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DESIGN AND EVALUATION OF 5'-O-DICARBOXYLIC AND POLYARGININE FATTY ACYL DERIVATIVES OF ANTI-HIV NUCLEOSIDES

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**DESIGN AND EVALUATION OF 5'-O-
DICARBOXYLIC AND POLYARGININE FATTY
ACYL DERIVATIVES OF ANTI-HIV NUCLEOSIDES**

BY

BHANU PRIYA, PEMMARAJU VENKATA

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE MASTER'S DEGREE
IN
BIOMEDICAL AND PHARMACEUTICAL SCIENCES**

UNIVERSITY OF RHODE ISLAND

2014

MASTER OF SCIENCE THESIS
OF
BHANU PRIYA, PEMMARAJU VENKATA

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2014

ABSTRACT

2',3'-Dideoxynucleoside (ddNs) analogs are the most widely used anti-HIV drugs in the market. Even though these drugs display very potent activities, they have a number of limitations when are used as therapeutic agents. The primary problem associated with ddNs is significant toxicity, such as neuropathy and bone marrow suppression. Due to their hydrophilic nature, they have poor cellular uptake. Some of these nucleoside analogs develop resistance over a period of time. Furthermore, these analogs themselves are not pharmacologically active, and undergo three-step phosphorylation in order to become active. Of these steps, the first step is usually a slow and rate-limiting process.

Herein, we report the synthesis and evaluation of 2',3'-dideoxynucleoside conjugates with fatty acids with varying chain length and fatty acyl poly-*L*-arginine derivatives. The hypothesis of this project was that conjugating the nucleosides with fatty acids or fatty acyl poly-*L*-arginine derivatives will generate multifunctional agents with enhanced anti-HIV activity and cellular uptake, and less drug resistance development when compared to that of the parent nucleoside alone. Furthermore, the compounds will also have longer duration of action through sustained intracellular release of the parent nucleosides. The fatty acid conjugates of nucleosides could have higher uptake into the cells because of their enhanced lipophilicity. This dissertation will be revealed in two manuscripts.

In the first **Manuscript**, the synthesis and anti-HIV activities of 5'-*O*-dicarboxylic fatty acyl monoester derivatives of 3'-azido-3'-deoxythymidine (zidovudine, AZT), 2',3'-didehydro-2',3'-dideoxythymidine (stavudine, d4T), and 3'-fluoro-3'-

deoxythymidine (alovudine, FLT) are discussed. The compounds were synthesized to improve the lipophilicity and potentially the cellular delivery of parent polar 2',3'-dideoxynucleoside (ddN) analogs. Three different fatty acids with varying chain length of suberic acid (octanedioic acid), sebacic acid (decanedioic acid), and dodecanedioic acid were used for the conjugation with the nucleosides. Among all the compounds, 5'-*O*-suberate derivative of AZT (**1**, EC₅₀ = 0.10 nM) exhibited the best anti-HIV profile when compared to other fatty acyl derivatives. The compound showed 80-fold higher anti-HIV activity than AZT without any significant toxicity (TC₅₀ > 500 nM). This work was published in *Tetrahedron Letters* (**2014**, <http://dx.doi.org/10.1016/j.tetlet.2014.02.001>).

In the second Manuscript, synthesis, anti-HIV activity, and preformulation tests of poly-*L*-arginine cell-penetrating peptides (CPPs) conjugated with fatty acyl derivatives of anti-HIV nucleosides, FLT, lamivudine (3TC) and emtricitabine (FTC) are discussed. All conjugates exhibited less anti-HIV activity when compared with the parent nucleoside analogues. For example, poly-*L*-arginine-fatty acyl derivative of 3'-fluoro-3'-deoxythymidine, FLT-CO-(CH₂)₁₂-CO-(Arg)₇, exhibited EC₅₀ values of 2.9 μM and 3.1 μM against X4 and R5 cell-free virus, respectively, while the FLT had EC₅₀ values of 0.2 μM and 0.1 μM, suggesting the limited uptake or intracellular hydrolysis to the parent analogue. Further preformulation studies were done on the FLT-conjugate by determining the lipid solubility (partition coefficient) and solution state degradation. The compound was found to be stable in acidic and alkaline conditions. The partition coefficient (Log P) of FLT conjugate was found to be -0.34. The derivative was also evaluated in dissolution studies using four different hydrogels

with and without a thermogelling polymer. Gel formulations of the compound were manufactured using non-ionic (HPC-SL) and anionic (Carbopol) polymers with and without the inclusion of a thermo-reversible gelling (Pluronic F-127) polymer. The compound was found to be unstable in the manufactured formulations as shown by HPLC profiles. This work has been submitted to a peer-reviewed journal.

In summary, the studies provided more insights in designing new generation of anti-HIV nucleoside conjugates with fatty acids and poly-arginine CPPs. Overall, we have established a strategy for generation more potent nucleoside conjugates as shown in **Manuscript I**. Furthermore, the data indicated that addition of positively-charged poly-arginine to the nucleoside analogs is not beneficial in generating more potent anti-HIV conjugates as demonstrated in **Manuscript II**, possibly because of the presence of the positively charged viral glycoprotein gp120. Further optimization of conjugated CPP-fatty acid-nucleoside conjugates is required to generate compounds with improved anti-HIV activity and optimized stability and formulation performance.

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PREFACE

This thesis is submitted for the degree of Master of Science at the University of Rhode Island and manuscript format is used to write the thesis. The research described herein was conducted under the supervision of Professor Keykavous Parang in the Department of Biomedical and Pharmaceutical Sciences. It is written and formatted following the guidelines provided by the University of Rhode Island Graduate School.

MANUSCRIPT I: Synthesis and Biological Evaluation of 5'-*O*-Dicarboxylic Fatty Acyl Monoester Derivatives of Anti-HIV Nucleoside Reverse Transcriptase Inhibitors

This manuscript was published in "Tetrahedron Letters", January 2014.

MANUSCRIPT II: Design, Synthesis, Antiviral Activity, and Pre-formulation Development of Poly-*L*-Arginine-Fatty acyl Derivatives of Nucleoside Reverse Transcriptase Inhibitors

This manuscript was submitted to a peer-reviewed journal, March 2014.

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INTRODUCTION

1. Acquired Immunodeficiency Syndrome (AIDS)

According to UNAIDS estimation, 2.6 million people were infected with Human Immunodeficiency Virus (HIV) infection and 1.7 million people died of Acquired Immunodeficiency Syndrome (AIDS) in 2011. This deadly syndrome is caused by infection by enveloped retrovirus HIV. HIV infects CD4⁺ T-lymphocytes, macrophages, monocytes and follicular dendritic cells, resulting in suppression of the host's immune system. Cell death, syncytia formation, neuronal cell damage, dementia and myelopathy are the consequences of HIV infection (Staprans, 1997). A person infected with HIV shows illness trajectory characterized by clinical latency and an asymptomatic infection caused due to persistent viremia and humoral immune response (Brennan et al., 1997).

2. Human Immunodeficiency Virus Structure

HIV is 100-120 nm in diameter and belongs to the Lentivirus family. There are two types of HIV, HIV-1 and HIV-2. HIV-1 is the most predominant type of the virus. HIV-1 is capable to mutate and has M (10 different subtypes) and O subtypes (Quinn, 1996).

HIV consists of central viral core and is surrounded by a lipid bilayer membrane (Figure 1). The membrane is interspersed with glycoprotein gp160 that is constituted from gp120 and gp41 glycoproteins. The gp120 trimer is non-covalently attached to the ectodomain gp41 transmembrane binding protein (Biscone et al.,

2002). These glycoproteins are responsible for the viral binding to the host CD4⁺ T lymphocytes. The core viral capsule consists of p24, p7, and p9 core proteins and is surrounded by p17 protein. The capsid also includes two RNA strands and various viral enzymes like reverse transcriptase RNase, polymerase, integrase, and protease, which are responsible for viral replication. The genes responsible for HIV-1 structural proteins and replication are *gag*, *pol*, *env*, *vif*, *vpu*, *vpr*, *tat*, *rev* and *nef*.

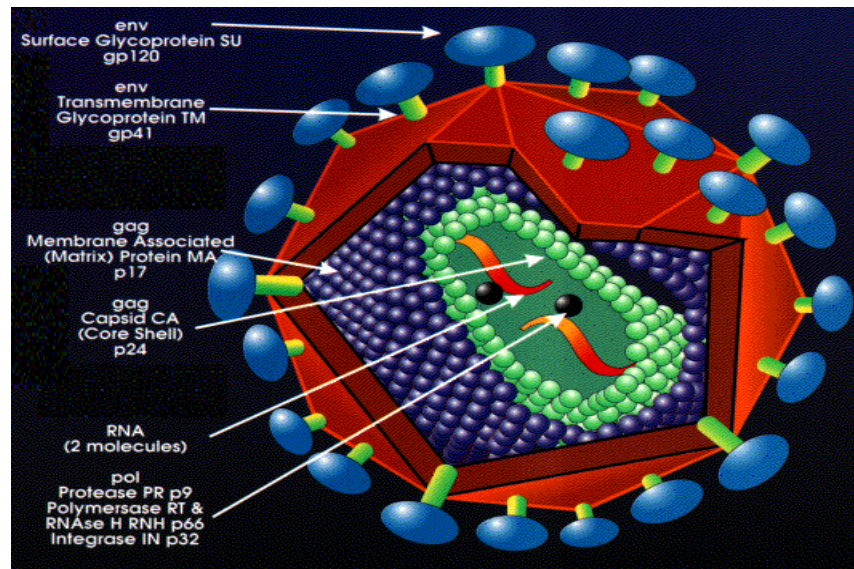


Figure 1. Structure of HIV-1

(Courtesy: <http://www.stanford.edu/~rabriggs/hiv/hiv.html> 9/15/12)

3. HIV Receptors and Strains

Various HIV-1 isolates recognize two different kinds of co-receptors, CCR5 and CXCR4. CCR5 co-receptors are expressed on macrophages and CD4 T cells and are called as R5 strains. CXCR4 co-receptors are expressed on CD4 T cells and are termed as X4 strains. CCR5 receptor plays a significant role in HIV transmission and R5 strains are the primary cause of HIV infection (O'Hara and Olson, 2002).

During the viral binding process, the positively charged viral glycoprotein gp120 interacts with negatively charged CD4 receptor, and CCR5- and CXCR4 coreceptors (Kajumo et al., 2000, Cheng-Mayer et al., 1997). These chemokine receptors are interspersed between the membranes and belong to two different classes of receptors, C-X-C (α -receptor) and C-C (β -receptor) (Deng et al., 1996). Therefore even HIV is divided into two types of strains, R5 and X4. R5 strains of virus uses CCR5 coreceptor and the X4 strains use the CXCR4 coreceptor to help attachment to the T lymphocytes.

4. HIV-1 Life Cycle

The life cycle of the HIV-1 is divided into two phases: HIV-1 entry and development of infection resulting in viral assembly and budding. CD4⁺ host cell marker is required for the HIV-1 to undergo replication. The viral envelope glycoprotein (gp160) binds to the CD4 receptor and coreceptor. This energetically unfavorable process is driven by the conformational changes in the viral glycoprotein during the fusion process (Wyatt and Sodroski, 1998). The viral glycoprotein gp120 helps in binding of the virus to the host CD4⁺ T lymphocytes. The fusion of the HIV-1 envelope to the host cell membrane is aided by gp41 glycoprotein. CD4 receptor viral attachment induces conformational changes in gp41 enabling the insertion of *N*-terminal hydrophobic peptide into the T-lymphocyte cell membrane. This is followed by rearrangement of the helical regions in ectodomain gp41 to form stable six-helix unit. The cofactors fusion and CC CKR5 are also essential for the viral entry. The virus penetrates into the cell and releases the viral content into the host cell cytoplasm

(Rowe, 1996). Hence, the viral receptors and the glycoprotein domain are major targets for the small molecule, entry, and/or fusion inhibitors.

The HIV-1 needs to make proviral DNA from RNA in order to undergo replication. Reverse transcriptase (RT) catalyzes the formation of viral DNA and it consists of two enzymes: RNase and polymerase enzymes. The RNA is converted to DNA in the presence of RNase of RT. The DNA is duplicated to form double stranded DNA by polymerase enzyme activity of RT. The newly formed DNA then integrates with the host DNA in the nucleus in the presence of HIV integrase enzyme to form HIV-1 provirus (Figure 2). The proviral DNA undergoes transcription by cellular polymerases to RNA and upon translation basal amounts of Tat, Rev and Nef are produced (Jordan et al., 2001).

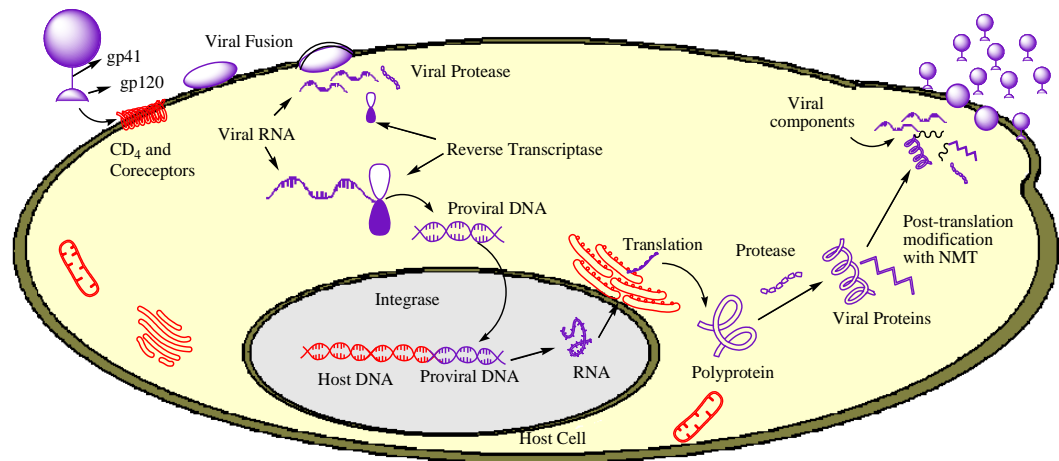


Figure 2. Life cycle of HIV (Agarwal, 2008).

