Two-Component Covalent Inhibitors (TCCI) of the Human Immunodeficiency Virus Reverse Transcriptase (HIV-RT)

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TWO-COMPONENT COVALENT INHIBITORS (TCCI) OF THE HUMAN IMMUNODEFICIENCY VIRUS REVERSE TRANSCRIPTASE (HIV-RT)

by

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ABSTRACT

The traditional design of nucleoside reverse transcriptase inhibitors (NRTI’s) involves the synthesis of chain-terminated nucleoside analogs. HIV-RT has relatively low fidelity which facilitates mutations that confer resistance towards NRTI’s, also, drug promiscuity from NRTI’s result in various side-effects that lead to poor patient adherence to treatment. We designed and tested two-component covalent inhibitors against HIV-RT. Our inhibitor design results in higher specificity due to its binary approach, which has previously been used in biosensing applications, where both components are necessary for therapeutic effect, and lower chances for mutagenesis because of its inhibitory action. The TCCI approach results in up to 93% inhibition of HIV-RT Furthermore, our inhibitor design is highly modular and can be adjusted towards the therapeutic targeting of other biopolymers.
ACKNOWLEDGMENTS

I am forever thankful to my advisor, Dmitry Kolpashchikov, Ph.D. Unlike many of my previous colleagues from the Diaz group, in the face of uncertainty, I decided to take a risk and change my career path. I would no longer synthesize materials for OLED's, I would explore medicinal chemistry and biochemistry, as these fields always interested me. I took a risk, and Dr. Koplashchikov took that risk with me. He made me a better scientist and changed my way of thinking. As they say, two heads are better than one, I have to add, two molecules are better than one. In a professional sense, Dr. Kolpashchikov will always be a part of my family.

I am also eternally grateful for the past and present members of the Kolpashchikov lab group. Especially Evan Cornett, Ph.D. who got the ball rolling on the TCCI project with his work on light activated TCCI’s and greatly contributed to the first generation of TCCI;s. Yulia Gerasimova, Ph.D. who greatly helped in my advancement as a scientist with her poignant questions (why?... what if?…) 

I also want to acknowledge my parents and grandparents for their support, and their contributions to my way of thinking, for always answering my “why?” questions, and always igniting my curiosity. I want to thank Adriana, my wife, for supporting my stubborn obsession with my career choices. I would also like to dedicate this dissertation to my son David who is too young to remember this date, in the end, I did it all for him.
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<table>
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<th>Description</th>
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<tr>
<td>6-Az</td>
<td>6-Azidohexanoic acid</td>
</tr>
<tr>
<td>AZT</td>
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<td>CA</td>
<td>Clavulanic acid</td>
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<tr>
<td>DBCO</td>
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<td>FBA</td>
<td>Filter Binding Assay</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>NMR</td>
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</tr>
<tr>
<td>NNRTI</td>
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<tr>
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CHAPTER 1: INTRODUCTION

In 1959, a Bantu man died in Kinshasa, Belgian Congo of unknown causes. A fellow female died of unknown causes in Kinshasa, Belgian Congo in 1960. Studies on preserved tissues from these two individuals in 2008 found that both had succumbed to HIV, making them the oldest proven cases of HIV infection. By modeling the genetic differences between the two viral genome samples, it was determined that the HIV epidemic started sometime in 1920’s Leopoldville (Kinshasa).1

In 1981, unusual cases of patients infected with *pneumocystis carinii*, and mucosal candidiasis were rapidly growing. All of these patients where immunocompromised and did not respond to treatment.2 It was not until 1982 that the CDC formally recognized AIDS, and scientists at the Pasteur Institute recognized HIV as the causing agent in 1984.3 In 1986, clinical trials for AZT, developed in 1964 as a chemotherapeutic anti-cancer agent,4 as an antiretroviral agent and is approved as a drug by the FDA in 1987. Initially, AZT appeared as the answer towards the containment and eventual defeat of the AIDS pandemic, but it was soon found that HIV mutates inducing resistance to AZT.5 AZT served as a mere life extension for HIV/AIDS infected patients until 1996 when highly active antiretroviral (HAART) therapy was introduced. For thirty years AZT, a nucleoside reverse transcriptase inhibitor, has been in the forefront in the fight against AIDS. While AZT taken in combination with other antiretroviral drugs has prolonged the life of HIV positive patients, however, HIV is still mutating and slowly making AZT obsolete. Other
NRTIs have been derived from AZT to combat AZT-resistant strands of HIV, yet these drugs act through the same mechanism as AZT.

**HIV Replication Cycle**

HIV has a replication cycle composed of ten steps, fusion, uncoating, reverse transcription, integration, transcription, RNA export, translation, assembly, release, and maturation. Current therapies are able to attack the replication cycle at four of these steps, fusion, reverse transcription, integration, and maturation. The most vulnerable steps for therapeutics are reverse transcription and maturation.

**HIV Fusion and Uncoating**

The viral envelope is spotted with glycoprotein spikes. Each spike has a trimeric structure composed of three surface proteins collectively known as GP120 and three transmembrane proteins known as GP41. GP120 is heavily glycosylated with N-linked mannose residues that form a protective armor over its sensitive active sites.

To enter the CD4+ t-helper cells, GP120 binds to the CD4 viral receptor and one chemokine co-receptor on the host cell. Then, the hydrophobic domains on GP41 aid in the fusion between the viral envelope and the host membrane. Upon fusion, the viral capsid containing the viral genome and viral enzymes is released. Reverse transcription is initiated within the viral capsid, where nucleotides from the host cell are imported by dynamic pores on the capsid surface. The capsid undergoes an uncoating event mechanically activated by the first strand transfer of reverse transcription, and by
cellular factors from activated CD4+ cells such as phosphorylation by MELK in the intracellular environment lead to the release of the viral genome and enzymes.\textsuperscript{15}

Reverse Transcription

The newly released viral RNA genome is transcribed into complementary DNA by HIV reverse transcriptase (HIV-RT). HIV-RT has two active sites, a polymerase site where complementary DNA is synthesized from either an RNA or DNA template and a RNase H site that degrades RNA only when present as a RNA-DNA duplex. HIV-RT being a well-studied enzyme is a prime target for therapeutics partly because it is present in the cytosol of the host cell and partly because it is a low-fidelity enzyme. Over half of currently approved anti-HIV drugs are reverse transcriptase inhibitors.\textsuperscript{16}

Integration

After the viral genomic RNA is transcribed into dsDNA, the pre-integration complex (PIC) is formed. The PIC consists of biomolecules of viral origin (viral DNA, Vpr, matrix, and integrase) and a barrier to autointegration factor 1 from the host cell. The PIC is able to enter the nucleus through the nuclear pore complex without interrupting the nuclear membrane. Upon entering the nucleus, integrase inserts the viral genome in the host genome, preferentially in areas of high transcription to form the provirus.\textsuperscript{17}

Transcription, Export, and Translation

Transcription of the provirus is regulated by the long terminal promoter (LTR) which possesses several binding sites for several cellular transcription factors necessary for T-
cell cellular activity. Transcription then results in the formation of full-length viral mRNA which is responsible for the formation of several structural HIV proteins.

RNA, full, and incompletely spliced are exported out of the nucleus by viral protein REV, while spliced RNA is transported via cellular mechanisms. REV is a viral protein with cellular localization signal that aids in the transport of unspliced RNA from the nucleus to the cytoplasm. Translation of viral RNA is initiated by the internal ribosome entry site (IRES) during which host cell translation is suppressed allowing the efficient synthesis of viral proteins.

Assembly, Budding, and Maturation

The viral proteins are assembled via viral HIV-1 gag protein mediation. Gag contains several binding sites for newly synthesized viral proteins and RNA to form the spherical gag-polyprotein. Gag then interacts with cellular motor protein KIF4 and other components of cellular trafficking pathways to exit the host cell through budding. During the budding process, the virion acquires its transmembrane glycoproteins. After budding, viral protease (HIV-PR) is activated, and the polyprotein is then cleaved which initiates the maturation of the viral particle. During the maturation process, the viral capsid formed, and viral RNA is reorganized into its dimeric form.

