vitamin B, 5 µL trace elements were combined and diluted with 500 mL of ddH₂O. Mixture was sterile filtered and stored until use.

Large-Scale Expression of Protein Product in Spectra 9 N¹⁵ from plates:
Colonies of previously transformed Origami cells were washed from plates into 500 mL of Spectra 9 (N¹⁵) media with 250 µL of carbenicillin. Reaction mixture was incubated at 37 °C at 250 rpms and bacterial growth was measured periodically using optical density. Cells were induced with 500 µL of IPTG and allowed to continue expression for 5 hours. Cell mixture was then spun down on a JLA-16 250 rotor at 7,000 rpms until the entire mixture was condensed to pelleted cells. Supernatant was discarded and the cell pellet was stored at -80 °C for at least 6 hours before beginning lysis.

General Protein Purification Procedure:
Pellet was transferred from -80 °C into a 50 mL conical tube on ice. 20 mL of 50 mM NaP buffer, pH 6.8 was added to the pellet and resuspended carefully to reduce bubble formation. 15 mg of lysozyme, 200 µL of 1M MgCl₂, and 20 µL of DNase were added. Mixture was allowed to incubate at 25 °C for 30 minutes. Mixture was sonicated at 50% output for 16 minutes, alternating 1 minute of sonication and 1 minute on ice. Centrifuge at 20,000 rpms in JA25.50 rotor for 30 minutes at 4 °C. Immediately separate the supernatant from the pellet and store at 4 °C until ready to be processed by FPLC.

General Procedure for FPLC:
Run using the absorbance mode at 0.05. Load filtered supernatant at 2 mL/min onto a 5 mL “HisTrap FF” (GE Healthcare, Cat. # 17-5255-01) nickel affinity column that has been equilibrated in Buffer A and collect 2 mL fractions. Save the flow through. Manually switch to 40% Buffer B at an A₂₈₀nm of about 0.5. Wash the column at 40% Buffer B until the A₂₈₀nm...
stabilizes and starts to slightly go up (approximately 10 mLs of total elution at 40% BufferB).

Manually switch to 100% Buffer B. Approximately 2 to 3 fractions later Scytovirin derivatives should elute as a symmetrical peak with an $A_{280}$ between 2000-2500 on an AKTA FPLC. Pool relevant fractions and dialyze in a 3 to 12 mL 10 kDa molecular weight cutoff cassette with 50 µL of thrombin versus 2 L of 20 mM Tris-HCl, pH 7.6 with 50 mM NaCl for 4 hours at 4 °C.

Transfer solution to a 2059 tube and add 1M MgCl$_2$ to a final concentration of 2 mM. Add 1.5 to 2 µL of rEK (Novagen, Cat. # 69066, ~ 2.1 U/µl) per mL of Scytovirin and incubate overnight at 32 °C.

**Reverse Phase HPLC Purification Procedure:**

1 µL of PMSF was added per 1 mL of Scytovirin solution. Centrifuge cut Scytovirin derivative at 20,000 rpms in a microfuge for 20 minutes. Load and inject 5 mL aliquots onto a C18 column (BioRad, HiPore RP318, 10mm X 250mm, Cat. # 155-9111) equilibrated in Buffer A. Run “sc2_improved” method: 0-24% Buffer B from 0 to 41 minutes, 24-57% Buffer B from 41 to 50 minutes, 57-90% Buffer B from 50 to 59 minutes, 90-0% Buffer B from 59 to 70 minutes. Collect 1 mL fractions. Properly folded Scytovirin derivatives elute at a retention time of 40 to 41 minutes. A smaller peak just after this elution at 41 to 43 minutes is misfolded protein. Pool relevant samples and equilibrate C18 column in methanol until next use. Freeze pooled samples in liquid nitrogen and lyophilize for 48 hours. Weigh dry weight of Scytovirin derivative. Store Scytovirin desiccated in foil at room temperature.

**Post-HPLC Dialysis for NMR Preparation:**

Pool relevant samples. 3.1 mL of 0.05% TFA buffer was added to pooled samples. 9.3 mL of Scytovirin NMR buffer was added to the pooled samples. Mixture was pipetted into dialysis tubing. Mixture was dialyzed versus 1 L of NMR buffer at 4 °C, overnight.
Scytovirin NMR Buffer:
4.264g of MES powder, 222 µL of 0.45M EDTA stock and 10 µL of 1M NaN₃ stock were combined and diluted with 1 L of ddH₂O. Final concentrations were 20 mM MES, pH 5.5, 100 µM EDTA, 10 µM NaN₃.