Translation is activated when Tat binds to the TAR portion of the LTR and messenger RNAs are produced (Sierra et al., 2005). This mRNA moves out of the nucleus to facilitate formation of the polyproteins and enzymes, which are the

precursors of the capsid components (Figure 2). The protease enzyme splits the large polyproteins resulting in the formation of smaller glycoproteins and enzymes of the virus core. These polyproteins undergo myristoylation at *N*-terminal glycine residue with the aid of *N*-myristoyl transferase (NMT) enzyme (Figure 2). This process makes the polyproteins more lipophilic and allows them to move towards the cell membrane. The components will bud out together to form HIV-1 virions, which will undergo more changes and mature into infectious HIV-1 virus.

5. Anti-Retroviral Drugs

The primary target for blocking HIV replication is the RT enzyme (Clercq, 1992). The main class of drugs acting against this enzyme is nucleoside reverse transcriptase enzyme inhibitors (NRTIs). Common anti-HIV NRTIs include 2',3'-dideoxynucleoside (ddN) analogs. Approved drugs by Food and Drug Administration (FDA) include 3'-azido-3'-deoxythymidine (zidovudine, AZT), 2',3'-dideoxy-3'-thiacytidine (lamivudine, 3TC), 2',3'-didehydro-2',3'-dideoxythymidine (stavudine, d4T), 2',3'-dideoxy-5-fluoro-3'-thiacytidine (emtricitabine, FTC), 2',3'-dideoxycytidine (zalcitabine, ddC), and 3'-fluoro-3'-deoxythymidine (FLT).

The mechanism of NRTIs is inhibiting of viral RNA conversion to proviral DNA. NRTIs interrupt RNA-DNA transcription in the virus-infected cells. These analogs themselves are not pharmacologically active, and undergo three-step phosphorylation to become active. The anti-HIV NRTIs are phosphorylated intracellularly to mono-, di- and triphosphates by cellular kinases.

This triphosphate form acts as reverse transcriptase substrate, competes with the naturally occurring nucleosides, and is incorporated into the growing proviral DNA chain (Tan et al., 1999). NRTIs do not have a hydroxyl group at 3' position of the ribose (Lee et al., 2001). The incorporation of nucleoside analogs in DNA causes chain termination and inhibition of elongation, and thereby inhibits viral replication.

Current antiretroviral nucleosides do not eliminate the virus thoroughly and complete restoration of the immune functions does not occur. There are several complications associated with antiretroviral therapy like poor adherence, limited access, low therapeutic index, and short duration of action due to the nucleoside short half-life, dependence on cellular kinases for activation and development of mutation in HIV-1 that causes viral resistance to these drugs (Parang et al., 2000). The primary problem associated with ddNs is significant toxicity such as neuropathy and bone marrow suppression.

Furthermore, NRTIs have limited cellular uptake and bioavailability owing to their hydrophilic nature. To overcome their limited cellular uptake, various prodrugs, ddN analogs have been synthesized with increased lipophilicity (Parang et al., 1998, 1997, Agarwal, 2011).

The so-called highly active antiretroviral therapy (HAART) includes at least two different classes of antiretroviral drugs. HAART has reduced plasma viremia to unnoticeable levels and increased CD4⁺ T cell count. This therapeutic strategy has many limitations that include serious side effects, development of viral resistance, and pharmacokinetic interactions (Duran et al., 2001).

It has been demonstrated that even under HAART therapy there is persistent viral replication undergoing in the lymph nodes and lymphoid tissues (Hamer, 2004). This is due to low levels of the drug and presence of residual virus in these tissues. Lymphoid and central nervous system (CNS) tissues serve as viral reservoirs. Thus, sufficient drug exposure and delivery to these tissues are critical regardless of their target of action. In order to maximize the antiviral potential of a particular therapy and to prevent recurring infections, development of novel, safe, less toxic, and more effective broad-spectrum anti-HIV drugs targeting different stages of the viral life cycle. Furthermore, drug delivery approaches that reduce the residual viral load are still needed to help prevent the progression of the HIV infection.

There are no candidate vaccines in the pipeline that can induce sterilizing immunity and protect against infection with HIV. Therefore, there is an urgent need to develop additional safe and effective treatment and preventative strategies. One of those strategies has become known as microbicides, topically applied agents to the vagina or rectum that prevent or reduce transmission of sexually transmitted organisms, in particular HIV/AIDS.

The development of a female controlled prevention method in the form of a topical microbicide to protect a woman from contracting HIV remains a high priority. Despite the successful outcome of the Caprisa 004 trial (Abdool Karaim et al., 2010), the most advanced product available (Tenofovir gel) is still only partially effective in preventing the transmission of HIV. For this reason, it is critical to continue the development of a pipeline of potent and novel inhibitors that have the potential to be effective as microbicides. In addition, it is critical to define optimized delivery

methods for these products that will place the microbicide at the right place, at the right time, and at the right concentration to prevent the initial infection events in the vagina and/or rectum.

Current development in the microbicide field has become highly focused on anti-HIV compounds approved for therapeutic use. These include the NRT inhibitor tenofovir, the non-nucleoside RT inhibitor dapivirine, and the CCR5 antagonist maraviroc, as well as various combinations of these drugs (Abdool Karim et al, 2010, Mertenskoetter et al., 2011). Since complete transmission inhibition has not yet been achieved with any microbicide product, it becomes even more important to continue to exploit the potential of other non-FDA approved antiretroviral compounds with varying mechanisms of action.

6. Objectives of Research

The conjugation of nucleosides with fatty acyl and polyarginine-fatty acyl residues are investigated to generate anti-HIV agents and to circumvent some of the problems associated with using parent anti-HIV nucleosides. This combination may result in development of anti-HIV microbicides having enhanced efficacy, longer duration of action by sustained intracellular release of active substrates at adequate concentrations, and/or higher uptake into the infected cells. The combination of these residues with NRTIs may reduce the toxicity associated with nucleosides. Furthermore, development of viral resistance to two active drugs would occur at lower rate than to either compound alone.

Manuscript I

The first **Manuscript** discusses the synthesis and biological evaluation of fatty acyl derivatives of antiretroviral nucleosides that include AZT, FLT and d4T. The fatty acyl conjugates of nucleosides are proposed to act against HIV by inhibiting the viral enzymes, RT and NMT, by nucleosides and fatty acid analogs, respectively. These bifunctional agents are expected to improve the cellular uptake by enhancing the lipophilicity of the conjugates, to reduce the cytotoxicity of the parent nucleosides, and to show higher genetic barrier to resistance through dual mechanism of antiviral action.

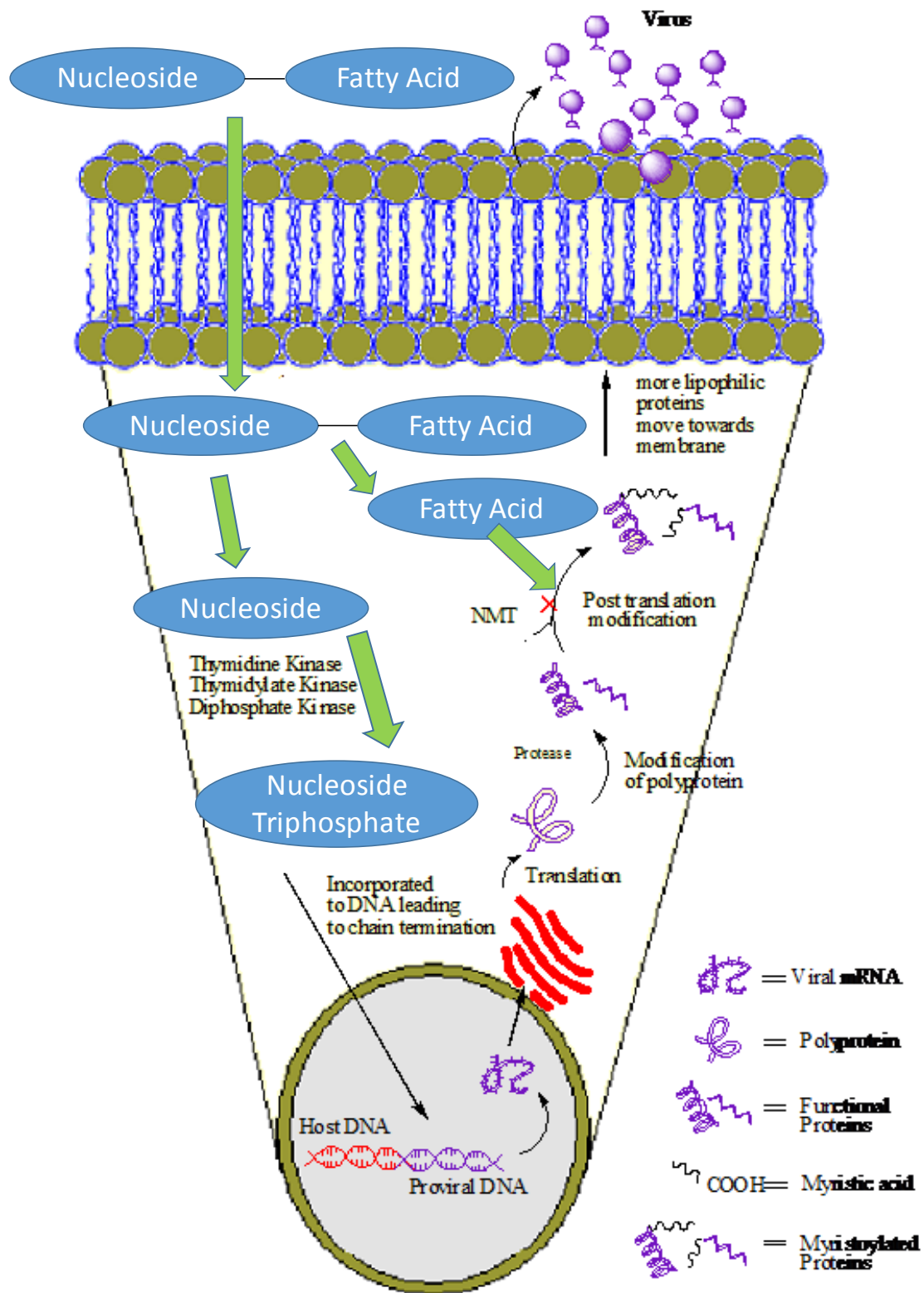


Figure 3. Proposed mechanism of action of 5'-O-fatty acyl derivatives of nucleosides.

The main purpose of this project is to develop bifunctional anti-HIV agents by covalently attaching nucleoside reverse transcriptase inhibitors to fatty acids with varying chain length (Figure 4). The fatty acid attachment will increase the lipophilicity and thereby improve the cellular uptake of these bifunctional conjugates. These specifically designed bifunctional agents provide a novel approach to design highly efficacious anti-HIV microbicides. Nucleosides and fatty acid act as reverse transcriptase inhibitors and viral NMT inhibitors, respectively (Fig 3). This bifunctional strategy yields antiviral agents with enhanced efficacy, longer duration of action provided by sustained intracellular drug release and higher cellular uptake.

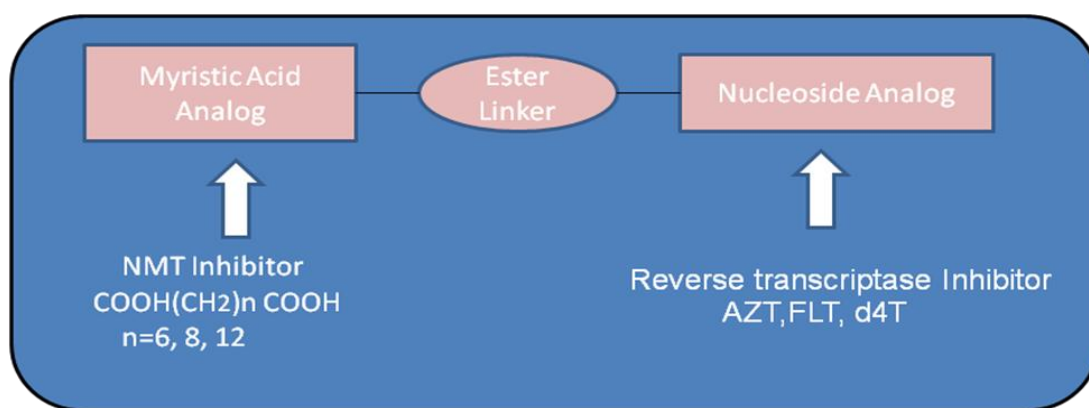


Figure 4. Design of 5'-O-(fatty acyl) ester nucleoside derivatives

Manuscript II

The second manuscript discusses the synthesis and biological evaluation of polyarginine-fatty-acyl derivatives of anti-HIV nucleosides, FLT, FTC and 3TC. The main objective of the conjugation of poly-L-arginine residues to fatty acylated nucleosides was to improve the efficacy and delivery of the agents in HIV-1-infected cells. Once it enters the cells, the conjugate undergoes intracellular hydrolysis to release the three components, polypeptide, fatty acid and the anti-HIV nucleoside. The

fatty acyl and nucleoside derivatives exert their action against reverse transcriptase (RT) and *N*-myristoyl transferase (NMT) viral enzymes. The fatty acyl moiety increases the lipophilicity and thereby improves the cellular uptake of the conjugates. The poly-L-arginine component is involved not only as a carrier but also acts as a cell-penetrating peptide due to the presence of many positively charged guanidinium groups.