HIV-1 Reverse Transcriptase Structure and Function

HIV-1 Reverse Transcriptase (HIV-RT) is a heterodimer composed of the p51 and p66 subunits, each named by their corresponding molecular weights. HIV-RT has two
enzymatic functions, a polymerase domain which copies DNA or RNA template and a RNase H domain which cleaves RNA when present as a DNA-RNA duplex. Like other DNA polymerases, HIV-RT requires the presence of both, a primer and a template.21

DNA synthesis is initiated by host tRNA$\text{lys}_3$ acting as a primer after hybridizing with the 18-nt primer binding site (PBS) on the viral RNA.22 The primer is then elongated to the 5'-R region which upon terminating forms the negative strand stop. This first primitive product is digested by RNase H, and the nascent DNA strand hybridizes to the 3'-R region. This process known as the first jump occurs either by intermolecular or intramolecular mechanisms. The primer then continues elongating along the viral RNA template until it passes the polypurine rich (PPT) region where RNase H cleaves the PPT region’s borders to create a unique positive RNA strand, and as elongation continues, RNase H removes the complementary template. The PPT region along with other small polypurine-rich RNA islands act as templates for the formation of the positive DNA strand which elongates until the annealed tRNA$\text{lys}_3$ is copied thus generating a positive DNA PBS region. This is known as the plus-strand strong stop, and RNase H then removes the tRNA. Positive DNA PBS then hybridizes with negative DNA PBS (second jump) forming cDNA and positive DNA elongation continues. Then, strand displacement and or repair and ligation results in the formation of a linear DNA duplex with long terminal repeats (LTR’s). Often, HIV-RT makes double-stranded DNA from two different RNA genomes resulting in viral recombination. This results in mutations that lead to multi-drug resistant HIV.23
Structurally, the p66 subunit contains both polymerase and RNase H active site. The p51 subunit is a structural subunit. The polymerase domain contains four subdomains, fingers, palm, thumb, and connection; the nucleic acid binding cleft includes the previously mentioned four domains plus the RNase H domain. The nucleic acid domain undergoes a conformation change when primer-template is present known as the primer grip. This grip is formed by a highly conserved region, and mutations in this region may affect polymerase and RNase H activity. The polymerase domain contains a triad of aspartic acid residues (D110, D185, and D186) complexed with magnesium where D185 and D186 are highly conserved. R72 and K65 bind to the β and γ phosphates from incoming dNTPs and are also highly conserved. Y115 discriminates between dNTPs and rNTPs, while Q151 interacts with the incoming dNTP’s 3’-OH.

**Nucleoside Reverse Transcriptase Inhibitors (NRTI’s)**

NRTI’s are the first line of defense against HIV infection developed. Currently, there are eight FDA approved NRTI’s: abacavir (ABC), didanosine (ddI), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T), zalcitabine (ddC), and zidovudine (AZT). The first NRTI approved for HIV was AZT, also known as azidothymidine. AZT, first synthesized in 1964²⁴ as a possible anti-cancer chemotherapeutic was approved by the FDA in 1987 for the treatment of HIV/AIDS.

AZT, as well as other NRTIs, act upon HIV-RT as chain terminators due to the lack of 3’-OH. When originally developed, AZT was used as a standalone antiretroviral; the efficacy of AZT declined shortly thereafter because of viral mutations.²⁵ With the
development of lamivudine, another NRTI, and protease, integrase, and non-nucleoside reverse transcriptase inhibitors, the highly-active antiretroviral therapy (HAART) drug regimen were introduced in 1996 to mitigate the proliferation of NRTI resistance and extend the patient’s life. HAART consists of a combination of two NRTI’s and one protease, integrase, or NNRT inhibitor. While HAART can suppress the virus for the lifetime of a patient, mutations can still arise. Also, patients must adhere to HAART for the rest of their lives.

Figure 1. Commonly used nucleoside reverse transcriptase inhibitors (NRTIs)
Resistance NRTI’s occurs via two mechanisms, pyrophosphorolysis mediated by ATP and increased discrimination between natural dNTPs and inhibitors. There are two types of mutations which lead to resistance nucleotide associated mutations (NAMs) and thymidine associated mutations (TAMs). TAMs are involved in the pyrophosphorolysis of AZT and d4T and there are two distinct pathways leading to TAMs. Pathway 1 (M41L, L210W, T215Y, and occasionally D67N) and pathway 2 (D67N, K70R, T215F, and 219E/Q). NAMs which discriminate between dNTPs and NRTIs arise from the M184V/I and the K65R mutations. The M184V mutation discriminated between dNTPs and 3TC or FTC. The K65R mutation discriminates against tenofovir, ddC, ddI, d4T, and ABC. These mutations decrease HIV-RT fitness, but the loss of activity can be mitigated by the appearance of secondary mutations. The drive behind the development of more NRTI’s lies in causing lethal mutagenesis, a viral particle that is no longer viable due to the amount of mutations present.

**The Two-Component Covalent Inhibitor (TCCI) Approach**

Most of the currently approved protein and nucleic acid inhibitors bind reversibly to their targets to decrease biological activity. Ideally, the inhibitors will selectively bind to their intended target only, but, they may also bind to non-specific targets and cause serious side effects. The reversibility of these inhibitors also increases the probability of mutations and development of drug-resistant pathogens. In this study, we propose to use a novel approach towards the design of irreversible covalent drugs by taking advantage of Two Component Covalent Inhibitors (TCCI’s) concept. TCCI’s show improved
specificity and irreversibility of inhibition. The main idea behind this approach is the use of two relatively unreactive chemical functional groups that would react with each other when in close proximity and correct orientation when bound to a specific biopolymer. The reaction between the pre-reactive groups forms a highly reactive electrophilic species which is then open to the attack by nearby nucleophiles on the target protein or nucleic acid. The specificity of the TCCI’s is determined by the chosen ligand analogs which can be modified accordingly by adding the respective pre-reactive groups. We demonstrate that the TCCI approach can be effectively used to covalently and selectively inhibit T4 DNA polymerase when using radiation activated TCCI, and HIV-RT when using chemically activated TCCI systems.

Figure 2. Light dependent TCCI dTTP analogs

The radiation activated TCCI was first developed by Evan Cornett at the Kolpashchikov lab in 2012. This system consisted of two “pre-reactive” dTTP analogs where the first analog contained a tetrafluoroazidobenzene (FAB) warhead, and the
second contained a pyrene warhead. When T4 DNA polymerase was exposed to radiation at 340 nm, the pyrene transferred energy to the FAB group generating a nitrene radical which cross-linked and inactivated the enzyme. By calculating the specificity factor for the TCCI approach and comparing the calculation for a single covalent inhibitor, it was found that the specificity was increased by a factor of 11. This specificity was demonstrated by an experiment where T4 DNA polymerase competed for a hairpin substrate with T7 RNA polymerase. Inhibition of T4 DNA polymerase varied little up to the presence of 4000:1 T7 RNA polymerase: T4 DNA polymerase, while single component covalent inhibition showed marked promiscuity where inhibition of T4 DNA polymerase dramatically decreased in the concentrations of T7 RNA polymerase became high enough to limit the availability of the hairpin substrate.

Figure 3. TCCI scheme
Figure 4. TCCI specificity assay
CHAPTER 2: FIRST GENERATION TWO-COMPONENT COVALENT INHIBITORS

Introduction

The radiation initiated TCCI has limited application due to the short penetration range of violet light; leading to an effort to find a chemically activated TCCI. The first generation of TCCIs was β-lactam derived dTTP analogs activated by nucleophiles. The β-lactam approach yielded good results, but β-lactam’s ring instability led to characterization problems. Both TCCI candidates were designed as HIV-RT inhibitors.

