Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

R. Trent Stubbs Date

Glyoxal-based Caging of Nucleoside Reverse Transcriptase Inhibitors for the Treatment of Viral Infections

By

R. Trent Stubbs
Master of Science

Chemistry

Jennifer M. Heemstra, PhD
Advisor

Frank McDonald, Ph.D.
Committee Member

Monika Raj, Ph.D.
Committee Member

Accepted:

Kimberly Jacob Arriola, Ph.D.
Dean of the James T. Laney School of Graduate Studies

Date

Glyoxal-based Caging of Nucleoside Reverse Transcriptase Inhibitors for the Treatment of Viral Infections

By

R. Trent Stubbs
B.S., Furman University, SC, 2020

Advisor: Jennifer M. Heemstra, PhD

An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science
in Chemistry
2022

Abstract

Glyoxal-based Caging of Nucleoside Reverse Transcriptase Inhibitors for the Treatment of Viral Infections
By R. Trent Stubbs

Viral infections pose a significant public health challenge, leading to both acute pandemic events such as the current COVID-19 outbreak and long-lasting endemic challenges such as HIV prevalence in Africa. Nucleoside reverse transcriptase inhibitors (NRTIs) are a leading class of antiretroviral compounds often prescribed as a first-line treatment for viral infections. However, they have inherent limitations such as low solubility and circulation lifetime that often necessitate multi-intraday dosing. These factors compromise patient adherence, in turn contributing to poor patient outcomes and increased antiretroviral drug resistance. Current solutions to combat these challenges have primarily focused on the development of novel pharmaceuticals; however, these efforts require extensive time and resources, and discoveries are specific to each chemical entity. A promising alternative is the elaboration of currently approved therapeutics into prodrug moieties, as simple modifications of existing antiretroviral therapeutics may simultaneously promote an extended-release mechanism and improve unfavorable pharmacokinetic parameters to combat poor patient adherence. We propose to explore the use of glyoxal for generating NRTI prodrugs that undergo spontaneous self-activation over hours to days, providing a mechanism for extended release. Glyoxal reacts with the nucleobases found in many NRTIs, and thus this approach is potentially generalizable to many FDA-approved drugs. Using a diverse NRTI library, we are exploring the substrate scope and kinetics for caging and subsequent activation, and investigating the pharmacokinetic properties of the most promising caged molecules. Together, the work presented here may provide a highly versatile method for achieving timed release activation of structurally diverse NRTIs, which is anticipated to advance treatment options for patients receiving antiretroviral therapies.

Glyoxal-based Caging of Nucleoside Reverse Transcriptase Inhibitors for the Treatment of Viral Infections

By

R. Trent Stubbs
B.S., Furman University, SC, 2020

Advisor: Jennifer M. Heemstra, PhD

A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science
in Chemistry
2022

Acknowledgments

This work was supported by the National Institutes of Health (R21GM134564, and R01GM140657 to J.M.H.). I acknowledge Dr. Jen Heemstra for the leadership and mentoring she has provided since I was admitted to Emory. I acknowledge all of my fellow labmates for their support and scientific advice. I acknowledge my Research Committee, Dr. Monika Raj and Dr. Frank McDonald, for their guidance and consultation on my research progress.

Contents

List of Figures

Chapter 1

Introduction

Nucleoside-based therapeutics offer a first-line treatment for viral infections; however, they can have undesirable properties that limit patient adherence to treatment, thus compromising patient outcomes and the management of global outbreaks such as the COVID-19 pandemic. Current drug discovery efforts to combat low adherence to chronic, antiviral disease therapies have primarily focused on the development of novel pharmaceuticals. Rather than expensive and labor-intensive efforts devoted to the discovery of these new compounds, simple modifications of existing antiretroviral drugs may unlock methods to promote an extended-release mechanism that combats existing shortcomings. Currently, limited investigations have explored the use of existing antivirals to generate prodrugs that contain an extended-release mechanism dependent upon base-pairing disruption. The research presented here addresses these challenges by exploring modified therapeutics, specifically nucleoside reverse transcriptase inhibitors (NRTIs), which are bound in a reversible adduct that disrupts base-pairing. Slow release from this adduct *in vivo* enables a time-dependent activation of the NRTI, improving their pharmacokinetic parameters and increasing patient adherence treatment options for viral infections.