Another objective of designing both classes of compounds in Manuscripts I and II is to develop anti-HIV microbicides for vaginal application. The conjugation of fatty acyl or poly-L-arginine-fatty acyl residue to the nucleoside may result in microbicides with enhanced efficacy and longer duration of action. Among compounds in class II, FLT conjugate was selected and its stability under different conditions was evaluated. Preformulation studies were conducted to determine the solution state degradation and partition coefficient of the selected conjugate. The dissolution studies were conducted by manufacturing four different hydrogels with or without polymer. These studies evaluate the stability and formulation performance of the compound in the vaginal hydrogels intended to act as microbicidal agents.

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Manuscript I

Synthesis and Biological Evaluation of 5'-O-Dicarboxylic Fatty Acyl Monoester Derivatives of Anti-HIV Nucleoside Reverse Transcriptase Inhibitors

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1.1. Abstract

A number of 5'-*O*-dicarboxylic fatty acyl monoester derivatives of 3'-azido-3'-deoxythymidine (zidovudine, AZT), 2',3'-didehydro-2',3'-dideoxythymidine (stavudine, d4T), and 3'-fluoro-3'-deoxythymidine (alovudine, FLT) were synthesized to improve the lipophilicity and potentially the cellular delivery of parent polar 2',3'-dideoxynucleoside (ddN) analogs. The compounds were evaluated for their anti-HIV activity. Three different fatty acids with varying chain length of suberic acid (octanedioic acid), sebacic acid (decanedioic acid), and dodecanedioic acid were used for the conjugation with the nucleosides. The compounds were evaluated for anti-HIV activity and cytotoxicity. All dicarboxylic ester conjugates of nucleosides exhibited significantly higher anti-HIV activity than that of the corresponding parent nucleoside analogs. Among all the tested conjugates, 5'-*O*-suberate derivative of AZT ($EC_{50} = 0.10$ nM) was found to be the most potent compound and showed 80-fold higher anti-HIV activity than AZT without any significant toxicity ($TC_{50} > 500$ nM).

1.2. Introduction

Highly Active Antiretroviral Therapy (HAART) for the treatment for human immunodeficiency virus (HIV) includes using different classes of anti-HIV agents, including nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) along with protease inhibitors (Agarwal, 2008). 2',3'-Dideoxynucleoside (ddN) analogs are similar to naturally occurring nucleosides, but they cannot be used for the synthesis of new DNA as they lack the 3'-hydroxyl group. These agents act as nucleoside reverse transcriptase inhibitors (NRTIs) and thereby cause DNA chain termination. NRTIs have shown severe adverse effects, mitochondrial toxicity, and resistance to multi-drug resistant HIV (Otto, 2004; Tan et al., 1999; Lewis et al., 2006; Lund et al., 2007). Furthermore, nucleoside analogs are polar in nature and have limited cellular uptake. Novel anti-HIV agents are urgently needed with a better safety and resistance profile for the prevention and treatment of HIV infection.

Prevention of HIV transmission to women is essential to avoid transmission of the virus to the newborn. Nevirapine, a non-nucleoside reverse transcriptase inhibitor is known to lower the risk of HIV-1 transmission in breastfeeding population by nearly 50% during the first 14-16 weeks of life (Laura et al., 1999). Currently there are no vaccines developed to provide protection against HIV. Thus, it is a necessity to develop additional safe, effective treatment and preventative strategies.

Microbicidal agents are agents which are topically applied to the vagina or rectum to prevent the transmission of sexually transmitted organisms. Microbicides which are focused on the prevention of HIV transmission target vaginal, cervical or rectal

mucosa and may act via several mechanisms, such as the non-specific direct inactivation of the virus (Balzarini and Damme, 2007). Currently known microbicides in development, such as UC-781 (thiocarboxanilide), TMC-120 (dapivirine), MIV-150, and tenofovir, act specifically via a single mechanism of the inhibition of reverse transcriptase enzyme (Mauck and Doncel, 2001). Developing new anti-HIV microbicides that are multifunctional anti-HIV agents is urgently needed to reduce the incidence of drug resistance. Furthermore, the lipophilic microbicides could bind tightly or irreversibly to the HIV envelope, leading to inactivation of cell-free, or cell-associated virus or both resulting in a significant decrease in virus transmission.

Furthermore, there have been several reports on lipids acting as antiviral and antibacterial agents (Thormar et al., 1987). The medium length chain saturated fatty acids are one of those agents that inhibit enveloped viruses like HIV and herpes simplex virus type 1 (HSV-1) (Kristmundsdottir, 1999). Microbicidal hydrogels of these fatty acids have been developed and evaluated, and they possibly act by disrupting the viral lipid membrane (Kristmundsdottir, 1999). HIV-1 replication is inhibited by heteroatom substituted myristic acid analogs without showing any significant cellular toxicity. Myristoylated HIV proteins include PR160*gag-pol*, Pr55*gag*, p17*gag*, and p27*nef*. Myristic acid analogs inhibit the *N*-myristoyl transferase (NMT) that catalyzes the *N*-myristoylation of HIV proteins (Langner et al., 1992). 2-Methoxydodecanoic acid, 4-oxatetradecanoic acid, and 12-thioethyldodecanoic acid have shown to reduce HIV-1 replication in acutely infected T-lymphocytes (Parang et al., 1997). For example, 12-thioethyldodecanoic acid

derivative was moderately active ($EC_{50} = 9.4 \mu M$) against HIV-infected T4 lymphocytes.

Several studies have demonstrated the use of lipids as active intravaginal microbicide agents to protect against various sexually transmitted infections (STIs) (Thormar et al., 1987; Langner et al., 1992; Kristmundsdottir, 1999). When lipid-associated drugs are administered subcutaneously, they are usually distributed throughout the lymphoid system with improved stability, and the drug can be delivered at higher concentrations (Kinman et al., 2003). This is a major advantage since HIV is hidden in the lymphatic system not accessible by many commercially available polar anti-HIV nucleosides.

Thus, designing lipophilic anti-HIV nucleosides for developing anti-HIV agents or anti-HIV microbicides is a subject of major interest. The most predominant approach to this strategy is the esterification strategy between nucleosides and fatty acids. The *in vitro* chemical stability of ester conjugates helps to produce formulations with adequate shelf lives. The esters also act as the substrates for esterase enzyme and, therefore, are labile *in vivo* (Sriram et al., 2004). The 5'-*O*-hydroxyl esterification of the anti-HIV nucleosides also provides a viable approach to increase cellular delivery of polar nucleosides. We have previously shown that fatty acyl monocarboxylic ester derivatives of 3'-azido-3'-deoxythymidine (zidovudine, AZT) (Parang et al., *Antiviral. Chem. Chemother.* **1998**, Parang et al., **2000**), 3'-fluoro-3'-deoxythymidine (alvudine, FLT) (Parang et al., *Nucleosides & Nucleotides* 1998), (-)-2',3'-dideoxy-3'-thiacytidine (lamivudine, 3TC) (Agarwal et al., 2012), 5-fluoro-(-)-2',3'-dideoxy-3'-thiacytidine (emtricitabine, FTC) (Agarwal et al., 2013), and 2',3'-didehydro-2',3'-

dideoxythymidine (stavudine, d4T) (Agarwal et al, 2011) with enhanced anti-HIV activity against CXCR4-tropic, CCR5-tropic cell-associated, and/or multi-drug resistant strains of virus. The fatty acid substitution with FTC and 3TC showed a significant increase in their cellular uptake in comparison to their parent nucleosides (Agarwal et al, 2012, 2013).

Herein, we report the synthesis and evaluation of novel 5'-*O*-fatty acyl ester derivatives of AZT, d4T, and FLT using dicarboxylic fatty acids. As shown before for other conjugates, fatty acids were expected to improve the lipophilicity of polar nucleoside analogs and cellular uptake and to generate lipophilic agents with higher anti-HIV activity. Dicarboxylic acids instead of monocarboxylic fatty acids were selected to generate more amphipathic property in the structure of conjugates due to the presence of additional polar free carboxylic acid.

1.3. Materials and Methods

1.3.1. Materials

FLT and AZT were purchased from Euro Asia Tran Continental (Bombay, India). Stavudine (d4T) was purchased from Kemprotec (Middlesbrough, U.K.). HBTU, HOBt, 1,6-octanedioic acid, 1,8-decanedioic acid, and 1,12-dodecanedioic acid were purchased from Sigma Aldrich Chemical Co. Solvents and all the other reagents were purchased from Fisher scientific. Phenomenex®Gemini 10 μ m ODS reversed-phase column (2.1 \times 25 cm) was used along with Hitachi HPLC system for the purification of the synthesized final products. The HPLC method was a gradient system at a constant flow rate of 18 ml/min (Table 1.1).

Hitachi analytical HPLC system was used to determine the purity of the compounds. Analytical C18 column (Grace Allsphere ODS 2-3 μ , 150 \times 4.6 mm) with water:acetonitrile (30:70 v/v) system at a constant flow rate of 1 ml/min was used to determine the purity of the final products. The compounds showed UV detection at 265 nm. A Bruker NMR spectrometer (400 MHz) was used for characterization, and the chemical shifts were reported in parts per millions (ppm). The chemical structures of the final products were confirmed by high-resolution PE Biosystems Mariner API time-of-flight electrospray mass spectrometry.

1.3.2. Chemistry

1.3.2.1. General method for the synthesis of 5'-*O*-(fatty Acyl) esters of FLT, d4T and AZT (1–7). Nucleoside (AZT, FLT, or d4T, 0.58 mmol), fatty acid ($\text{HOOC}(\text{CH}_2)_n\text{COOH}$, (suberic acid (octanedioic acid, $n = 8$), sebacic acid (decanedioic, $n = 10$), and dodecanedioic acid ($n = 12$), 3.8 mmol), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 720 mg, 1.9 mmol), 1-hydroxybenzotriazole (HOBt (255 mg, 1.2 mmol), diisopropylcarbodiimide (DIC, 200 μ L, 1.15 mmol), and *N,N*-diisopropylethylamine (DIPEA, 2 mL, 15 mmol) were dissolved in dry *N,N*-dimethylformamide (DMF, 10 mL). The reaction mixture was stirred at room temperature overnight. The completion of reaction was confirmed by TLC. The mixture was concentrated and dried under vacuum. The residues were purified with reversed phase HPLC using C₁₈ column and water/acetonitrile as solvents (Table 1.1). The purity of the final products (>95%) was confirmed by using a Hitachi analytical HPLC system on a C18 column using

water:acetonitrile (30:70 v/v) at a constant flow rate of 1 mL/min with UV detection at 265 nm.

Table 1.1. HPLC method used for purification of the final products (**1-7**).

Time (min)	Water Concentration A (%)	Acetonitrile Concentration B (%)	Flow rate (mL/min)
0.00	100.0	0.0	1.0
1.00	100.0	0.0	18.0
45.0	40.0	60.0	18.0
50.0	0.0	100.0	18.0
55.0	0.0	100.0	18.0
60.0	100.0	0.0	18.0
60.1	100.0	0.0	1.0

8-[(3'-Azido-2',3'-dideoxythymidiny)-5'-yl]octandioate (1). Yield (100 mg, 20%); ¹H NMR (400 MHz, CD₃OD, δ ppm): 7.40 (s, 1H, H-6), 6.10 (t, *J* = 6.0 Hz, 1H, H-1'), 4.20-4.40 (m, 3H, H-3', H-5', H-5''), 4.00-4.15 (m, 1H, H-4'), 2.90-3.00 (m, 2H, -CH₂COOH), 2.80-2.90 (m, 2H, -CH₂COO), 2.20-2.50 (m, 4H, H-2' and H-2'', CH₂CH₂COOH and -CH₂CH₂COO), 1.85 (s, 3H, 5-CH₃), 1.50-1.80 (m, 2H, -CH₂CH₂CH₂COOH), 1.22-1.40 (m, 2H, -CH₂CH₂CH₂COO); HR-MS (ESI-TOF) (*m/z*): C₁₈H₂₅N₅O₇: calcd, 423.1754; found, 424.2143 [M + H]⁺.

10-[(3'-Azido-2',3'-dideoxythymidiny)-5'-yl]decandioate (2). Yield (120 mg, 24%); ^1H NMR (400 MHz, CD_3OD , δ ppm): 7.47 (s, 1H, H-6), 6.14 (t, $J = 6.4$ Hz, 1H, H-1'), 4.32-4.42 (m, 3H, H-3', H-5'), 4.29 (dd, $J = 4.0$ and 12.0 Hz, 1H, H-5''), 4.03 (dd, $J = 4.8$ and 9.2 Hz, 1H, H-4'), 2.34-2.50 (m, 4H, $-\text{CH}_2\text{COOH}$, H-2' and H-2''), 2.27 (t, $J = 7.4$ Hz, 2H, $-\text{CH}_2\text{COO}$), 1.89 (s, 3H, 5- CH_3), 1.50-1.70 (m, 4H, $\text{CH}_2\text{CH}_2\text{COOH}$, $\text{CH}_2\text{CH}_2\text{COO}$), 1.25-1.40 (br m, 8H, methylene protons); ^{13}C NMR (CD_3OD , 100 MHz, δ ppm): 177.56 (COOH), 174.75 (COOAZT), 166.15 (C-4 C=O), 152.06 (C-2 C=O), 137.73 (C-6), 111.76 (C-5), 86.54 (C-1'), 83.03 (C-4'), 64.51 (C-5'), 62.17 (C-3'), 37.77 (C-2'), 34.87 (CH_2COO), 30.21, 31.18, 30.12, 30.06, 26.00, 25.94, 25.89 (methylene carbons), 12.64 (5- CH_3); HR-MS (ESI-TOF) (m/z): $\text{C}_{20}\text{H}_{29}\text{N}_5\text{O}_7$: calcd, 451.2067; found, 452.2699 $[\text{M} + \text{H}]^+$.