The β-lactam approach consisted of a dTTP analog containing clavulanic acid first, but instability led to the design of a sulbactam derivative. Sulbactam required less processing prior to amidation, and is reportedly more stable than clavulanic acid\textsuperscript{38–41}. The second dTTP analog containing a nucleophile (imidazole, thiol, or phenol). This design was based on the mechanism of action of clavulanic acid and sulbactam were a β-lactamase nucleophilic residue attacks the four-membered β-lactam ring and forms a Michael acceptor that is readily attacked by a second nucleophilic residue as shown in figure 5.\textsuperscript{42} We propose an analogous mechanism for the inactivation of HIV-RT, where a nucleophilic dTTP analog recreates the nucleophilic attack by the serine residue on either clavulanic acid or sulbactam dTTP analogs (figure 6). Several nucleophilic residues where designed for this purpose, allylpropioimidazolamide dTTP (IM-dTTP),
allyl-(2,3,5,6-tetrafluoro-4-hydroxy)benzamide dTTP (TF-dTTP), and 3-mercaptopropionamide dTTP (3M-dTTP).

Figure 5. Mechanism of action for clavulanic acid. A serine residue on β-lactamase attacks the carbonyl on the β-lactam ring resulting in the opening of the penam structure, this results in the formation of a Michael acceptor that is readily attacked by a second nucleophile on β-lactamase resulting in covalent inhibition.
Figure 6. Proposed mechanism of action for the inhibition of HIV-RT by the β-lactam/nucleophile TCCI pair.

Materials and Methods

Synthesis of 5-allylamine dTTP\textsuperscript{43}: 5-allylamine dUTP was synthesized by using adding dUTP (200mg, 0.36 mmol) and mercuric acetate (574 mg, 1.8 mmol) were added to a 40 mL solution of 0.1 M sodium acetate at pH 6 and stirred for 4 hours at 50 °C. The solution was then cooled over ice, and lithium chloride (142 mg, 3.25 mmol) was added and stirred for 15 minutes to produce mercuric chloride. The mercuric chloride was then removed by washing the aqueous layer six times with 40 mL of ethyl
acetate, and the mercurated dUTP was precipitated by cold ethanol overnight. The mercurated dUTP pellets were then dried and resuspended in 50 mL of 0.1 M sodium acetate at pH 5. 1.5 mL of pure allylamine (13.3 M) was slowly added to 8.5 mL of cold 4 M acetic acid. Neutralized allylamine (1.1 mL, 2.2 mmol) were then added to the mercurated dUTP solution, followed by the addition of potassium tetrachloropalladate (0.5 mmol) previously prepared by the addition of palladium (II) chloride (90 mg, 0.5 mmol) to a 4 mL solution containing potassium chloride (150 mg, 2 mmol). As the solution progressed, palladium and palladium oxide precipitated turning the solution metallic black. The reaction was allowed to stir at room temperature for 20 hours. Then, the solution was filtered by a 0.45 mm-membrane syringe filter resulting in a yellow filtrate. The filtrate was then diluted five times, and purified by anion exchange chromatography while using DEAE Sephadex A-25 (General Electric) and a 0 – 0.6 M gradient of triethylammonium bicarbonate (TEAB) buffer. The desired product eluted between 0.25 M and 0.45 M TEAB. The eluant was then concentrated under vacuum at 40 °C and resuspended in N,N-dimethylformamide. The concentration of allylamine was then determined by measuring absorbance at 290 nM while using $\varepsilon_{290} = 8100$. 
Transformation of clavulanic acid, potassium salt: For DCC couplings, free acids or ammonium salts are required. Potassium clavulanate was then transformed into ammonium clavulanate by cation exchange chromatography. 10.2 mg of potassium clavulanate (Sigma) was dissolved in water and added to a column containing Amberlite IR-120 cation exchange previously treated with 1M ammonium chloride (Fisher). Elution of ammonium clavulanate was monitored by TLC. The resulting ammonium clavulanate solution was then lyophilized to obtain a light-yellow powder.

Figure 7. Synthesis of 5-allylamine dUTP

Figure 8. Transformation of potassium clavulanate into ammonium clavulanate
**Synthesis of 5-allylclavulanamide dTTP:** 1 equivalent of ammonium clavulanate was dissolved in 1 mL of N,N-dimethylformamide (Fisher) followed by the addition of 1.1 equivalents of N-hydroxysuccinimide (ACROS) and 1.2 equivalents of dicyclohexylcarbodiimide and shaken until the formation of dicyclohexylurea (ACROS) crystals were observed. The solution was then centrifuged at 12000 RPM at 4 °C, and the supernatant was transferred to a fresh microcentrifuge tube containing 0.5 equivalents of 5-allylamine dTTP and 4 equivalents of DIPEA (Fisher) in N,N-dimethylformamide. This solution was shaken at room temperature until the disappearance of the amine monitored by TLC/Ninhydrin stain. The resulting product migrated to a Rf = 0.32 on aluminum backed, silica gel TLC plates when using a 3:1:1 water:n-propanol: ammonium hydroxide mobile phase. The product was precipitated by dividing the solution into 200 mL aliquots in 2 mL microcentrifuge tubes and filling the tubes with 2% lithium perchlorate in acetone. The tubes were then centrifuged at 12000 RPM at 4 °C, and the resulting pellets were washed three times with cold absolute ethanol. The pellets were individually resuspended in 1 mL of water, and purified by HPLC (JASCO) equipped with a C-18 reverse phase column (Phenomenex). The product was eluted using a gradient 0.05M lithium perchlorate 0 – 30% acetonitrile in water buffer, with the desired product eluting at 4% of the gradient. The fractions containing the product of interest were combined and lyophilized overnight after flash-freezing in a round bottom flask. The product was then resuspended in a minimal amount of water, transferred to a 2 mL microcentrifuge tube and precipitated with lithium
perchlorate in acetone followed by two cold absolute ethanol washes and two cold acetone washes to remove excess lithium perchlorate, and three washes with 1,1,2-trichloro-1,2,2-trifluoroethane to remove remaining acetone and dried in a vacuum desiccator for 30 minutes. Afterwards, the pellet was resuspended in 55 mL of deuterium oxide (Sigma), and the sample was characterized by $^1$H-NMR, where possible traces of the product of interest were observed along with degradation of the product.

![Chemical structure](image)

**Figure 9. Synthesis of 5-allylclavulanamide dTTP**

*Synthesis of 5-allylaminosulbactamide dTTP:* Sulbactam (TCI America) 1 equivalent was dissolved in 1 mL of previously dried N,N-dimethylformamide containing 1.1 equivalents of N-hydroxysuccinimide and 1.2 equivalents of dicyclohexylcarbodiimide and shaken for 30 – 60 minutes. The sample was then centrifuged to separate the dicyclohexylurea side-product, and the supernatant was transferred to a 2 mL microcentrifuge tube containing 0.5 equivalents of 5-allylamine dTTP and 4 eq. of DIPEA. The mixture was allowed to react by shaking at room
temperature for 2 to 4 hours, and the reaction was monitored by TLC and ninhydrin stain, and the product was observed $R_f = 0.33$ when using the same system as above. Upon the consumption of 5-allylamine dTTP, the reaction mixture was divided into 200 mL aliquots and precipitated by 2% lithium perchlorate in acetone. The tubes were centrifuged at 12000 RPM at 4 °C for 5 minutes, and the supernatant was discarded. Then, the pellets were washed three times with absolute ethanol and resuspended in water for RP-HPLC purification using the same gradient system as with clavulanamide dTTP. The product eluted on average at 19.5% (5.9% acetonitrile) of the gradient, $t_R = 9.9$. After purification, the product was processed in the same fashion as the clavulanic acid derivative. 5-allylsulbactamide dTTP was then characterized by $^1$H-NMR where a mixture of decomposition products and traces of the product of interest were observed.