Competent Origami Cell Procedure:
Inoculate 50 mL LB with a single colony of *E. coli*. Grow overnight at 37 °C and 250 rpms.
Inoculate 4 mL culture into 400 mL LB in a sterile, 2-liter flask. Grow at 37 °C, 250 rpms, to an OD₅₉₀ of 0.375. Aliquot culture into 8 50-mL prechilled sterile polypropylene tubes and leave on ice for 5 to 10 minutes. Centrifuge tubes for 7 minutes at 16,000 x g at 4 °C. Discard supernatant and gently resuspend pellet in 10 mL ice-cold CaCl₂ solution. Centrifuge 5 minutes at 1,100 x g at 4 °C. Discard supernatant and resuspend in 10 mL CaCl₂. Keep on ice for 30 minutes.
Centrifuge for 5 minutes at 1,100 x g at 4 °C. Discard supernatant and resuspend pellet in 2 mL CaCl₂. Dispense 250 µL aliquots into prechilled, sterile polypropylene tubes and freeze immediately at -80 °C.

1% DNA Agarose Gel:
35 mL TAE 1x buffer, 0.35g agarose gel, and 12 µL ethidium bromide were combined in a sterile, 50 mL plastic beaker and microwaved on high for 45 seconds. Mixture was poured into DNA gel mold, comb inserted and allowed to set. Polymerized gel was placed in electrophoresis cell. 1x TAE buffer was used to cover the gel. All gels run at 120 mA.

50 mM NaP, 300 mM NaCl pH 7.5:
100 mL of 500 mM stock NaP, and 70 mL of 4.2 M NaCl filled to 1 liter with ddH₂O, pH adjusted to 7.5. Stock used for making buffers for FPLC, 600 mL used for Buffer A without derivation and 400 mL used for Buffer B.

Buffer for Removal of Imidazole from Scytovirin:
40 mL of 1M Tris, and 24 mL of 4.2 M NaCl filled to 2 liters with ddH₂O. pH adjusted to 7.6.
**FPLC Buffer B:**

400 mL of 50 mM NaP 300 mM NaCl pH 7.5 was added to 4.085 g of Imidazole (fw = 68.08 g).

**LB Media:**

8.00 g of LB agar into a sterile, 600 mL glass beaker. ddH$_2$O added to the 400 mL mark. Place on stirrer/hotplate until agar fully dissolves. Separate into sterile glass bottles and autoclave on the liquid setting.

**50 mg/mL X-Gal:**

0.05 g of X-Gal into 1 mL DMSO.

**NZY$^+$ Broth:**

10 g of NZ amine (casein hyrdolysate), 5 g of yeast extract, 5 g of NaCl, and fill to 1 liter with ddH$_2$O. Adjust pH to 7.5 using NaOH. Autoclave on the liquid setting. Before use add: 12.5 mL of 1M MgCl$_2$, 12.5 mL of 1M MgSO$_4$, 10 mL of a 2M filter-sterilized glucose solution and then filter sterilize.

**10x Reaction Buffer for QuikChange site-directed Mutagenesis:**

100 mM KCl, 100 mM (NH$_4$)$_2$SO$_4$, 200 mM Tris-HCl (pH 8.8), 20 mM MgSO$_4$, 1% Triton X-100, 1 mg/mL nuclease-free bovine serum albumin (BSA).

**TE Buffer:**

10 mM Tris-HCl pH 7.5, 1 mM EDTA

**HPLC Buffer A:**

HPLC grade H$_2$O that is 0.05% TFA.

**HPLC Buffer B:**

90% HPLC grade acetonitrile, 10% HPLC grade H$_2$O solution that is 0.05% TFA.
BIBLIOGRAPHY


surface envelope glycoprotein gp120: potential application to microbicide development. 


Appendix A: Experimental Data
Scytovirin_C - Vial 1 Inj 1 SD1SD2SD2_WT - Channel 1

Current Data Path: C:\WIN32APP\D2000HSM\Test\DATA\0260
Data Desc.: Ch1(2D)Data
Vial Number: 1   Inj Number: 1   Sample Name: SD1SD2SD2_WT_Linker_062211
Scytovirin_C - Vial 1 Inj 1 SD1SD2SD2_wt - Channel 1

Current Data Path: C:\WIN32APP\D2000HSM\Test\DATA\0411
Data Desc.: Ch1(2D)Data
Vial Number: 1    Inj Number: 1    Sample Name: SD1SD2SD2_wt_rEK2thr_7_25_12
SD1 - SD2 - SD2 WT

100% @ 23

14½ @ 12

SD1 - SD2 - SD2 WT M.K. 7/20/11

50 mV
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