12-[(3'-Azido-2',3'-dideoxythymidiny)-5'-yl]dodecandioate (3). Yield (115 mg, 23%); ^1H NMR (400 MHz, CD_3OD , δ ppm): 7.48 (s, 1H, H-6), 6.14 (t, $J = 6.3$ Hz, 1H, H-1'), 4.20-4.50 (m, 3H, H-3', H-5' and H-5''), 3.98-4.20 (m, 1H, H-4'), 3.55-3.75 (m, 4H, $-\text{CH}_2\text{COOH}$, $-\text{CH}_2\text{COO}$), 2.20-2.50 (m, 4H, $\text{CH}_2\text{CH}_2\text{COOH}$, H-2' and H-2''), 1.89 (s, 3H, 5- CH_3), 1.48-1.65 (m, 2H, $\text{CH}_2\text{CH}_2\text{COO}$), 1.25-1.35 (br m, 12H, methylene protons); ^{13}C NMR (CD_3OD , 100 MHz, δ ppm): 177.66 (COOH), 174.78 (COOAZT), 166.22 (C-4 C=O), 152.10 (C-2 C=O), 137.78 (C-6), 111.79 (C-5), 86.61 (C-1'), 83.11 (C-4'), 64.55 (C-5'), 62.22 (C-3'), 37.77 (C-2'), 34.98, 34.94, 34.84, 30.72, 30.64, 30.62, 30.42, 30.28, 30.23, 30.20, 26.12, 26.05, 25.99 (methylene carbons), 12.66 (5- CH_3); HR-MS (ESI-TOF) (m/z): $\text{C}_{22}\text{H}_{33}\text{N}_5\text{O}_7$: calcd, 479.2380; found, 480.2448 $[\text{M} + \text{H}]^+$.

10-[5'-O-(3'-Fluoro-2',3'-dideoxythymidiny)]decandioate (4). Yield (200 mg, 40%); ^1H NMR (400 MHz, CD_3CN , δ ppm): 7.44 (s, 1H, H-6), 6.18 (dd, $J = 5.6$ and 8.9 Hz, 1H, H-1'), 5.30 (dd, $J = 5.0$ and 53.2 Hz, 1H, H-3'), 4.47 (dt, $J = 3.5$ and 26.7 Hz, 1H, H-4'), 4.36 (dd, $J = 4.4$ and 12.1 Hz, 1H, H-5'), 4.24 (dd, $J = 3.5$ and 12.1 Hz, 1H, H-5''), 2.50-2.70 (m, 1H, H-2''), 2.20-2.40 (m, 5H, CH_2COOH , CH_2COO , H-2'), 1.87 (s, 3H, 5- CH_3), 1.50-1.65 (m, 4H, $\text{CH}_2\text{CH}_2\text{COO}$), 1.20-1.40 (br m, 8H, methylene protons); ^{13}C NMR (CD_3CN , 100 MHz, δ ppm): 177.45 (COOH), 174.88 (COOFLT), 165.82 (C-4 C=O), 151.73 (C-2 C=O), 136.81 (C-6), 111.77 (C-5), 95.00 ($J = 175.9$ Hz, C-3'), 86.01 (C-1'), 83.24 ($J = 26.4$ Hz, C-4'), 64.29 ($J = 11.0$ Hz, C-5'), 38.37 ($J = 20.9$ Hz, C-2'), 34.54, 34.51 (CH_2COO), 29.54, 29.49, 29.46, 29.43, 29.39, 25.38, 25.31 (methylene carbons), 12.55 (5- CH_3); HR-MS (ESI-TOF) (m/z): $\text{C}_{20}\text{H}_{29}\text{FN}_2\text{O}_7$: calcd, 428.1959; found, 427.197 [$\text{M} - \text{H}$] $^+$.

12-[5'-O-(3'-Fluoro-2',3'-dideoxythymidiny)]dodecandioate (5). Yield (200 mg, 40%); ^1H NMR (400 MHz, CD_3OD , δ ppm): 7.36 (s, 1H, H-6), 6.28 (dd, $J = 5.5$ and 8.7 Hz, 1H, H-1'), 5.20 (dd, $J = 4.4$ and 53.4 Hz, 1H, H-3'), 4.30-4.50 (m, 2H, H-4', H-5'), 4.18-4.30 (m, 1H, H-5''), 2.53-2.70 (m, 1H, H-2''), 2.20-2.40 (m, 5H, CH_2COOH , CH_2COO , H-2'), 1.88 (s, 3H, 5- CH_3), 1.50-1.70 (m, 4H, $\text{CH}_2\text{CH}_2\text{COO}$), 1.15-1.40 (br m, 12H, methylene protons); ^{13}C NMR (CD_3OD , 100 MHz, δ ppm): 177.33 (COOH), 174.16 (COOFLT), 165.42 (C-4 C=O), 151.44 (C-2 C=O), 136.06 (C-6), 111.82 (C-5), 94.23 ($J = 178.9$ Hz, C-3'), 86.00 (C-1'), 83.17 ($J = 26.5$ Hz, C-4'), 64.03 ($J = 10.6$ Hz, C-5'), 38.55 ($J = 21.1$ Hz, C-2'), 34.66, 34.63 (CH_2COO), 30.08, 30.00, 29.97,

29.85, 29.78, 29.70, 29.64, 25.52, 25.40 (methylene carbons), 12.78 (5-CH₃); HR-MS (ESI-TOF) (m/z): C₂₂H₃₃FN₂O₇: calcd, 456.2272; found, 495.1764 [M + K]⁺.

10-[(2',3'-Didehydro-2',3'-dideoxythymidine)-5'-yl]decandioate (6). Yield (220 mg, 44%); ¹H NMR (400 MHz, CD₃OD, δ ppm): 7.41 (s, 1H, H-6), 6.85-6.95 (m, 1H, H-1'), 6.39 (d, *J* = 5.8 Hz, 1H, H-3'), 5.99 (d, *J* = 5.9 Hz, 1H, H-2'), 5.00-5.10 (m, 1H, H-4'), 4.39 (dd, *J* = 4.2 and 12.4 Hz, 1H, H-5''), 4.21 (dd, *J* = 2.9 and 12.4 Hz, 1H, H-5'), 2.24-2.42 (m, 4H, CH₂COOH and CH₂COO), 1.88 (s, 3H, 5-CH₃), 1.50-1.68 (m, 4H, CH₂CH₂COOH and CH₂CH₂COO), 1.16-1.44 (br m, 8H, methylene protons); ¹³C NMR (CDCl₃, 100 MHz, δ ppm): 177.62 (COOH), 174.87 (COO), 166.33 (C-4 C=O), 152.70 (C-2 C=O), 137.83 (C-6), 134.87 (C-3'), 127.83 (C-2'), 111.51 (C-5), 91.36 (C-1'), 85.83 (C-4'), 65.77 (C-5'), 34.92, 34.78 (CH₂COOH and CH₂COO), 30.24, 30.22, 30.15, 30.07, 26.05, 25.98, 25.92 (methylene carbons), 12.72 (CH₃); HR-MS (ESI-TOF) (m/z): C₂₀H₂₈N₂O₇, calcd, 408.1897; found, 407.186 [M - H]⁺.

12-[(2',3'-Didehydro-2',3'-dideoxythymidine)-5'-yl]dodecandioate (7). Yield (180 mg, 36%); ¹H NMR (400 MHz, CD₃OD, δ ppm): 7.30 (s, 1H, H-6), 6.90-6.95 (m, 1H, H-1'), 6.33 (d, *J* = 6.0 Hz, 1H, H-3'), 5.76 (d, *J* = 5.8 Hz, 1H, H-2'), 5.00-5.10 (m, 1H, H-4'), 4.38 (dd, *J* = 4.0 and 12.4 Hz, 1H, H-5''), 4.21 (dd, *J* = 2.9 and 12.4 Hz, 1H, H-5'), 2.28 (dt, *J* = 7.5 and 22.2 Hz, 4H, CH₂COOH and CH₂COO), 1.88 (s, 3H, 5-CH₃), 1.50-1.68 (m, 4H, CH₂CH₂COOH and CH₂CH₂CO), 1.16-1.34 (br m, 12H, methylene protons); ¹³C NMR (CDCl₃, 100 MHz, δ ppm): 177.35 (COOH), 174.44 (COO), 165.61 (C-4 C=O), 152.06 (C-2 C=O), 136.76 (C-6), 134.21 (C-3'), 127.52 (C-2'),

111.44 (C-5), 90.58 (C-1'), 85.06 (C-4'), 65.27 (C-5'), 34.67 (CH₂COOH and CH₂COO), 30.11, 30.03, 30.00, 29.87, 29.82, 29.73, 29.66, 25.55, 25.39 (methylene carbons), 12.82 (CH₃); HR-MS (ESI-TOF) (m/z): C₂₂H₃₂N₂O₇, calcd, 436.2210; found, 435.216 [M - H]⁺.

1.3.3. Anti-HIV Assays

Anti-HIV-1 Evaluation in PMBC Assay. PMBC based anti-HIV assays were performed as previously described (Watson et al., 2008). Briefly, PHA-stimulated PBMCs cultured in the presence of IL-2 were suspended at 1×10^6 cells/mL and were added to a 96-well round-bottom plate. Serially diluted test materials were added to the plate in triplicate followed by the appropriate pre-titered strain of HIV. The culture was incubated for 7 days at 37 °C/5% CO₂. Following the incubation, supernatants were collected for analysis of virus replication by supernatant RT activity and cells analyzed for viability by XTT dye reduction. AZT was used as an internal assay standard. All the assays were carried out in triplicate.

1.4. Results and Discussion

1.4.1. Chemistry

The synthesis of seven mono-substituted 5'-*O*-(fatty acyl)esters of nucleosides is shown in Figure 1.1. Three nucleosides, FLT, AZT, and d4T, and three different dicarboxylic fatty acids were used for esterification. The conjugates were synthesized by reacting nucleosides and dicarboxylic fatty acids in *N,N*-dimethylformamide (DMF) in the presence of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium

hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBT) and diisopropylcarbodiimide (DIC) as coupling reagents and *N,N*-diisopropylethylamine (DIPEA) as a base. The reaction mixtures were stirred at room temperature overnight. The final products were purified by HPLC on C-18 column using water and acetonitrile as the solvent system in order to achieve more than 95% purity. The chemical structures of the final products were characterized by nuclear magnetic resonance spectrometry (^1H NMR and ^{13}C NMR), and were confirmed by a high-resolution time-of-flight electrospray mass spectrometer.

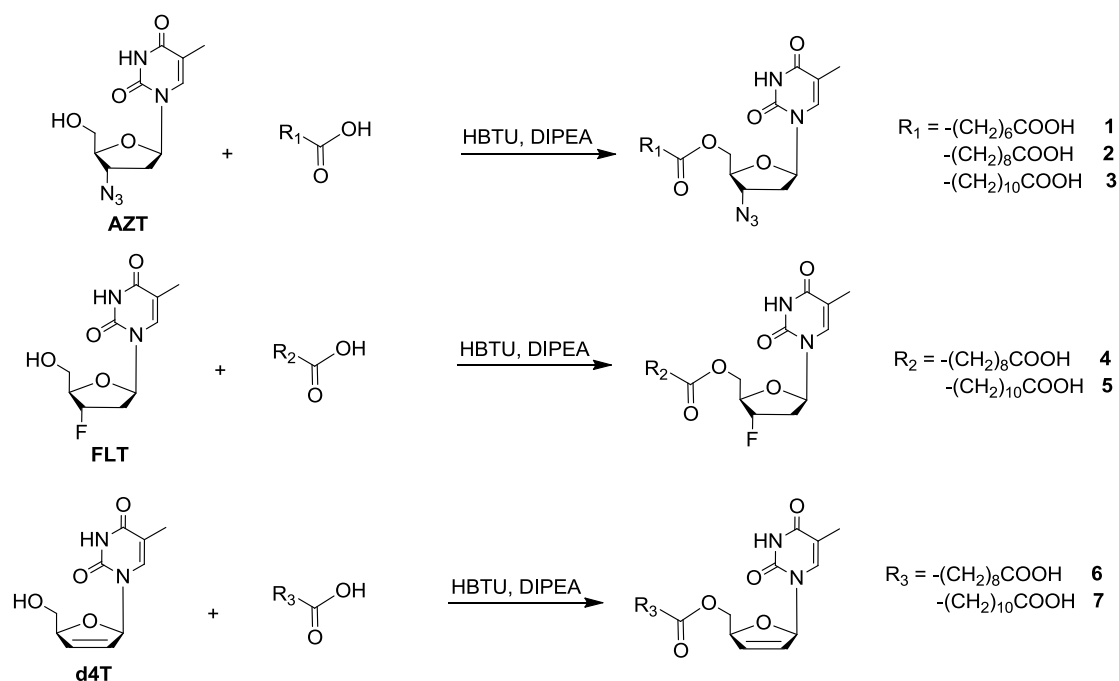


Figure 1.1. Synthesis of 5'-mono-substituted fatty acyl ester nucleoside conjugates of FLT, AZT, and d4T (**1-6**).

1.4.2. Biological Evaluation

All the synthesized conjugates were evaluated for their inhibitory activity of HIV-1 (subtype B, US/92/727) replication in human peripheral blood mononuclear

(PBMC) cells (Watson et. al., 2008) Table 1.2 illustrates the anti-HIV-1 activity (EC_{50}) and cytotoxicity (TC_{50}) of the nucleoside ester conjugates compared with their corresponding parent nucleosides. No cytotoxicity was observed up to the highest tested concentration for both the parent nucleosides and the synthesized conjugates ($TC_{50} > 500$ nM) (**1-7**).