![Figure 10. Synthesis of allylsulbactamide dTTP](image)

**Synthesis of 5-allylpropioimidazolamide dTTP**: 1 equivalent of Imidazole propionic acid (TCI America) was dissolved in 1 mL of previously dried N,N-

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dimethylformamide containing 1.1 equivalents of N-hydroxysuccinimide and 1.2 equivalents of dicyclohexylcarbodiimide and shaken for 30 – 60 minutes. The sample was then centrifuged to separate the dicyclohexylurea side-product, and the supernatant was transferred to a 2 mL microcentrifuge tube containing 0.5 equivalents of 5-allylamine dTTP and 4 eq. of DIPEA. The mixture was allowed to react by shaking at room temperature for 2 to 4 hours, and the reaction was monitored by TLC and ninhydrin stain, and the product was observed Rf = 0.29 when using the same system as above. Upon the consumption of 5-allylamine dTTP, the reaction mixture was divided into 200 mL aliquots and precipitated by 2% lithium perchlorate in acetone. The tubes were centrifuged at 12000 RPM at 4 °C for 5 minutes, and the supernatant was discarded. Then, the pellets were washed three times with absolute ethanol and resuspended in water for RP-HPLC purification using the same gradient system as with clavulanamide dTTP. The product eluted on average at 68% of the gradient (9.6% acetonitrile) of the gradient, tR= 13 minutes. After purification, the product was processed in the same fashion as above and was then characterized by 1H-NMR.

Figure 11. Synthesis of allylpropioimidazolamide dTTP
Synthesis of 5-allyl-(2,3,5,6-tetrafluoro-4-hydroxy)benzamide dTTP (TF): 1 equivalent of 2,3,5,6-tetrafluoro-4-hydroxybenzoic acid (Sigma) was dissolved in 1 mL of previously dried N,N-dimethylformamide containing 1.1 equivalents of N-hydroxysuccinimide and 1.2 equivalents of dicyclohexylcarbodiimide and shaken for 45 minutes. The sample was then centrifuged to separate the dicyclohexylurea side-product, and the supernatant was transferred to a 2 mL microcentrifuge tube containing 0.5 equivalents of 5-allylamine dTTP and 4 eq. of DIPEA. The mixture was allowed to react by shaking at room temperature for 16 hours, and the reaction was monitored by TLC and ninhydrin stain, and the product was observed $R_f = 0.47$ when using the same system as above. Upon the consumption of 5-allylamine dTTP, the reaction mixture was divided into 200 $\mu$L aliquots and precipitated by 2% lithium perchlorate in acetone. The tubes were centrifuged at 12000 RPM at 4 °C for 5 minutes, and the supernatant was discarded. Then, the pellets were washed three times with absolute ethanol and resuspended in water for RP-HPLC purification using the same gradient system as with clavulanamide dTTP. The product eluted on average at 56% of the gradient (13.2% acetonitrile) of the gradient, $t_R = 14$ minutes. After purification, the product was processed in the same fashion as previously reported dTTP analogs and characterized by $^1$H-NMR, $^{19}$F-NMR, and $^{31}$P-NMR.
Figure 12. Synthesis of allyl-(2,3,5,6-tetrafluoro-4-hydroxy)benzamide dTTP

Synthesis of 5-allyl-(3-mercapto)propionamide dTTP: 1 equivalent of 3-mercaptopropionic acid (Sigma) was dissolved in 1 mL of previously dried N,N-dimethylformamide containing 1.1 equivalents of N-hydroxysuccinimide and 1.2 equivalents of dicyclohexylcarbodiimide and shaken for 60 minutes. The sample was then centrifuged to separate the dicyclohexylurea side-product, and the supernatant was transferred to a 2 mL microcentrifuge tube containing 0.5 equivalents of 5-allylamine dTTP and 4 eq. of DIPEA. The mixture was allowed to react by shaking at room temperature for 2 hours, and the reaction was monitored by TLC and ninhydrin stain, and the product was observed Rf = 0.47 when using the same system as above. Upon the consumption of 5-allylamine dTTP, the reaction mixture was divided into 200 mL aliquots and precipitated by 2% lithium perchlorate in acetone. The tubes were centrifuged at 12000 RPM at 4 °C for 5 minutes, and the supernatant was discarded. Then, the pellets were washed three times with absolute ethanol and resuspended in water for RP-HPLC purification using the same gradient system as with clavulanamide.
dTTP. The product eluted on average at 56% (13.2% acetonitrile) of the gradient, $t_R= 16$ minutes. After purification, the product was processed in the same fashion as the clavulanic acid derivative. 5-allylsulbactamide dTTP was then characterized by $^1$H-NMR and $^{31}$P-NMR.

![Chemical structure of dTTP](image)

Figure 13. Synthesis of allylmercaptopropionamide

Substrate Properties and Inhibition

All substrate properties and inhibition assays were designed and implemented by Evan Cornett at the Kolpashchikov lab. The following is a summary of those results. Both, CL with IM and SU with TF showed significant attenuation of HIV-RT activity. When CL and IM were used as TCCI pair, an average of 78% inhibition was observed, while for the SU – TF pair, similar inhibition was observed.

Gel-Analysis of reactive pairs. 15 μL samples containing 50nM of HIV-1 RT (Worthington Biochemical Corporation), 1 μM of FAM-primer (FAM-Primer: 5'/FAM/ GTC CCT GTT CGG GCG CCA) and template (5'-C TGT GTG GTC GTT CTC TTG CTA TGG GCG CCG AAC AGG GAC) in HIV RT RXN buffer (50 mM Tris pH8.3, 8 mM
\[ \text{MgCl}_2, 40 \text{ mM KCl} \). FAM-primer was purified by HPLC. For CA - TF experiments, 5\( \mu \text{M} \) of each CA, TF, or dTTP analog was added to the corresponding microtube, followed by incubation at 37 °C. Aliquots of each sample were sequentially removed at different time points (5, 10, 20 and 30 minutes) and 500\( \mu \text{M} \) of natural dNTPs were added. After incubation at 37 °C for an additional hour, samples were quenched by addition of an equal volume of 2X dPAGE Loading Buffer (LB) (95\% Formamide, 20 mM EDTA (pH 8.0), 0.05\% bromophenol blue). For SB-TF experiments the first analog was added to a final concentration of 10 \( \mu \text{M} \) followed by 30 minute incubation at 37 °C. The second analog was added to a final concentration of 100 \( \mu \text{M} \) followed by 60 minute incubation at 37 °C. Next, 500 \( \mu \text{M} \) of all four natural dNTPs (dATP, dCTP, dGTP, dTTP) were added to each sample, as indicated, and incubated for 60 minutes at 37 °C. To stop the polymerase reaction, an equal volume of 2X dPAGE LB was added to each sample. Control samples to evaluate elongation by one base were quenched with dPAGE LB after the initial incubation period. For both CA and SB experiments, all samples were boiled for 5 minutes prior to loading 15 \( \mu \text{L} \) onto a 15\% 7 M urea denaturing PAGE gel that was typically run for 45 minutes (or until sufficient separation as indicated by migration of bromophenol blue) at 400 V with 55 °C water running through a Thermo Owl Electrophoresis apparatus. Gels were rinsed in deionized water and visualized using a Syngene U:Genius Gel documentation instrument which allowed detection of the fluorescently labeled FAM-primer with no additional staining. The resulting gels were analyzed by fluorescence of the corresponding analog or TCCI pair relative to the fluorescence of the natural substrate elongation.
Results and Discussion

The β-lactam derivatives were difficult to characterize due to their inherent instability, especially in the presence of nucleophiles under the basic reaction condition used for the N-acylation of allylamine dUTP. This led to poor proton NMR’s where not all the expected signals were present. The analogs were still analyzed under dPAGE and tested as possible candidates as TCCIs while awaiting improved synthetic pathways.

The dPAGE analysis of the analogs shows that both β-lactam modified dTTP analogs are efficient nucleotide analogs yielding approximately 82% product elongation relative to dTTP incorporation after 30 minutes, while TF shows 84% elongation and IM shows moderate inhibition at 45% elongation. When analogs were combined, a marked inhibitory effect is noticed where the CL-TF pair shows only 25% elongation, IM-CL 16% elongation, TF-SB 11% elongation.

The above results show that dTTP analog pairs (Nucleophile - β-lactam) inhibit HIV-RT, but the β-lactam moiety is unstable. This inherent instability facilitates the covalent inhibition of β-lactamases and possibly the inhibition of HIV-RT when used as a TCCI, but hinders the synthesis of dTTP analogs via traditional DCC/NHS coupling and subsequent product characterization.

Possible solutions for overcoming current synthetic difficulties are the transformation of sulbactam or clavulanic acid into acyl chlorides followed by acylation
of allylamine dideoxyuridine. The acyl chloride is more reactive than the strained β-lactam and the lack of hydroxide groups on the nucleic acid analog eliminates competing reactions. The drawback from this proposal is that substrate properties for the analog cannot be tested because it would act as a chain terminator.
CHAPTER 3: SECOND GENERATION TWO-COMPONENT COVALENT INHIBITORS

Introduction

Azides and strained cyclooctynes are well known biorthogonal reagents used for the labeling of proteins,\textsuperscript{44} glycosides,\textsuperscript{45} and nucleic acids.\textsuperscript{46} This led to the utilization of 6-Azidohexanamide dTTP (6Az-dTTP) and Dibenzocyclooctyne-amide dTTP (DBCO-dTTP) as pre-reactive analogs against HIV-RT. The available literature does not indicate any reactivity by azides, DBCO, or triazole click-product; except for a technical brief from Glen Research that shows cleavage of DBCO from its internal amide linker in the presence of iodine.

While testing this TCCI pair, inhibition rates between above 90\% were observed independent of the order of addition while individual analogs show little inhibition. Also, this TCCI pair was well characterized and showed covalent cross-linking by Western blot.

Methods and Materials

\textit{Synthesis of 6-azidohexanoic acid:} 1.94g (10 mmol) of 6-bromohexanoic acid (ACROS) and 1.3g (20 mmol) of sodium azide (ACROS) were added to 10 mL of previously dried N,N-dimethylformamide (ACROS). The solution was stirred at room temperature for 36h. The product was extracted with 15 mL of dichloromethane, and the organic layer was washed three times with 20\% aqueous lithium chloride and dried over
anhydrous magnesium sulfate overnight at 4 °C. The solvent was then evaporated under vacuum and the product characterized by $^1$H-NMR (ppm): 1.38 (m, 2H); 1.59 (m, 4H); 2.32 (m, 2H); 3.23 (m, 2H); 10.47 (s, 1H) and $^{13}$C-NMR (ppm): 24.48; 26.47; 28.85; 34.18; 51.9; 180.00.

![Chemical structure](image)

Figure 14. Synthesis of 6-azidohexanoic acid

*Synthesis of 5-allyl(6-azidohexanamide) dTTP (6-Az dTTP):* 6-azidohexanoic acid (mmol) was added to a 1 mL solution containing N-hydroxysuccinimide (mmol) and dicyclohexylcarbodiimide (mmol) in N,N-dimethylformamide. The solution was rocked for 30 minutes, and the solution was separated from dicyclohexylurea by centrifugation at 10000 rpm for 5 minutes at 4 °C. The supernatant was then transferred to a tube containing 200 µL 5-allylamine dUTP (46.8 mM solution in DMF), and trimethylamine (mmol). The new reaction mixture was rocked for approximately 2h when consumption of allylamine was observed by ninhydrin stain on TLC, $R_t = 0.36$. The resulting product was purified by RP-HPLC, $t_r = 12$ min (%), and characterized by $^1$H-NMR (ppm): 1.41 (m, 2H); 1.63 (m, 4H); 2.34 (m, 2H); 2.43 (m, 2H); 3.35 (m, 3H); 3.94 (m, 1H); 4.24 (m,
3H); 4.70 (m, 2H); 6.34 (m, 2H); 6.44 (m, 1H); 7.93 (s, 1H). $^{31}$P-NMR (ppm): -19.94 ($\beta$); -10.71 ($\alpha$); -4.83 ($\gamma$).

**Figure 15. Synthesis of allyl(6-azidohexanamide) dTTP**

*Synthesis of 5-allyl(6-(11,12-Didehydro-5,6-dihydrodibenzo[b,f]azocine-5-yl)-6-oxohexanamide) dTTP (DBCO-dTTP)*: Dibenzocyclooctyne-acid (mmol) was added to a 1 mL solution containing N-hydroxysuccinimide (mmol) and dicyclohexylcarbodiimide (mmol) in N,N-dimethylformamide. The solution was rocked for 30 minutes, and the solution was separated from dicyclohexylurea by centrifugation at 10000 rpm for 5 minutes at 4 °C. The supernatant was then transferred to a tube containing 200 µL 5-allylamine dUTP (46.8 mM solution in DMF), and diisopropylethylamine (mmol). The new reaction mixture was rocked for approximately 2h when consumption of allylamine was observed by ninhydrin stain on TLC, $R_t = 0.36$. The resulting product was purified by RP-HPLC, $t_r = 33$ min (30% acetonitrile), and characterized by $^1$H-NMR (ppm): 1.13 (m, 4H); 1.91 – 2.04 (m, 4H); 2.33 (m, 2H); 4.16 (m, 3H); 4.60 (m, 1H); 6.25 (m, 2H);
7.12 (m, 1H); 7.35 (m, 4H); 7.56 (m, 1H); 7.76 (m, 1H). $^{31}$P-NMR (ppm): -19.97 ($\beta$); -10.74 ($\alpha$); -5.01 ($\gamma$).

![Chemical structure]

**Figure 16. Synthesis of DBCO-dTTP**

**Substrate Properties and Inhibition**

Initial substrate properties and inhibitory action by the 6-Az dTTP and DBCO dTTP TCCI was investigated by dPAGE of a fluorescein labeled primer/template, where elongation of the primer was achieved by utilizing 6-Az dTTP and DBCO dTTP with little loss of activity relative to dTTP, however, enzyme activity was lost upon loading of both 6-Az dTTP and DBCO dTTP analogs prior to the addition of dNTPs.

The substrate properties were tested by incubating each sample containing 5µM dTTP or dTTP analog for 30 minutes at 37 °C in a solution containing fluorescein.
labeled primer/template (1 mM), HIV-RT (50 nM), BSA 100 µg/mL, DTT (2 mM), and HIV-RT buffer (50 mM Tris-HCl pH = 8, 100 mM KCl, and 8 mM MgCl₂), followed by the addition of 1.5 µL of 5 mM dNTPs (dTTP, dCTP, and dGTP) to an aliquot (13.5 mL) of the previously incubated mixture. The reaction mixtures were quenched by the addition of loading buffer (70% formamide, 0.05% bromophenol blue, 29.95% glycerol, and 20 mM EDTA). The reaction mixtures were then boiled for 5 minutes in a water bath and added to a 15% dPAGE. The gel was run at 400 V for 50 minutes.

Similarly, inhibition assays were prepared by incubating either 6-Az dTTP or DBCO dTTP added as the first analog and incubated for 30 minutes at 37 °C, followed by the addition of the second analog and incubation for an additional 20 minutes. At the conclusion of the second incubation, dNTPs were added followed by a 45-minute incubation. The reaction mixtures were quenched by the addition of loading buffer, then, the reaction mixtures were then boiled for 5 minutes in a water bath, and added to a 15% dPAGE. The gel was run at 400 V for 50 minutes.