The AZT conjugates (**1-3**, $EC_{50} = 0.1-0.3$ nM) exhibited consistently higher anti-HIV activity than that of AZT ($EC_{50} = 8.0$ nM). For example, octandioate (suberate) ester derivative of AZT (**1**, $EC_{50} = 0.1$ nM) showed 80 times higher anti-HIV activity than the parent nucleoside. AZT conjugates having longer chain fatty acids also showed enhancement in anti-HIV activity than AZT while the ratio of improvement was less than that of compound **1**. The decandioate ester of AZT (**2**, $EC_{50} = 0.31$ nM) was 26-fold more potent than that of AZT. The activity of dodecandioate ester of AZT (**3**) was 24 times higher when compared to AZT. Among the AZT conjugates, AZT-suberate conjugate (**1**) showed the highest anti-HIV activity. These data suggest that conjugation of AZT with dicarboxylic acids significantly enhances the anti-HIV activity with higher potency seen in conjugates with shorter chain length.

Similarly, dicarboxylic ester conjugates of d4T (**6** and **7**, $EC_{50} = 1.98-18.3$ nM) showed better anti-HIV activity from that of d4T ($EC_{50} = 90$ nM) in the PBMC assay against HIV-1_{US/92/727}. The decanedioate ester of d4T (**6**, $EC_{50} = 1.98$ nM) exhibited 45 times more anti-HIV activity than d4T. The dodecandioate ester of d4T (**7**, $EC_{50} = 18.3$ nM) showed 5 times higher anti-HIV activity when compared to that of its parent nucleoside. These results indicate that the anti-HIV activity of the carboxylic esters of

anti-HIV nucleoside depends on the chain length of the carboxylic acid, and shorter length dicarboxylate monoester conjugates are more potent.

A similar trend was observed for FLT ester conjugates compared to the parent nucleoside. Amongst all the FLT conjugates ($EC_{50} = 0.25-0.26$ nM), the decanedioate ester of FLT (**4**, $EC_{50} = 0.26$ nM) and dodecanedioate ester of FLT (**5**, $EC_{50} = 0.25$ nM) showed 8-fold increase in anti-HIV activity when compared to FLT ($EC_{50} = 2$ nM), but the difference in anti-HIV activity between the two conjugates was not significant.

There was a significant increase in the therapeutic index of AZT and FLT derivatives when compared to that of the respective parent nucleosides. The increased therapeutic index indicates synergistic effect of conjugation of dicarboxylic acids with ddNs. Among all the synthesized dicarboxylic acid ester derivatives of AZT, FLT, and d4T, the decandioate monoester conjugate of AZT (**1**) showed the highest anti-HIV activity.

This study indicates that NRTIs when in conjugation with long chain dicarboxylic acids exhibit higher anti-HIV activity possibly due to the improved lipophilicity thereby increasing the cellular uptake of NRTIs. As shown by calculated partition coefficient values (Table 1), the ester conjugates were more lipophilic than their parent analogs. We have shown previously for other fatty acyl derivatives of nucleosides that the highly lipophilic conjugates could have higher cellular uptake contributing to their improved anti-HIV activity (Agarwal 2012, 2013). However, for dicarboxylic monoester conjugates, an appropriate lipophilicity is required for an optimal anti-HIV activity since highly lipophilic conjugates with longer chain length

were less potent than those with shorter chain length. We have previously shown higher cellular uptake and the intracellular hydrolysis of several fatty acyl ester derivatives of 3TC, d4T, and FTC (Agarwal et al, 2011, 2012, 2013). Similarly, it is expected that when the conjugate esters enter the cells, they undergo intracellular hydrolysis by esterases and release the anti-HIV nucleosides and fatty acids targeting different stages of the HIV life cycle possibly contributing to enhanced anti-HIV activity. Nucleoside and fatty acid analogs are known to target the reverse transcriptase and the NMT enzymes. More mechanistic studies are required to determine the exact mechanism of activity of the ester conjugates of the nucleosides.

Table 1.2. Anti-HIV activity of dicarboxylic acid ester conjugates of nucleoside conjugates (**1-7**).

Com pd.	Chemical Name	PBMC/HIV-1 _{US/92/727}			Log P ^d
		EC ₅₀ (nM) ^a	TC ₅₀ (nM) ^b	TI ^c	
AZT	3'-azido-2',3'-dideoxythymidine	8.00	>1000	>125	-0.24 ^e
FLT	3'-fluoro-2',3'-deoxythymidine	2.00	>500	>250	-0.41
d4T	2',3'-didehydro-2',3'- dideoxythymidine	90.0	>500	>5.6	-0.34
1	8-[(3'-azido-2',3'- dideoxythymidiny)-5'- yl]octandioate	0.10	>500	>5000	1.97 ^e
2	10-[(3'-azido-2',3'- dideoxythymidiny)-5'- yl]decandioate	0.31	>500	>1613	3.03 ^e
3	12-[(3'-azido-2',3'- dideoxythymidiny)-5'- yl]dodecandioate	0.33	>500	>1516	4.09 ^e
4	10-[5'-O-(3'-fluoro-2',3'- dideoxythymidiny)]decandioate	0.26	>500	>1923	1.99
5	12-[5'-O-(3'-fluoro-2',3'- dideoxythymidiny)]dodecandioate	0.25	>500	>2000	2.83
6	10-[(2',3'-didehydro-2',3'- dideoxythymidine)-5'- yl]decandioate	1.98	>500	>253	2.06
7	12-[(2',3'-didehydro-2',3'- dideoxythymidine)-5'- yl]dodecandioate	18.30	>500	>27	2.90

^aEC₅₀ (50% effective concentration), ^bTC₅₀ (50% toxic concentration), ^cTherapeutic index(TC₅₀/EC₅₀); ^dCalculated Partition coefficient by ChemDraw Ultra 12.0; ^eCLogP calculated by ChemDraw Ultra 12.0.

1.5. Conclusions

Several lipophilic 5'-*O*-fatty acyl dicarboxylic monoester derivatives of the nucleoside reverse transcriptase inhibitors, AZT, FLT, and d4T were synthesized and evaluated for their anti-HIV activity. The fatty acid substitution at the 5'-*O*-position enhanced the lipophilicity of the anti-HIV nucleoside analogs. All conjugates exhibited higher anti-HIV activity when compared to their parent nucleosides. The improved viral inhibition by the AZT, d4T, and FLT fatty acyl derivatives versus the parent nucleoside is presumably due to the enhanced cellular uptake of the lipophilic conjugates. These conjugates have the potential to be used as potent anti-HIV agents and/or lipophilic anti-HIV microbicides after further optimization.

1.6. Acknowledgments

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Manuscript II

Design, Synthesis, Antiviral Activity, and Pre-formulation Development of Poly-L-Arginine-Fatty acyl Derivatives of Nucleoside Reverse Transcriptase Inhibitors

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2.1. Abstract

The objective of this study was to design, synthesize, and evaluate poly-*L*-arginine cell-penetrating peptides (CPPs) conjugated with fatty acyl derivatives of anti-HIV nucleosides. Three conjugates of alovudine (FLT), lamivudine (3TC) and emtricitabine (FTC) were synthesized through solid-phase synthesis from the conjugation of Wang-resin bound fatty acyl poly-*L*-arginine with FLT and N4-protected 3TC and FTC, followed by cleavage and deprotection. The final products were purified by HPLC and characterized by ^1H -NMR, ^{13}C -NMR, and mass spectroscopy, and were evaluated for cytotoxicity and anti-HIV activity. All conjugates exhibited less anti-HIV activity when compared with the parent nucleoside analogues. For example, poly-*L*-arginine-fatty acyl derivative of 3'-fluoro-3'-deoxythymidine, FLT-CO-(CH₂)₁₂-CO-(Arg)₇, exhibited EC₅₀ values of 2.9 μM and 3.1 μM against X4 and R5 cell-free virus, respectively, while the FLT had EC₅₀ values of 0.2 μM and 0.1 μM , suggesting the limited uptake or intracellular hydrolysis to the parent analogue. The FLT-conjugate was selected for further preformulation studies by determination of solution state degradation and lipid solubility (partition coefficient). The compound was found to be stable in neutral and oxidative conditions, moderately stable in heated conditions, and unstable in acidic and alkaline conditions. The FLT conjugate was hydrophilic as the partition coefficient (Log P) was found to be -0.34. The stability of the compound was evaluated using HPLC. The derivative was evaluated in dissolution studies using four different hydrogels gels with and without a thermogelling polymer. Gel formulations of the compound were manufactured using non-ionic (HPC-SL) and anionic (Carbopol) polymers with and

without the inclusion of a thermo-reversible gelling (Pluronic F-127) polymer. The compound was found to be unstable in the manufactured formulations as shown by HPLC profiles. These data suggest that the conjugation of poly-Arg to fatty acyl derivatives of anti-HIV nucleosides do not generate conjugates with improved anti-HIV activity versus the parent nucleosides and they lack adequate stability. Further investigation is required in order to generate nucleoside-fatty acid-CPP conjugates with improved anti-HIV activity and optimized dissolution and stability in formulations.

2.2. Introduction

According to UNAIDS estimation, 35.3 million people are living with Acquired Immunodeficiency Syndrome (AIDS) in 2013 (www.unaids.org). High Activity Antiretroviral Therapy (HAART) involves the use of a minimum of three types of antiretroviral drugs including protease inhibitors, non-nucleoside reverse transcriptase inhibitors, and nucleoside reverse transcriptase inhibitors. HAART has increased the lifespan of HIV-infected patients and has converted this terminal illness into a chronic and manageable disease (Clercq, 2007). Although the FDA approved anti-HIV nucleosides zidovudine (AZT), lamivudine (3TC), and emtricitabine (FTC) have reduced the mortality rate in patients, their use is still complicated because of toxicity, chronic intake, development of resistant virus, and incomplete elimination of the viral reservoirs. These anti-HIV nucleosides have also limited cellular uptake due to their hydrophilic nature. Upon intracellular uptake, they need to undergo three phosphorylation steps in order to become active nucleoside triphosphate analogues (Clercq, 2007). Thus, novel drug delivery systems are urgently needed in order to generate nucleoside conjugates with better anti-HIV profile, such as higher cellular uptake and anti-HIV activity.

The primary hypothesis of this work was to design conjugates of anti-HIV nucleosides conjugated with fatty acids and cell penetrating polyArg peptides. Herein, we designed polyArg-fatty acyl derivatives of anti-HIV nucleosides with the expectation to overcome their limited cellular uptake. The objective was to design multifunctional anti-HIV drug conjugates where:

1. Nucleosides act as reverse transcriptase inhibitors (NRTIs);

2. Long chain fatty acyl component acts as *N*-myristoyl transferase (NMT) inhibitor and also improves the cellular uptake; and
3. The Poly-*L*-arginine component plays a crucial role as a cell-penetrating peptide.

Long chain Carboxylic acids as NMT Inhibitors: Long chain carboxylic acids are known to inhibit NMT, which is responsible for the myristoylation of various HIV proteins, such as P17 capsid protein, Pr55^{gag}, Pr160^{gag-pol}, and p27^{nef} in the virus infected host cells (Langner et al., 1992). Myristoylation of viral proteins allows the viral protein components to become more hydrophobic (Farazi et al., 2001). Certain heteroatom-containing myristic acid analogues, such as 12-thioethyldodecanoic acid, 4-oxatetradecanoic acid and 2-methoxydodecanoic acid derivatives, inhibit HIV-1 replication in acutely infected T-lymphocytes (Bryant et al., 1993; Takamune et al., 2002). 12-Thioethyldodecanoic acid was found to be moderately active against HIV infected T4 lymphocytes with an EC₅₀ value of 9.4 μ M (Parang et al., 1997).

Various 5'-*O*-fatty acyl derivatives of nucleoside reverse transcriptase inhibitors (3'-azido-3'-deoxythymidine (zidovudine, AZT), 3'-fluoro-3'-deoxythymidine (alovudine, FLT), (-)-2',3'-dideoxy-3'-thiacytidine (lamivudine, 3TC), 5-fluoro-(-)-2',3'-dideoxy-3'-thiacytidine (emtricitabine, FTC), 2',3'-didehydro-2',3'-dideoxythymidine (stavudine, d4T)) were found to have enhanced anti-HIV activity profiles as compared to their parent nucleosides against X4, R5 strains, cell-associated, and/or multi-drug resistant virus, presumably due to increased cellular uptake caused by their high lipophilic profile (Parang et al., 1996, 1997, 1998, 2000;

Agarwal et al, 2011, 2012, 2013). These ester conjugates were shown to act as bifunctional agents through intracellular hydrolysis by esterases to release nucleoside reverse transcriptase inhibitors, and the fatty acids.

Poly-L-Arginine as a Cell Penetrating Peptide: An important subclass of these molecular transporters are CPPs, which contain guanidinium-rich transporters (GRTs). The positively charged guanidinium groups on the arginine side chains are responsible for its penetrating ability through interaction with the phospholipid bilayer. The development of these polyarginine transporter molecules was inspired by the lead HIV-1 transcription transactivator protein (Tat) made up of repeated arginine and lysine residues (Wender et al., 2008). This transporter is highly polar, readily soluble in water, and unlike other polar drugs travels across the lipid bilayer membrane (Wender et al., 2008). Studies have shown that peptides with 6-20 arginine residues pass through the membrane showing rapid uptake across the membrane and into the nucleus without any signs of acute toxicity (Wender et al., 2008). Molecular transporters when attached to poorly bioavailable drugs could enable them to pass through the biological membrane as shown previously (Rothbard et al, 2000).

Anti-HIV microbicides are topically applied agents that prevent or reduce transmission of infectious disease, particularly HIV/AIDS (Lederman et al., 2006). One of the major issues of anti-HIV drug delivery is to obtain selective uptake of the drug at the targeted site, and one of the strategies for selectivity is local or direct administration of the drug. GRTs such as polyarginine peptide penetrate both the epidermal and dermal layers of the skin upon topical application (Wender et al., 2008).