Figure 17. Substrate and inhibition properties of 6-Az dTTP and DBCO-dTTP
To determine the formation of protein-primer crosslink, Western blot was employed. dTTP and two sets of analogs were added to solutions containing biotin labeled hairpin substrate (1mM), HIV-RT (100 nM), BSA 100 µg/mL, DTT (2 mM), and HIV-RT buffer. After 30 minutes of incubation at 37 °C, DBCO dTTP was added to one of the solutions containing 6-Az dTTP, while 6-Az dTTP was added to one solution containing DBCO dTTP. The mixtures were further incubated for 30 minutes.

![Modified Western blot of HIV-RT upon the incorporation of 6-Az dTTP and DBCO dTTP into a biotinylated DNA hairpin substrate](image)

Figure 18. Modified Western blot of HIV-RT upon the incorporation of 6-Az dTTP and DBCO dTTP into a biotinylated DNA hairpin substrate

A filter binding assay (FBA)\textsuperscript{47} was employed to quantify primer elongation by dNTPs after the addition of dTTP, analogs, and TCCI combinations. For the FBA, a master mix containing fluorescein labeled primer/template (1mM), HIV-RT (50 nM), BSA
100 µg/mL, DTT (2 mM), and HIV-RT buffer was aliquoted into six 15 mL portions: Sample 1 (negative control), samples 2, 3, and 4 (dTTP, 6-Az dTTP, and DBCO dTTP respectively), and samples 5 and 6 (6-Az-dTTP and DBCO dTTP). The samples were incubated for 45 minutes at 37 °C. After the first step elongation, 15 mL of FBA master mix (1 mM dTTP, 1 mM dATP, 1 mM dCTP, and 10 mM 3H-dGTP) was added to samples 1 through 4 and incubated for an additional 45 minutes, while DBCO dTTP was added to sample 5 and 6-Az dTTP was added to sample 6, and incubated for 30 minutes. After the 30 minutes elapsed, FBA master mix was added to samples 5 and 6, and further incubated for 45 minutes, while samples 1 through 4 were quenched by the addition of 30 mL of 0.5M EDTA and placed in ice. After finalizing the incubation of samples 5 and 6, the samples were also quenched by the addition of 30 mL of 0.5 M EDTA. All samples were then added to anion exchange filter papers (Whatman, DE81) and allowed to air dry. The dried filter papers were then washed twice with two 50 mL portions of 0.5 M sodium phosphate buffer at pH 6.8, followed by one ethanol wash. After washing, the filter papers were oven dried. Once dry, the filter papers were all placed in 25 mL scintillation vials, and 7 mL of BD scintillation cocktail was added. Radiation was then measured by a scintillation counter (Beckmann 5900), and the reported values in counts per minute (CPM) were normalized to the value given by sample 2 which contains natural substrates. The experiment was run in triplicate to ensure statistical significance, and normalized to the elongation of natural dNTPs.
Figure 19. Filter binding assay measuring the incorporation of tritiated dGTP

A study comparing the click-TCCI with AZT was conducted where HIV-RT was incubated with dTTP for 45 minutes followed by the addition of 5mM dNTPs containing 10 µM of tritiated dGTP and further incubated for three hours to serve as a positive control. Two TCCI samples prepared by the addition of 6-Az dTTP as the first analog and DBCO dTTP as the second analog after a 45 minute incubation were incubated for 30 minutes to ensure maximum cross-linking; one 1.5 µL aliquot of dNTPs were then added to the first sample, while a 3 µL aliquot was added to the second and the
template concentration was doubled. Similarly, two samples were incubated containing AZT for 45 minutes followed by the addition of dNTPs for the first AZT sample or doubled addition of dNTPs and template for the second sample. Like the inhibition assay above, the samples were quenched, transferred to anion exchange filter papers and radiation counts were measured.

Figure 20. FBA comparing the efficacy of AZT against the click-TCCI system

The specificity of the inhibitor was studied by a filter binding assay. Samples containing 50 nM HIV-RT or 50nM HIV-RT plus either 1000 nM, 5000 nM, or 10000 nM T7 RNA polymerase were incubated in the presence of dTTP (50 nM HIV-RT only),
DBCO dTTP, and TCCI when 6-Az dTTP was added first. The samples were first incubated for 30 minutes with dTTP, DBCO dTTP, or 6-Az dTTP. Then, DBCO dTTP was added to the sample containing 6-Az dTTP, and all samples were further incubated for 30 minutes. FBA master mix was then added to all samples and incubated for 30 minutes. The reaction was quenched by adding an equivalent volume of 0.5M EDTA. The samples were then transferred to a Whatman DE81 anion exchange filter disc and dried in the oven for 20 minutes at 50 °C. The dried filters were then washed five times in 50 mL of pH 6.8 sodium phosphate buffer followed by two ethanol washes and oven dried for 20 minutes. The filter discs were then placed in 25 mL glass scintillation vials, and 7 mL of scintillation cocktail was added. The samples were then analyzed by using a Beckmann 5000 liquid scintillation counter.

Figure 21. Specificity assay for the click-TCCI system, where the click-TCCI inhibition is unaffected by the presence of T7 RNA polymerase
Results and Discussion

Substrate properties of the individual analogs show equivalence to dTTP as they are efficiently incorporated into the template, and the template is fully elongated following analog incorporation even in the absence of dTTP. However, when both analogs are used in conjunction, enzyme activity is lost and no other dNTPs are able to incorporate even when dTTP is present in excess concentrations independent of the order of addition. We believe that this is due to a combination of cross-linking of the DBCO-dTTP analog and the formation of a bulky click-product in the presence of the 6Az-dTTP analog. The selectivity of the TCCI is demonstrated in the presence of T7 RNA polymerase where inhibition was unaffected by the presence of up to 200 times excess T7 RNA polymerase. Another important advantage of the TCCI is the demonstrated superiority over AZT. When the TCCI was incubated after the increased concentration of primer-template and dNTPs, the activity of HIV-RT did not increase whereas the activity of HIV-RT increased almost threefold in the presence of AZT. HIV-RT shows ~7% activity in the presence of the TCCI independent of the order of addition which is comparable to the ~5% activity when HIV-RT is incubated with AZT.

The DBCO dTTP/6-Az dTTP TCCI demonstrate the potential application of TCCIs as anti-retroviral therapeutics. The inference from the dPAGE experiments shows that the mechanism of action likely proceeds via nucleophilic acyl substitution of the amide on the DBCO linker adjacent to cyclooctyne.
CHAPTER 4: CONCLUSION

Reagent-based TCCIs are attractive alternatives to the traditional design of drugs. While the current study is limited to nucleotide analogs, the system is highly modular and can be applied towards the design of therapeutics against other biologically relevant targets.

Some of the challenges of TCCI development lie in the reactivity of the analogs with the amine linker, as in the case of the β-lactams. This problem may be avoided by transforming the carboxylic acid into an acyl chloride. This approach would require protection of any hydroxides on the thymidine derivative.

The click-reaction-based TCCI clears some the hurdles above by using seemingly unreactive analog warheads which unexpectedly inhibited the intended target. While these analogs function as TCCI's, it is not known if inhibition is caused by the covalent cross-link, or if the covalent cross-link merely aids with the inhibition by providing an anchoring site for a bulky click triazole product to form which inherently covalently blocks the DNA polymerase active site. Yet, the click-reactive analogs act specifically against HIV-RT since there is little deviation in inhibition when in the presence of up to 200 time excess T7 RNA Polymerase, and the complete elongation of the substrate is unchanged when DBCO dTTP is added without 6-Az dTTP.
CHAPTER 5: PROPOSED RESEARCH

Introduction

Chain-terminating nucleotide analogs must meet five requirements to be considered as good therapeutic candidates, they must possess high catalytic efficiency, no potential for elongation following incorporation, poor excision upon incorporation, high selectivity towards target DNA polymerase, and effective metabolism of parent nucleoside. While there are two main pitfalls concerning chain-terminators, resistance development, and promiscuity leading to side effects.

The inherent side-effects of chain terminators, in general, arise from incorporation by mitochondrial DNA polymerase-\(\gamma\), which shares many conserved regions with more primitive viral polymerases. A comparative study shows that Lamiduvine, a chain-terminating NRTI used against HIV-RT, has an effective incorporation rate of 0.019 against HIV-RT, while polymerase-\(\gamma\) shows an effective rate of 0.009. This means that Lamivudine is only 2.1 times more selective towards HIV-RT.\(^{48}\) Other side-effects are caused by poor metabolism, for example, the AZT-MP is a competitive inhibitor of thymidine kinase which leads to depletion of dTTP. This inhibitory effect is caused by the bulkiness of the 3'-azide group.\(^{49,50}\)

We propose to synthesize chain-terminating 2',3'-dideoxythymidine two-component covalent inhibitors based on the click-TCCI system. The TCCI system shows that selectivity is greatly enhanced when a binary reagent is used, therefore
reducing the possibility of promiscuity. The analogs presented in this publication also show that they are high catalytic efficiency based on their substrate properties, low concern for excision due to covalent inhibition. Scale synthesis of analogs and HIV-RT-TCCI adduct can be utilized to confirm the mechanism of action of the TCCI (citation).

**Materials and Methods**

5-base-modified-2',3'-dideoxythymidine analogs are synthesized from their parent nucleoside 2',3'-dideoxy-5-iodouridine which acts as a good substrate for either palladium coupling with allylamine or propargyl amine.\(^{51,52}\)

*Preparation of 5-iodo-2',3'-dideoxyuridine(I-ddU):* To a stirred solution of 2',3'-dideoxyuridine (500 mg, 2.36 mmol) in 10 mL of DMF, add N-iodosuccinimide (740 mg, 3.21 mmol), and lithium azide from 20% aqueous solution by wt. (1.44 mL, 6.42 mmol) and stirred for 16 hours. The reaction is then quenched by the addition of aqueous sodium nitrite, concentrated under vacuum, and purified by silica gel chromatography by using a methanol: dichloromethane solvent system.

![Chemical reaction diagram](image)

*Figure 22. Synthesis of 5-iodo ddU*
Preparation of 5-allylamine-2',3'-dideoxyuridine (aa-ddU): To a solution of 1-ddU (338 mg, 1.0 mmol) with cuprous iodide (38 mg, 0.2 mmol) in 5 mL of DMF, add allylamine (225 µL, 3.0 mmol), triethylamine (279 µL, 2.0 mmol), and tetrakis(triphenylphosphine)palladium (0) (116 mg, 0.1 mmol) and stir at room temperature for 4 hours. After reaction completion, the product is concentrated under vacuum and purified by silica gel chromatography.

![Reaction Diagram]

**Figure 23. Synthesis of 5-allylamine ddU**

*Synthesis of 5-allyl(6-(11,12-Didehydro-5,6-dihydrodibenzo[b,f]azocine-5-yl)-6-oxohexamamide-2',3'-dideoxythymidine: DBCO-acid (40 µmol), N-hydroxysuccinimide (44 µmol), and dicyclohexylcarbodiimide (48 µmol) are added to a microcentrifuge tube containing 800 µL of dry DMF and placed on a shaker for 10 minutes. The supernatant is then transferred to another tube containing aa-ddU (10 µmol) and DIPEA (20 µmol) in 200 µL of dry DMF. The reaction is then allowed to shake at room temperature for 1 – 5h while monitored by the disappearance of amine by TLC/ninhydrin stain. The product is then purified by HPLC equipped with a SHARC-1*
hydrogen bonding column utilizing a 100% to 75% acetonitrile – methanol gradient containing 0.5% formic acid and 0.05% ammonium formate. The purified product is then characterized by H-NMR and overnight C-NMR.

Figure 24. Synthesis of DBCO-ddT

**Synthesis of 5-allyl(6-azidohexanamide)-2',3'-dideoxythymidine:** 6-azidohexanoic acid (40 \( \mu \)mol), N-hydroxysuccinimide (44 \( \mu \)mol), and dicyclohexylcarbodiimide (48 \( \mu \)mol) are added to a microcentrifuge tube containing 800 \( \mu \)L of dry DMF and placed on a shaker for 10 minutes. The supernatant is then transferred to another tube containing aa-ddU (10 \( \mu \)mol) and DIPEA (20 \( \mu \)mol) in 200 \( \mu \)L of dry DMF. The reaction is then allowed to shake at room temperature for 1 – 5h while monitored by the disappearance of amine by TLC/ninhydrin stain. The product is then purified by HPLC equipped with a SHARC-1 hydrogen bonding column utilizing a 100% to 75% acetonitrile – methanol gradient containing 0.5% formic acid and 0.05% ammonium formate. The purified product is then characterized by H-NMR and overnight C-NMR.
Figure 25. Synthesis of allyl(6-azidohexanamide) ddT

Preparation of 5- N-Allyl-N,N-bis(trimethylsilyl)amine-2’,3’-dideoxyuridine(aaSi-ddU): To a solution of I-ddU (338 mg, 1.0 mmol) with cuprous iodide (38 mg, 0.2 mmol) in 5 mL of DMF, add N-Allyl-N,N-bis(trimethylsilyl)amine (741 µL, 3.0 mmol), triethylamine (279 µL, 2.0 mmol), and tetrakis(triphenylphosphine)palladium (0) (116 mg, 0.1 mmol) and stir at room temperature for 4 hours. After reaction completion, the product is concentrated under vacuum and purified by silica gel chromatography.

Figure 26. Synthesis of 5- N-Allyl-N,N-bis(trimethylsilyl)amine-2’,3’-dideoxyuridine
**Preparation of 5’-O-protected aaSi-ddU:** aaSi-ddU (100 mg, 0.244 mmol) is dissolved in 1 mL dry DMF with DIPEA (85 µL, 0.488 mmol). 2-(2-Nitrophenyl)propyl chloroformate (119 mg, 0.488 mmol), a photolytic protecting group, is added to the reaction vessel and allow to react for 30 minutes at room temperature in the dark. The product is then purified by column chromatography in a dark environment and the solvent is evaporated under vacuum. The product is then characterized by H-NMR and C-13 NMR.

![Synthesis of 5’-O-protected aaSi-ddU](image)

**Figure 27. Synthesis of 5’-O-protected aaSi-ddU**

**Removal of trimethylsylil-N-protecting groups:** O-protected aaSi-ddU is placed in a 1M TBAF/THF solution for 72h under vacuum in the dark, and purified by silica gel column chromatography to yield deprotected allylamine. The product is then characterized by H-NMR.
Figure 28. N-deprotection of 5'-O-protected aaSI-ddU

**Synthesis of sulbactam acyl chloride:** Sulbactam (117 mg, 0.5 mmol) is dissolved in 1 mL of dichloromethane and cooled to -20 °C. 2 µL of DMF is added to the reaction vessel, followed by the slow addition of oxalyl chloride (42.3 µL, 0.5 mmol) over 15 minutes. The reaction is then allowed to stir for 5h to afford the acyl chloride derivative.

Figure 29. Synthesis of sulbactam, acyl chloride

**Synthesis of 5-allylsulbactamide-2',3'-dideoxythymidine:** Sulbactam acyl chloride (20 µmol) is dissolved in 1 mL dry DMF containing O-protected aa-ddU (10 µmol) and DIPEA (11 µmol) as a chloride scavenger. The reaction is allowed to shake in the dark
at -20 °C until the disappearance of amine by TLC/ninhydrin stain. Upon reaction completion, the reaction is quenched by the slow addition of 1 mL of aqueous 5% sodium bicarbonate. The derivative is then deprotected by irradiation at 365 nm for 30 minutes at 0 °C. The product is then purified by HPLC equipped with a SHARC-1 hydrogen bonding column utilizing a 100% to 75% acetonitrile – methanol gradient containing 0.5% formic acid and 0.05% ammonium formate at 4 °C. The purified product is then characterized by H-NMR and overnight C-NMR.