Thus, the poly-*L*-arginine peptide was expected to act in an anti-HIV microbicidal vaginal application. The compounds were expected to have potential to be used for the development of anti-HIV microbicides.

Polyarginine-Fatty Acyl Conjugates. Myristoylated polyarginine peptide (MPAP) has a noninvasive and non-disruptive mode of transportation across the blood brain barrier (BBB) wherein the myristoyl group promotes membrane association and the positively charged polyArg undergoes electrostatic interactions with the negative charges of the BBB cell membranes (Pham et al., 2005). MPAP was attached to an optically detectable dye, and *in vivo* imaging showed accumulation in the mouse brain especially in the neurons (Pham et al., 2005). Thus, CPP-fatty acyl conjugates of nucleoside could have also application for targeting HIV infected brain cells.

Various peptide-derived HIV entry/fusion inhibitors of CXCR4, a chemokine co-receptor found on CD4⁺ T lymphocytes, have been reported to mimic the HIV-1 Tat basic domain designed to target the transactivator response element (TAR) RNA of HIV-1 (Borkow et al., 2003). For example, the nona-arginine peptide binds TAR RNA with high affinity *in vitro* (Hamy et al., 1997). Thus, we hypothesized that when poly-*L*-arginine is attached to fatty acylated nucleosides, it could also act as possible CXCR4 HIV entry inhibitor. Upon cellular entry, the peptide linker is cleaved by protease, thereby allowing the fatty acylated nucleoside to localize in the nucleus and other cellular components.

Herein, we report the design, synthesis, and biological activities of three poly-*L*-arginine linked poly-Arg 1,12-dodecanedicarboxylate derivatives of anti-HIV nucleosides (FLT, 3TC, and FTC (Figure 2.1) and their application as multifunctional

anti-HIV agents. It was expected that the conjugation of nucleosides to the poly-*L*-arginine-fatty acyl residue could result in the development of anti-HIV agents or microbicides with enhanced efficacy, longer duration of action by sustained intracellular release of active substrates at adequate concentrations, and/or higher uptake into infected cells. Furthermore, the development of viral resistance to two or more active drugs might be expected to occur at a lower rate than to either compound alone.

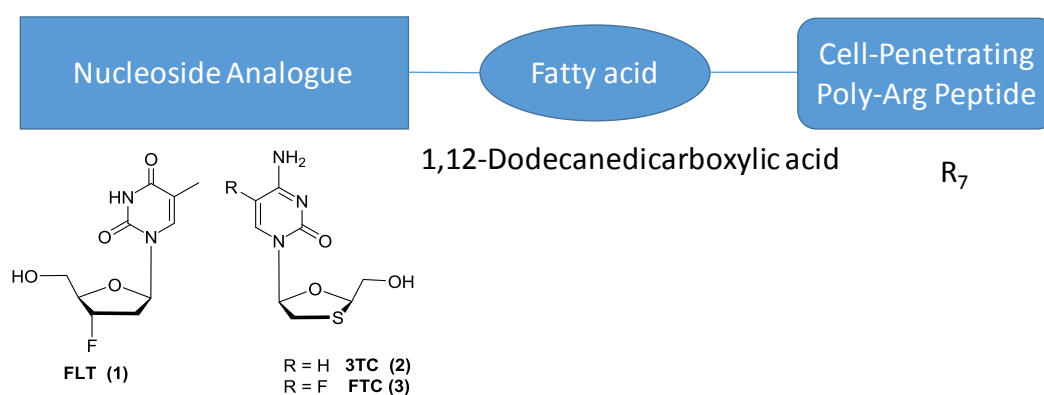


Figure 2.1. Poly-*L*-arginine linked fatty acylated nucleosides.

2.3. Materials and Methods

2.3.1. Materials

Nucleosides (**1-3**) were purchased from Euro Asia Trans Continental (Bombay, India) for the nucleoside ester conjugate synthesis. [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate] (HBTU), *N,N*-diisopropylethylamine (DIPEA), and 1,12-dodecanedicarboxylic acid were bought from Sigma Aldrich Chemical Co. Solvents and all other reagents were purchased from Fisher Scientific. The products were purified using a Phenomenex-Gemini C18 column (10 μ m, 250 \times 21.2 mm) with Hitachi HPLC system using a gradient system at a constant flow rate of

10 ml/min (Table 2.1). The purity of the final products was confirmed by using a Hitachi analytical HPLC system on a C18 column using a gradient system (water:acetonitrile 30:70 v/v) at a constant flow rate of 1 ml/min with UV detection at 220 nm and 265 nm. The chemical structures of the final products were determined by nuclear magnetic resonance spectrometry (^1H NMR and ^{13}C NMR) on a Bruker NMR spectrometer (400 MHz) and confirmed by a SELDI-TOF mass spectrometer on a Ciphergen protein chip instrument using α -cyano-4-hydroxycinnamic acid as a matrix.

Table 2.1. HPLC method used for purification of the final compounds.

Time (min)	Water Concentration A (%)	Acetonitrile Concentration B (%)	Flow rate (mL/min)
0.00	100.0	0.0	1.0
1.00	100.0	0.0	10.0
5.0	90.0	10.0	10.0
25.0	70.0	30.0	10.0
35.0	60.0	40.0	10.0
60.0	0.0	100.0	10.0
65.0	0.0	100.0	10.0
70.0	100.0	0.0	1.0

2.3.2. Chemistry

Synthesis of Polyarginine (11). The peptide synthesis was carried out in Bio-Rad polypropylene columns by shaking and mixing using a Glass Col small tube rotator or on a PS3 automated peptide synthesizer (Rainin Instrument Co., Inc.) at room

temperature unless otherwise stated. Fmoc-Arg(Pbf)-Wang resin (**10**, 1.1 mmole, 2.97 g, 0.37 mmole/g) was placed in manual peptide synthesis container (250 mL) equipped with a three way stopper. Resin was swelled in *N,N*-dimethylformamide (DMF, 150 mL) with constant N₂ bubbling for 30 min (2 times). The Fmoc group was removed from the resin in the presence of piperidine (20% v/v in DMF, 2 × 50 mL) for 15 min. The resin was washed with DMF (5 × 50 mL). Fmoc-Arg(Pbf)-OH (3 equiv, 3.3 mmol, 2.14 g) and HBTU (3 equiv, 1.25 g, 3.3 mmol) were added to the reaction vessel, followed by the addition of DMF (25 mL) and DIPEA (6 equiv, 6.6 mmol, 1.15 mL) (Scheme 2) and bubbled with N₂ for 2.5 h. The resin was washed with DMF (5 × 25 mL) to remove unreacted starting materials, impurity, and reagents. The deprotection and coupling cycles were repeated five more times to add a total of 7 arginine residues on the resin. *N*-Terminal Fmoc was deprotected by using piperidine (20 % v/v in DMF) to afford resin **11**. Resin **11** was washed with DMF (5 × 100 mL). The structure of **11** was confirmed by cleaving a small amount of the resin in the presence of reagent R (TFA/thioanisole/1,2-ethanedithiol/anisole, 90:5:3:2 v/v/v/v, 2 mL) for 4 h. The crude peptide was precipitated in the presence of cold diethyl ether (Et₂O, 150 mL), and separated and washed by centrifugation (3 × 15 mL) at 4000 rpm for 5 min. The crude peptide was dissolved in water (0.1%, TFA). The molecular weight of poly-L-arginine (R₇) was confirmed by a SELDI-TOF mass spectrometer on a Ciphergen protein chip instrument. MS (SELDI-TOF) (m/z): C₄₂H₈₆N₂₈O₈, calcd, 1111.3; found, 1111.1 [M]⁺.

Synthesis of Polyarginine 1,12-Dodecanedicarboxylate (12). 1,2-Dodecanedicarboxylic acid (1.43 g, 5.5 mmol), HBTU (2.09 g, 5.5 mmol), and 1-hydroxy-7-azabenzotriazole (HOAt, 0.74 g, 5.5 mmol) were added to the reaction vessel containing polyarginine resin **11** in anhydrous DMF (200 mL), followed by the addition of DIPEA (8.1 mmol, 1.42 mL) and *N,N'*-diisopropylcarbodiimide (DIC, 9.0 mmol, 1.4 mL) (Scheme 2). The peptidyl resin was bubbled with N₂ for 7 h and then washed with DMF (5 × 100 mL). The *N*-terminal Fmoc group was deprotected by using piperidine (20 % v/v in DMF) to yield **12**. Resin **12** was finally washed with DMF (5 × 150 mL). A small amount of the resin was cleaved using reagent R (TFA/thioanisole/1,2-ethanedithiol/anisole, 90:5:3:2 v/v/v/v, 2 mL) and precipitated with cold diethyl ether (200 mL) and finally centrifuged at 4000 rpm for 5 min. The residue was dissolved in water and the formation of product **12** was confirmed with SELDI-TOF mass spectrometer. MS (SELDI-TOF) (*m/z*): C₅₆H₁₁₀N₂₈O₁₁, calcd, 1351.7; found, 1352.0 [M + H]⁺.

General Procedure for the Synthesis of Polyarginine-1,12-Dodecanedicarboxylate Acylated Nucleoside Analogues (13-15). N4-4,4'-Dimethoxytrityl (DMTr)-3TC (**8**) and N4-DMTr-FTC (**9**) were synthesized as described in the previously published procedure (Agarwal et al., 2012). Peptidyl polyarginine dodecanedicarboxylate resin **12** (0.7 g), the nucleoside analogues (**1**, **8**, or **9**, 1.1 mmol), HBTU (0.417 mg, 1.1 mmol), and HOAt (148 mg, 1.1 mmol) were dissolved in anhydrous DMF (25 mL) in a round bottom flask (100 mL) followed by the addition of DIPEA (1.95 mmol, 342 µL) and

DIC (2.5 mmol, 383 μ L) (Scheme 2). The reaction vessels were flushed with N₂ and placed on a shaker at 4000 rpm to mix the reagents.

The reaction mixture was filtered off, and the resin was washed with DMF (3 \times 20 mL), methanol (3 \times 20 mL), and dichloromethane (DCM, 3 \times 20 mL). The resin was cleaved along with deprotection of Pbf and DMTr protective groups from the side chain of arginine by and corresponding nucleosides, respectively, using reagent R (20 mL) for 4 h at room temperature. The resin was filtered, and the filtrate was added drop wise to cold diethyl ether (50 mL) for precipitation followed by centrifugation at 4000 rpm. The precipitates were washed with diethyl ether (2 \times 50 mL) to afford solid crude peptide-nucleoside conjugates (**13-15**). The precipitates were dried and dissolved in 50% acetonitrile and water. The conjugates were purified by preparative reverse-phase HPLC (Shimadzu LC-8A preparative liquid chromatograph) on a Phenomenex-Gemini C18 column (10 μ m, 250 \times 21.2 mm) at 10.0 mL/min flow rate using a gradient of 10% acetonitrile (0.1% TFA) in water (0.1% TFA) to 100% acetonitrile (0.1% TFA) (Table 2.1). Chromatograms were recorded at 220 and 265 nm using a UV detector. The purity of final products (>95%) was confirmed by analytical HPLC. The chemical structures of compounds were determined by a SELDI-TOF mass spectrometer on a Ciphergen protein chip instrument using α -cyano-4-hydroxycinnamic as a matrix and by ¹H and ¹³C NMR.

1-[5'-O-(3'-Fluoro-2',3'-dideoxythymidinyl)]tetradecan-1,14-dioate conjugate of PolyArg (FLT-OCO(CH₂)₁₂CONH-RRRRRRR-OH, **13).** Yield (210 mg, 60.5%). ¹H NMR (400 MHz, CD₃OD, δ ppm): 7.30 (s, 1H, H-6), 6.12 (dd, *J* = 5.7 and 8.6 Hz,

1H, H-1'), 5.15 (dd, $J = 4.3$ and 53.2 Hz, 1H, H-3'), 4.04-4.26 (m, 7H, -NH-CH-CO), 3.90-4.00 (m, 1H, H-4'), 3.52-3.66 (m, 2H, H-5' and H-5''), 3.26-3.35 (m, 2H, =NH-CH₂), 2.95-3.10 (m, 10H, =NH-CH₂), 2.70-2.90 (m, 2H, =NH-CH₂), 2.40-2.55 (m, 1H, H-2''), 2.05-2.25 (m, 5H, CH₂O and H-2'), 1.84-1.90 (br s, 7H, 5-CH₃ and CH₂CH₂CO), 1.35-1.80 (m, 32H, =NH-CH₂-CH₂), 1.08-1.20 (br s, 16H, methylene protons). ¹³C NMR (CD₃CN, 100 MHz, δ ppm): 175.87, 173.26, 172.79, 172.69, 156.36, 135.79, 118.26, 114.78, 84.89, 82.22, 63.19, 52.82, 51.83, 48.74, 40.22, 37.14, 33.16, 27.49, 24.07, 19.98, 19.25, 13.83, 11.43, 0.73, 0.52, 0.31, 0.11, 0.10, 0.36. MS (SELDI-TOF) (m/z): C₆₆H₁₂₁FN₃₀O₁₄, calcd, 1576.97; found, 1577.5 [M + H]⁺.