![Figure 30. N-acylation of 5'-O-protected aaSI-ddU with sulbactam followed by O-deprotection](image)

**Synthesis of 5-allylimidazole propionamide-2',3'-dideoxythymidine:** Imidazole-4-acetic acid (40 µmol) is added to a microcentrifuge tube containing NHS (44 µmol) and DCC (48 µmol) in 800 µL of dry DMF. The reaction is placed on a shaker for 10 minutes, and the supernatant is transferred to a tube containing aa-ddU (10 µmol) in 200 µL of dry DMF and DIPEA (40 µmol). The reaction is allowed to shake while monitored by TLC/ninhydrin stain. The product is then purified by HPLC equipped with a
SHARC-1 hydrogen bonding column utilizing a 100% to 75% acetonitrile – methanol gradient containing 0.5% formic acid and 0.05% ammonium formate. The purified product is then characterized by H-NMR and overnight C-NMR.

\[
\begin{align*}
\text{H}_2\text{N} &\xrightarrow[\text{DMF, RT}]{1.1 \text{ eq. NHS}} \text{HN} \text{N} \xrightarrow[2.2 \text{ eq. DIPA}]{1.2 \text{ eq. DCC}} \text{O} \\
\text{O} &\xrightarrow[\text{HO}]{\text{HO}} \text{NH} \\
\text{OH} &\xrightarrow[\text{N}]{\text{N}} \text{O} \\
\text{NH}_2 &\xrightarrow[\text{N}]{\text{O}} \text{NH} \\
\text{O} &\xrightarrow[\text{HO}]{\text{HO}} \\
\end{align*}
\]

Figure 31. Synthesis of allyl(3-imidazolamide) ddT

**Determination of Mechanism of Action**

We propose to study the mechanism of action of TCCIs on HIV-RT via two approaches: molecular docking and MALDI-TOF analysis of HIV-RT digestion fragments. Protein mass fingerprinting (PMF) is a mass spectrometry technique commonly used to determine the amino acid sequence of proteins.\textsuperscript{53–55} Proteins are chemically digested by proteases to give hydrolyzed fragments that can be analyzed with the software aid. In recent years, there has been an increase of utilizing the same protein sequencing techniques to determine covalently modified residues in proteins.\textsuperscript{56} When comparing the data obtained from HIV-RT covalently modified with TCCIs, the resulting pattern is compared to known fragmentation patterns from databases. The modified residues result in higher molecular weights relative to the fragments from the database. These higher molecular weight fragments are then studied to determine the
presence of possible nucleophilic amino acid residues. The determination of the mechanism is further complimented by molecular modeling of the reaction within the catalytic core of HIV-RT. Existing models from X-ray crystalline structure data are used as a base for the docking of each of the TCCI components. The data obtained from this experiment is used to determine why is inhibition achieved only when both TCCI reagents are used, and why does the cyclooctyne dTTP analog form a reversible covalent bond. The study can also serve as a platform for the in-silico development and validation of other TCCIs.

**Inhibitory Assays with Cell Lines**

HIV-RT can serve as a substitute for DNA polymerase I in *E. coli*.\(^{57–59}\) This enables the study of HIV-RT inhibitors in-vivo without the need for high biosafety level clearance while minimizing the exposure to HIV infected CD4 cells. By inhibiting HIV-RT in transformed cells, an increase in apoptosis would be observed, and the effectiveness of the TCCI can then be determined and compared to the effectiveness AZT without further modification. This assay also allows for the testing of the TCCI against mutant HIV-RT strains that show resistance towards the current line of nucleoside reverse transcriptase inhibitors. Also, limited toxicity studies on the TCCI can be studied by testing the nucleoside analogs on wild-type *E. coli*.

Transformation of cells: The JS 200 strain of *E. coli*. Carries a temperature sensitive allele of DNA polymerase I (Pol I\(^\text{ts}\)) where the cells can grow at 30 °C, but not 37 °C or above. By complementing the cells with viral, eukaryotic, or prokaryotic DNA
polymerases, the transformed *E. coli* cells can survive at higher temperatures. An aliquot of JS 200 cells are electroporated in the presence of the pHIV_pTp66p51 plasmid kit from addgene. The cells are then resuspended in LB media and placed in a shaker for 1h at 30 °C. Plate 1:0 and 1:1000 dilutions of cells on LB tetracycline chloramphenicol plates and incubate for 1h. Remove two single colonies from each plate and add to 5 mL of LB broth containing tetracycline and chloramphenicol and incubate overnight at 30 °C followed by shaking at 30 °C for 1 to 2 hours. To test for temperature sensitivity, inoculate a spiral of cell dilutions on two LB tetracycline chloramphenicol X-Gal agar plates where one plate is preheated at 30 °C and the second at 37 °C. Then both plates are incubated at their respective temperatures. Successful plasmid incorporation is indicated by growth at 37 °C and blue coloration of the cells.

![Figure 32. Transformation of competent JS200 e.coli cells. A) A microcentrifuge tube containing competent JS200 cells and pHIV_pTp66p51 plasmid kit are electroporated to obtain B. Solution B is then inoculated on an LB plate C with X-gal, and incubated for 1h at 30 °C to obtain D. Viable colonies with distinct blue coloration are then collected in microcentrifuge tubes and frozen for later use E.](image-url)
Inhibition Assays: Batches of LB media with tetracycline, chloramphenicol, and X-Gal are prepared to contain no inhibitor, AZT, or TCCI nucleosides. Aliquots of transformed cells are inoculated in a spiral and incubated for 1 – 24h at 37 °C. Normal cell growth occurs in plates containing no inhibitor, but the presence of inhibitors results in apoptosis.

Figure 33. In-vivo testing of TCCI. Three LB plates are inoculated and incubated at 37 °C with previously transformed JS200 cells A to give bacterial plates B. The plates are then incubated in the presence of no inhibitor (top), AZT (middle), and TCCI (bottom) to give plates C, where a decrease in number of colonies should be observed.
APPENDIX A: NMR DATA
Figure 34. Proton NMR for potassium clavulanate
Figure 35. Proton NMR of 5-allylclavulanamide dTTP
Figure 36. Proton NMR of sulbactam
Figure 37. Proton NMR of 5-allylsulbactamide dTTP
Figure 38. Proton NMR of 5-allyl-(2,3,5,6-tetrafluoro-4-hydroxy)benzamide dTTP
Figure 39. Fluorine-19 NMR of 5-allyl-(2,3,5,6-tetrafluoro-4-hydroxy)benzamide dTTP
Figure 40. Proton NMR of 6-azidohexanoic acid
Figure 41. Proton NMR of 5-allyl(6-azidohexamamide) dTTP
Figure 42. Phosphorus 31 NMR of 5-allyl(6-azidohexanamide) dTTP
Figure 43. Proton NMR of 5-allyl(6-(11,12-Didehydro-5,6-dihydrodibenzo[b,f]azocine-5-yl)-6-oxohexanamide) dTTP
Figure 44. Phosphorus 31 NMR of 5-allyl(6-(11,12-Didehydro-5,6-dihydropyridobenzofajazocine-5-yl)-6-oxohexanamide) dTTP
REFERENCES


(32) García-Lerma, J. G.; MacInnes, H.; Bennett, D.; Reid, P.; Nidtha, S.; Weinstock,


2532.

(53) Webster, J.; Oxley, D. Methods Mol. Biol. 800.