1-[(−)-2',3'-Dideoxy-3'-thiacytidine]tetradecan-1,14-dioate conjugate of PolyArg (3TC-OCO(CH₂)₁₂CONH-RRRRRRR-OH, 14). Yield (180 mg, 52.3%). ¹H NMR (400 MHz, CD₃OD, δ ppm): 8.27 (d, $J = 7.8$ Hz, 1H, H-6), 6.42-6.48 (m, 1H, H-1'), 6.34 (d, $J = 7.5$ Hz, 1H, H-5), 5.60-5.80 (m, 1H, H-4'), 4.58-4.68 (m, 1H, -NH-CH-CO), 4.40-4.50 (br s, 5H, -NH-CH-CO), 4.30-4.40 (m 1H, -NH-CH-CO), 3.78 (dd, $J = 12.8$ and 5.4 Hz, 1H, H-5''), 3.61 (d, $J = 12.8$ Hz, 1H, H-5'), 3.42-3.52 (m, 1H, H-2''), 3.22-3.40 (br s, 15H, H-2', =NH-CH₂), 2.52-5.64 (m, 2H, CH₂CONH-), 2.36-2.44 (m, 2H, CH₂COO), 1.65-2.10 (m, 32H, =NH-CH₂-CH₂), 1.30–1.50 (br s, 16H, methylene protons). ¹³C NMR (CD₃CN, 100 MHz, δ ppm): 176.50, 176.46, 174.91, 173.47, 172.76, 172.69, 172.65, 172.59, 172.48, 162.08, 161.74, 159.40, 156.29, 148.20, 143.62, 94.00, 86.78, 84.18, 63.21, 53.17, 52.83, 52.77, 52.43, 40.17, 36.95, 35.03, 33.33, 28.51, 28.38, 28.30, 28.11, 28.02, 27.91, 27.67, 27.57, 24.96, 24.21, 24.07,

24.01, 23.92. MS (SELDI-TOF) (m/z): C₆₄H₁₁₉N₃₁O₁₃S, calcd, 1561.93; found, 1562.7 [M + H]⁺.

1-[(–)-2',3'-dideoxy-5-fluoro-3'-thiacytidine]tetradecan-1,14-dioate conjugate of PolyArg (FTC-OCO(CH₂)₁₂CONH-RRRRRRR-OH, **15).** Yield (130 mg, 37.5%); ¹H NMR (400 MHz, CD₃OD, δ ppm): 8.11 (d, *J* = 6.7 Hz, 1H, H-6), 6.22-6.27 (m, 1H, H-1'), 5.44 (dd, *J* = 2.9 and 4.3 Hz, 1H, H-4'), 4.68 (dd, *J* = 12.6 and 4.4 Hz, 1H, H-5''), 4.30-4.48 (m, 7H, H-5', -NH-CH-CO), 4.20-4.30 (m, 1H, -NH-CH-CO), 3.45-4.65 (m, 1H, H-2''), 3.15-3.28 (m, 15H, H-2', CH₂NH), 2.13-2.38 (m, 4H, CH₂COO and CH₂CONH), 1.40-1.90 (m, 32H, methylene protons), 1.10-1.30 (br s, 16H, methylene proton); ¹³C NMR (CD₃CN, 100 MHz, δ ppm): 172.88, 172.41, 171.79, 171.21, 159.47, 158.84, 158.52, 156.76, 124.80, 99.43, 86.63, 82.02, 63.77, 52.02, 40.04, 39.83, 39.62, 39.41, 39.20, 38.99, 38.78, 36.06, 35.05, 33.16, 28.87, 28.79, 28.35, 28.26, 25.15, 24.86, 24.29. MS (MALDI-TOF) (m/z): C₆₄H₁₁₈FN₃₁O₁₃S, calcd, 1580.91; found, 1581.5 [M + H]⁺.

Stability and Degradation Studies. The stability of **13** was evaluated by using HPLC. All degradation studies were carried out at a drug concentration of 1 mg/mL. The solution stability studies were conducted by using the stock solution of compound **13** in the presence of room temperature (25 °C), heat (40 °C), neutral (water), acidic (1N HCl), alkaline (1N NaOH) and oxidation (3% H₂O₂) conditions at 40 °C. The compound was incubated in the above solutions for 24 hours. All the samples were kept at room temperature for 1h and the analytical HPLC was run for 35 min. The

solvent system used was water:acetonitrile (0.1% trifluoroacetic acid) and the HPLC was run at a flow rate of 1 mL/min at 220 nm and 256 nm wavelengths (Table 2.2).

Table 2.2. HPLC method used for Stability and Degradation Studies.

Time (min)	Water Concentration A (%)	Acetonitrile Concentration B (%)	Flow rate (mL/min)
0.00	100.0	0.0	1.0
1.00	100.0	0.0	1.0
20.0	0.0	100.0	1.0
25.0	0.0	100.0	1.0
30.0	100.0	0.0	1.0
35.0	100.0	0.0	1.0

2.3.3. Partition Coefficient (Log P). Log P HPLC studies were carried out by distributing 21.9 mg of compound **13** in 250 μ L each of *n*-octanol (organic) and pH 4 acetate buffer (aqueous). The mixture was stirred for 2 days at room temperature. The analytical HPLC was run using water:acetonitrile (0.1% TFA) as a solvent system at a flow rate of 1 mL/min at 220 nm and 256 nm wavelengths for each collected fraction (Table 2.2).

2.3.4. Gel Formulations. Vaginal gel formulations of compound **13** were manufactured using non-ionic (HPC-SL) and anionic (Carbopol) polymers with and without the inclusion of thermo-reversible gelling (Pluronic F-127) polymer. The HPC-SL formulation consisted of 2.25% w/v in water while Carbopol consisted of

0.2% w/v. For thermogelling formulations, the aforementioned HPC-SL and Carbopol gels were mixed with a 20% solution of Pluronic F-127 (3:1 v/v ratio). A drug load comprising 1.6% (w/v) of FLT conjugate was loaded in the gels and was sealed in SpectraPor dialysis tubing with MWCO of 3500 Da. The tubes were suspended in 70 ml of dissolution media (Table 2.3) held at 37 ± 0.5 °C with a stirring speed of 75 rpm using a 0.5 inch stir bar. The dissolution media consisted of a simulated vaginal fluid (Table 2.3). These dissolution samples were transferred to UV 96 well plates (Costar®,) and were then analyzed using a SpectraMax M2 UV detector (Molecular Devices, PA, USA).

Table 2.3. Dissolution media composition for (6 liters)

Chemicals	Acetic acid	Urea	Glucose	Lactic acid	Glycerol	Potassium hydroxide	Calcium hydroxide	Sodium chloride	pH
Weight (grams)	6	24	30	12	0.96	8.4	1.3	21	4.2

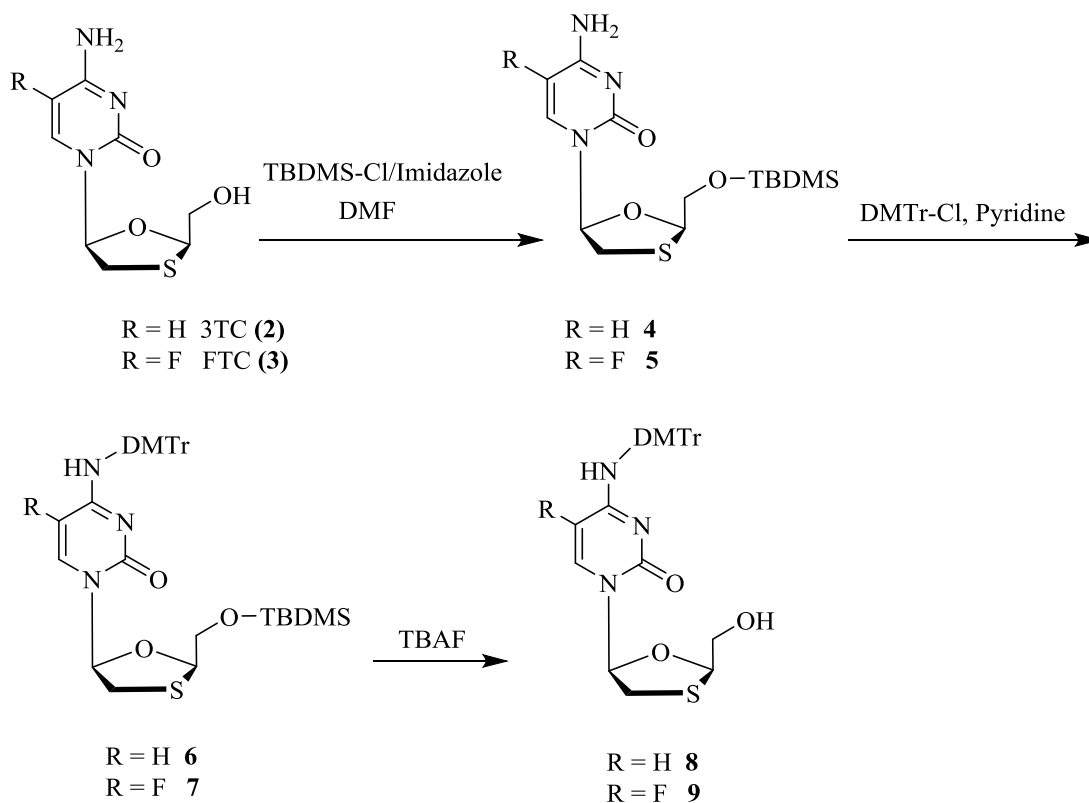
2.3.5. Anti-HIV Assays. The anti-HIV activity of the compounds was evaluated according to the previously reported procedure (Agarwal et al, 2010, 2011, Ahmadibeni, 2011, and Krebs, 2005). Compound anti-HIV activity was evaluated in single-round (MAGI) infection assays using X4 (IIIB) and R5 (BaL) HIV-1 and P4R5 cells expressing CD4 and coreceptors. In summary, P4R5MAGI cells were cultured at a density of 1.2×10^4 cells/well in a 96 well plate approximately 18 h prior to infection. Cells were incubated for 2 h at 37 °C with purified, cell-free HIV-1 laboratory strains IIIB or BaL (Advanced Biotechnologies, Inc., Columbia, MD) in the

absence or presence of each agent. After 2 h, cells were washed, cultured for an additional 46 h, and subsequently assayed for HIV-1 infection using the Galacto-Star β -Galactosidase Reporter Gene Assay System for Mammalian Cells (Applied Biosystems, Bedford, MA). Reductions in infection were calculated as a percentage relative to the level of infection in the absence of agents, and 50% inhibitory concentrations (EC_{50}) were derived from regression analysis. Each compound concentration was tested in triplicate wells. Cell toxicity was evaluated using the same experimental design but without the addition of virus. The impact of compounds on cell viability was assessed using an MTT (reduction of tetrazolium salts) assay (Invitrogen, Carlsbad, CA).

2.4. Results and Discussion

2.4.1. Chemistry

FLT (**1**), N₄-amino protected 3TC (**8**), N₄-amino protected FTC (**9**), and 1,12-dodecandicarboxylate-polyargine resin were used as the building blocks. N₄-DMTr-3TC (**8**) and N₄-DMTr-FTC (**9**) were synthesized according to the previously reported procedure (Agarwal, 2012). First, *tert*-butyldimethylsilyl chloride (TBDMS-Cl) was reacted with 3TC (**2**) or FTC (**3**) in the presence of imidazole to afford 5'-O-TBDMS-3TC (**4**) or 5'-O-TBDMS-FTC (**5**). Next, the N₄-amino group of **4** and **5** was protected with 4,4'-dimethoxytrityl (DMTr) protecting group, by reaction with DMTr-Cl in the presence of pyridine to yield **6** and **7**, respectively. Finally, TBDMS was removed by using tetrabutylammonium fluoride (TBAF) to yield N₄-DMTr-3TC (**8**) and N₄-DMTr-FTC (**9**) (Scheme 1).



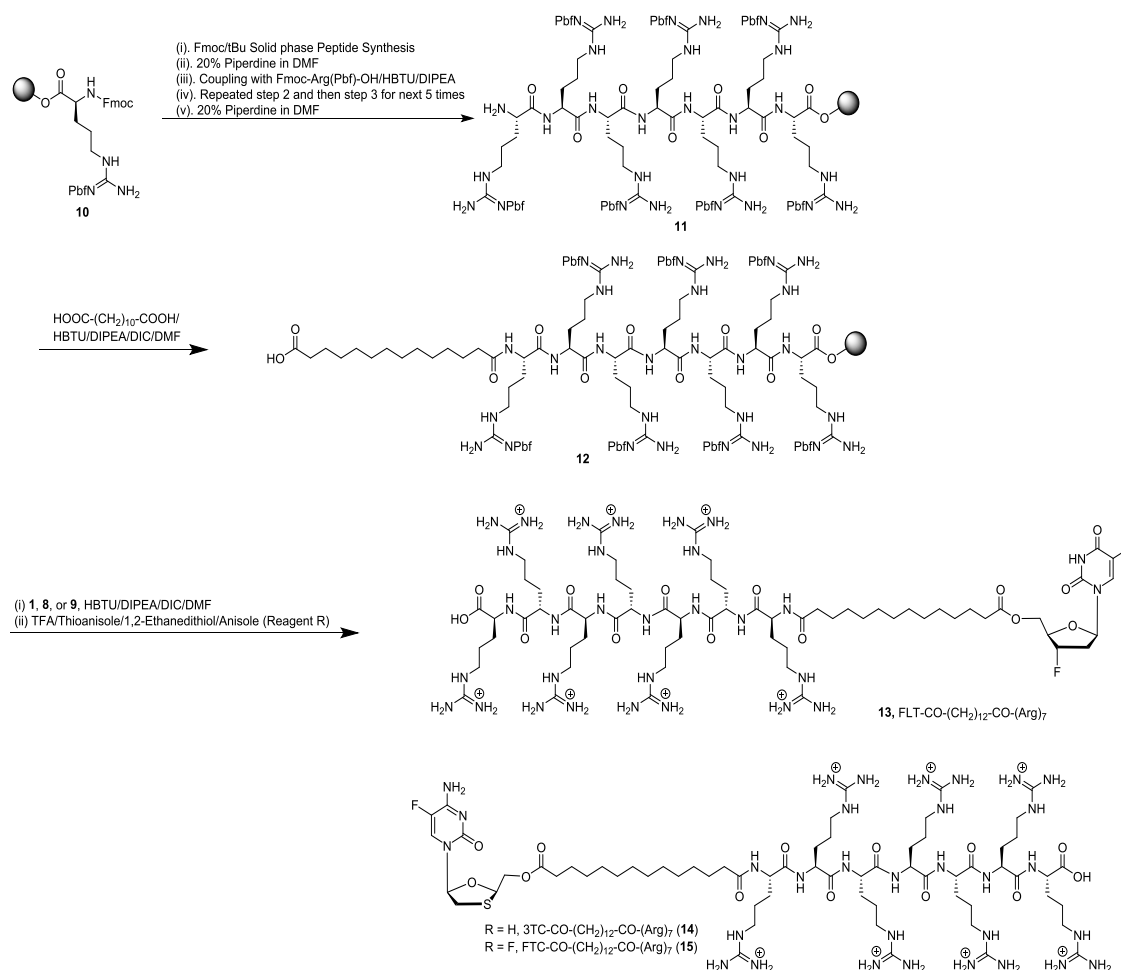
Scheme 2.1. Synthesis of N4-DMTr protected of FTC and 3TC nucleosides

(Agarwal, 2012).

Second, the polyarginine peptide (R_7) was manually synthesized by agitation of resin using nitrogen gas by using solid-phase Fmoc/*t*Bu strategy. The preloaded Fmoc-*L*-arginine(Pbf) Wang resin (**10**) was swelled in DMF followed by deprotection of Fmoc group by using 20% piperidine in DMF. The resin was washed with DMF and coupled with Fmoc-Arg(Pbf)-OH in the presence of coupling reagents 1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N,N*-diisopropylethylamine (DIPEA) as a base, respectively, in DMF at room temperature under nitrogen. The deprotection and coupling cycles were repeated 5 more times followed by the final *N*-

terminal Fmoc deprotection using 20% piperidine in DMF to yield NH₂-polyarginine Wang resin **11**. A small amount of resin cleavage confirmed the assembly of polyarginine peptide on Wang resin. 1,12-Dodecane dicarboxylic acid was coupled to NH₂-polyarginine Wang resin **11** to yield 1,12-dodecanedicarboxylate-polyarginine resin **12** in the presence of combination of coupling reagents (HBTU, HOAt and DIC) (Scheme 2.2).

The conjugation reaction between FLT (**1**) and N⁴-DMTr protected of 3TC and FTC nucleosides (**8** and **9**) and the resin-bound R₇-dodecanoate (**12**) was then carried out through conjugation of 5'-OH of nucleoside and free carboxylic acid of 1,12-dodecanedicarboxylate-polyarginine peptidyl resin in the presence of combination of coupling reagents. The nucleoside conjugated compounds were then cleaved and deprotected by using reagent R to yield the final products (**13-15**) (Scheme 2.2).



Scheme 2.2. Synthesis of hepta-*L*-arginyl-1,12-dodecanedicarboxylate-nucleoside conjugates **13-15**.

2.4.2. Biological Evaluation

The anti-HIV activity of the compounds was evaluated according to the previously reported procedure (Agarwal et al, 2010, 2011, Ahmadibeni, 2011, and Krebs, 2005). Table 2.4 shows the anti-HIV activities of the conjugates compared to their parent analogues. No cellular cytotoxicity was observed up to the highest tested concentration for the conjugates ($EC_{50} > 30 \mu\text{M}$). The conjugates showed no

significant anti-HIV activity against cell associated virus ($EC_{50} > 30 \mu M$). All conjugates exhibited less anti-HIV activity when compared with the parent nucleoside analogues against cell free virus. FLT conjugate **13** ($EC_{50} = 2.9-3.1 \mu M$) showed less potency than its parent nucleoside ($EC_{50} = 0.1-0.2 \mu M$) against X4 and R5 cell free virus. FTC conjugate **15** ($EC_{50} = 1.5-3.0 \mu M$) showed less anti-HIV-1 activity when compared to that of FTC ($EC_{50} = 0.18-0.48 \mu M$) against X4 and R5 cell free virus. 3TC conjugate **14** showed approximately 2-fold higher inhibition against X4 virus ($EC_{50} = 8.7 \mu M$) than the R5 virus strain ($EC_{50} = 16.6 \mu M$) but exhibited also less potency than that of 3TC ($EC_{50} = 2.6-7.5 \mu M$). The conjugates were less potent than their parent structures possibly due to the limited uptake. Different strains of HIV are known to have different gp120 V3 loops. Both X4 and R5 strains of HIV possess a high positive charge density on their V3 loop (Meylan et al., 1994; Maulard et al, 2000), suggesting that the presence of positive charge on the conjugates could block the interaction with gp120.

Table 2.4. Anti-HIV activity of hepta-*L*-arginyl-1,12-dodecanedicarboxylate-nucleoside conjugates.

Compound	Chemical Name	Cytotoxicity	Cell- Free Virus		Cell-Associated Virus
		CTS ^a EC ₅₀ ^b (μM)	X4 ^c EC ₅₀ (μM)	R5 ^d EC ₅₀ (μM)	CTC ^e EC ₅₀ (μM)
FLT (1)	3'-Fluoro-2',3'-deoxythymidine	>100	0.2	0.1	>100
3TC (2)	(-)-2',3'-Dideoxy-3'-thiacytidine	>100	7.5	2.6	18.4
FTC (3)	(-) 2',3'-Dideoxy-5-fluoro-3'-thiacytidine	>100	0.48	0.18	21.9
13	FLT-CO-(CH ₂) ₁₂ -CO-R ₇	>30	2.9	3.1	>30
14	3TC-CO-(CH ₂) ₁₂ -CO-R ₇	>30	8.7	16.6	>30
15	FTC-CO-(CH ₂) ₁₂ -CO-R ₇	>30	3.0	1.5	>30

^aCytotoxicity assay (MTS); ^b50% Effective concentration; ^cSingle-round infection assay (lymphocytotropic strain, X4); ^dSingle-round infection assay (monocytotropic strain, R5); ^eCell-associated transmission assay (X4).

2.4.3. Forced Degradation, Log P Determination, and Gel formulation of FLT

conjugate. These conjugates were designed to be used in areas where shipping and storage conditions are not ideal in terms of temperature and humidity. Thus, it was necessary to determine the relative stability of these compounds under different conditions. Compound **13** was selected for further evaluation in solution state degradation studies and determination of lipid solubility (partition coefficient). Since FLT-conjugate contain several potentially labile bonds, forced degradation of an aqueous solution of **13** at elevated temperature, in acid, base, and under oxidizing conditions, were conducted.

At heated (panel B) conditions, significant degradation of the compound **13** was observed resulting in a second HPLC peak at 9 min. Under both acidic and alkaline conditions, the compound degraded to a great extent resulting in a different peak at 7 min (panels C and D). Minimal degradation of the drug was noted at neutral (panel E)

and oxidative (H_2O_2) (panel F) conditions. (Figure 2.2). Thus, the FLT conjugate was relatively stable in neutral and oxidative conditions, but less stable in heated, acidic, and alkaline conditions.

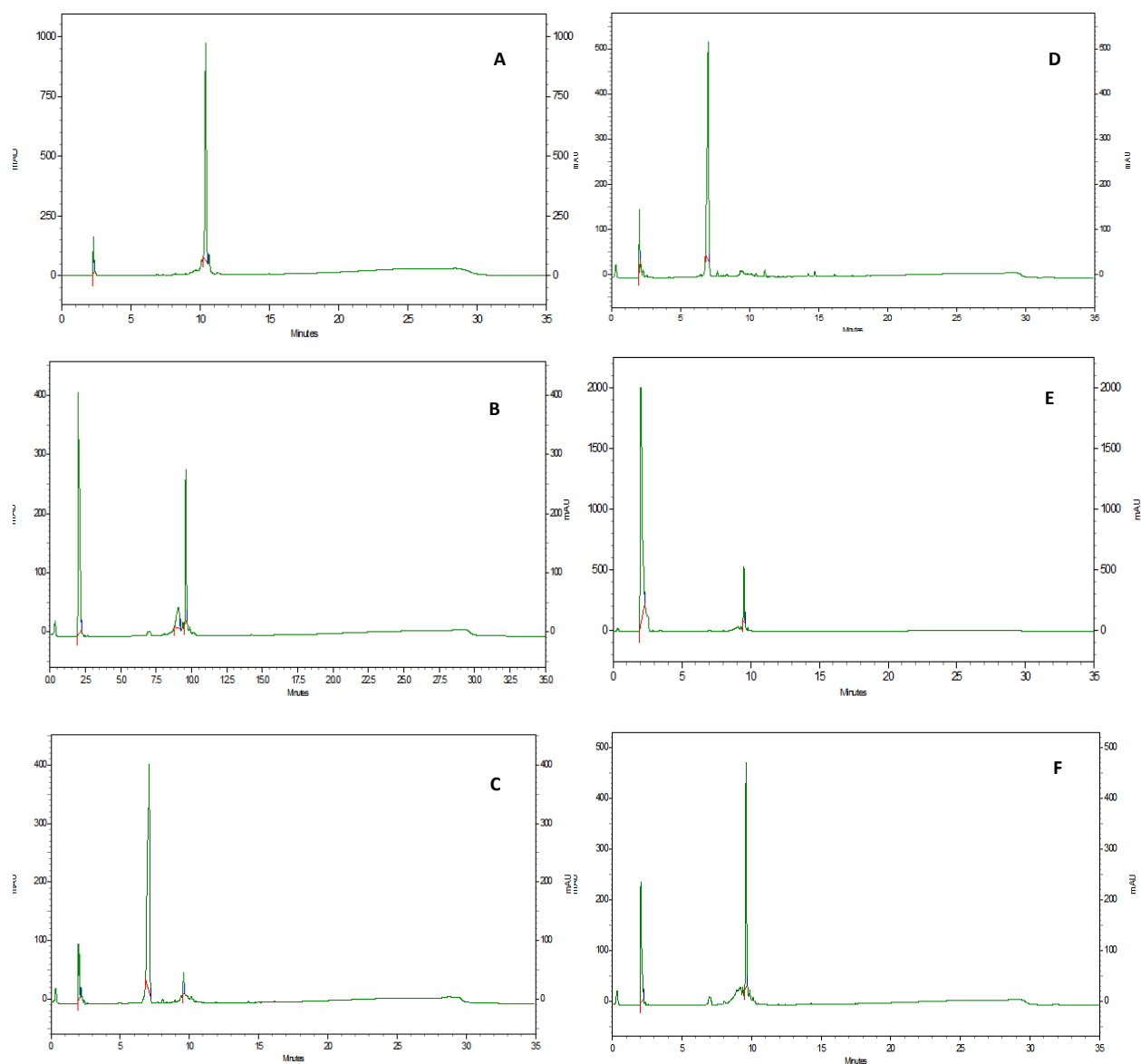


Figure 2.2. Degradation Studies of FLT conjugate **13**. **A:** Standard **13**, Retention time 9.7 min; **B:** Heat: 1 mL stock + 1 mL methanol at 40 °C; **C:** Acid: 1 mL stock + 1 mL 1N HCl at 40 °C; **D:** Base: 1 mL stock + 1 mL 1N NaOH at 40 °C;

E: Oxidation: 1 mL stock + 1 mL H₂O₂ at 40 °C; **F:** Water: 1 mL stock + 1 mL H₂O at 40 °C.

The Log P of the compound was determined by distributing the compound between equal volume of *n*-octanol (organic) and pH 4 acetate buffer (aqueous) with stirring at room temperature for 2 days, followed by HPLC studies. The Log P of the compound was found to be -0.34, indicating that the compound is hydrophilic, most likely due to the presence of several guanidinium moieties from hepta-*L*-arginine. These data suggest that the compound is still very hydrophilic despite the presence of the long 1,12-dodecane dicarboxylic acid linker.

Various gel formulations of the compound were manufactured using non-ionic (HPC-SL) and anionic (Carbopol) polymers with and without the inclusion of the thermo-reversible gelling (Pluronic F-127) polymer. The derivative was used for dissolution studies using four different gels with and without the thermogelling polymer. The HPC-SL formulation consisted of 2.25% w/v in water while Carbopol consisted of 0.2% w/v. For the thermogelling formulations, the HPC-SL and Carbopol gels were mixed with 20% w/v solution of Pluronic F-127(3:1 v/v ratio) in water. The formulations were then sealed in dialysis tubes and the rate and extent of compound dissolution were determined in simulated vaginal fluid at 37°C. There was no observed drug release that could be identified by UV spectrophotometry at 220 nm and 256 nm, suggesting that the compound was either unstable in these formulations.

2.5. Conclusions

Three poly-*L*-arginyl-1,12-dodecanedicarboxylate nucleoside conjugates of FLT, 3TC, and FTC were designed and synthesized using solid-phase chemistry. The structures of the compounds were confirmed by NMR and mass spectroscopy. The compounds were evaluated for their anti-HIV activity against cell-free and cell-associated virus. FLT conjugate **13** showed EC₅₀ values of 2.9-3.1 μ M against X4 and R5 virus. The compound was relatively stable in neutral and oxidative conditions, and unstable in heated, acidic, and alkaline conditions. FLT conjugate was hydrophilic as the Log P was found to be -0.34. The compound was unstable or did not undergo release from the tested hydrogel formulations. These data indicate that the presence of positively-charged CPP could impede the interactions between positively charged V3 loop in gp120 and the conjugates. Further optimization of conjugated CPP-fatty acid-nucleoside conjugates is required to generate compounds with improved anti-HIV activity and optimized stability and formulation performance.

2.6. Acknowledgments

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