1.1 Overview

Viral infections pose a significant threat to global public health, which can be manifested in both acute pandemic events like Covid-19 and long-lasting endemic challenges such as HIV prevalence in sub-Saharan Africa. Among the most effective treatments for viral infections are NRTIs, which mimic naturally occurring nucleosides, but lack a 3'-hydroxyl resulting in chain termination during reverse transcription of viral RNA (Figure 1.1). Even if the resulting truncated viral DNA is subsequently incorporated into the host genome, the fragments will not be transcribed and translated into functional viral proteins. Despite their efficacy in antiretroviral therapy, many NRTIs have a short mean plasma half-life, leading to dosing requirements of up to 5 times per day. As a result, patient adherence to NRTI therapeutic regimens is often inadequate, which compromises patient outcomes and contributes to a greater probability of developing viral drug resistance.

Extended-release antiretroviral therapies offer a promising solution to combat the challenges that lead to poor drug adherence, as they can obviate the need for multi-intraday dosing. Prodrug moieties capable of providing an extended-release mechanism may also provide additional functionality by improving drug pharmacokinetic properties such as solubility, permeability, and bioavailability. This is particularly attractive for NRTIs, as the therapeutic window for many of these drugs is limited by solubility and permeability constraints, contributing to undesirable side effects including nephrotoxicity.[1] Developing an extended-release strategy for NRTIs necessitates addressing both circulation life-time and time-controlled release of the active therapeutic. While limited generalizable approaches are available for extending circulation half-life of small-molecule therapeutics [2], few viable options have been reported for controlling the activity of NRTIs on the timescale needed to overcome current limitations associated with their dosing regimen. In this work, a novel prodrug strategy is presented using glyoxal to generate reversibly caged NRTIs that undergo spontaneous

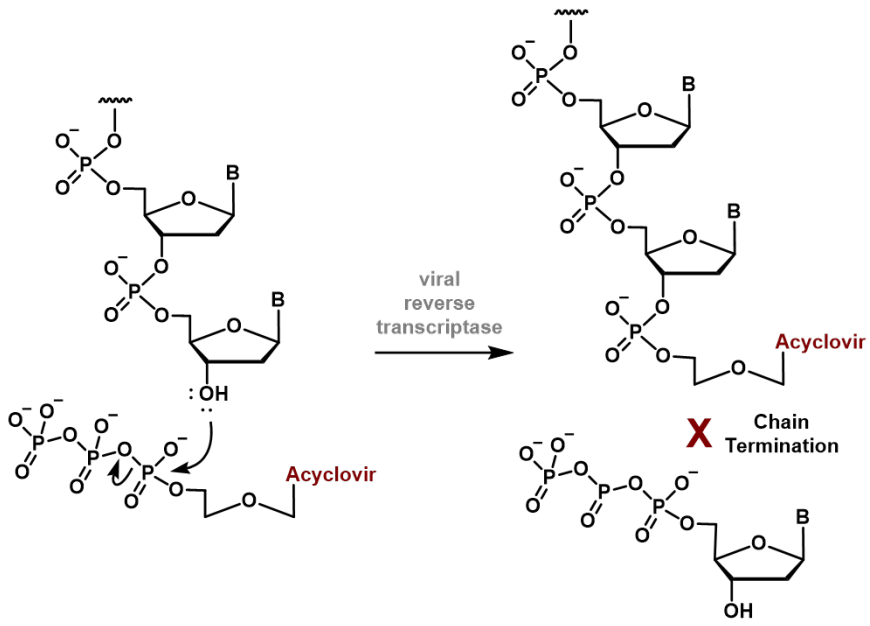

Figure 1.1: Chain termination of viral DNA replication by acyclovir.

release and activation on the timescale of hours to days, thus providing a foundation for the development of extended-release formulations.

Glyoxal, the simplest dialdehyde, is highly electron deficient and thus susceptible to nucleophilic addition under mild aqueous conditions. Amidine and guanidine functional groups (such as those found in guanine, adenine, and cytosine nucleobases) are particularly reactive toward glyoxal, as this produces a 5-membered bis-hemiaminal ring by nucleophilic addition (Figure 1.2). Importantly, the addition of glyoxal ("glyoxylation") blocks the Watson-Crick-Franklin face of nucleobases in the adduct, thus preventing them from engaging in base-pairing interactions, including those needed for incorporation by viral reverse transcriptase (RT) enzymes. Given the prevalence of nucleobases in existing antiviral pharmaceuticals, we envision that this approach will be highly generalizable. Reversal of the glyoxal cage is spontaneous under physiological conditions in the absence of external catalysis, with the rate of the reaction varying as a function of temperature.[3] Fortuitously, the decaging reaction at physiological temperature and pH has a half-life that is well-suited for extended release therapeutics over the course of hours to days. Previously, our lab demonstrated the

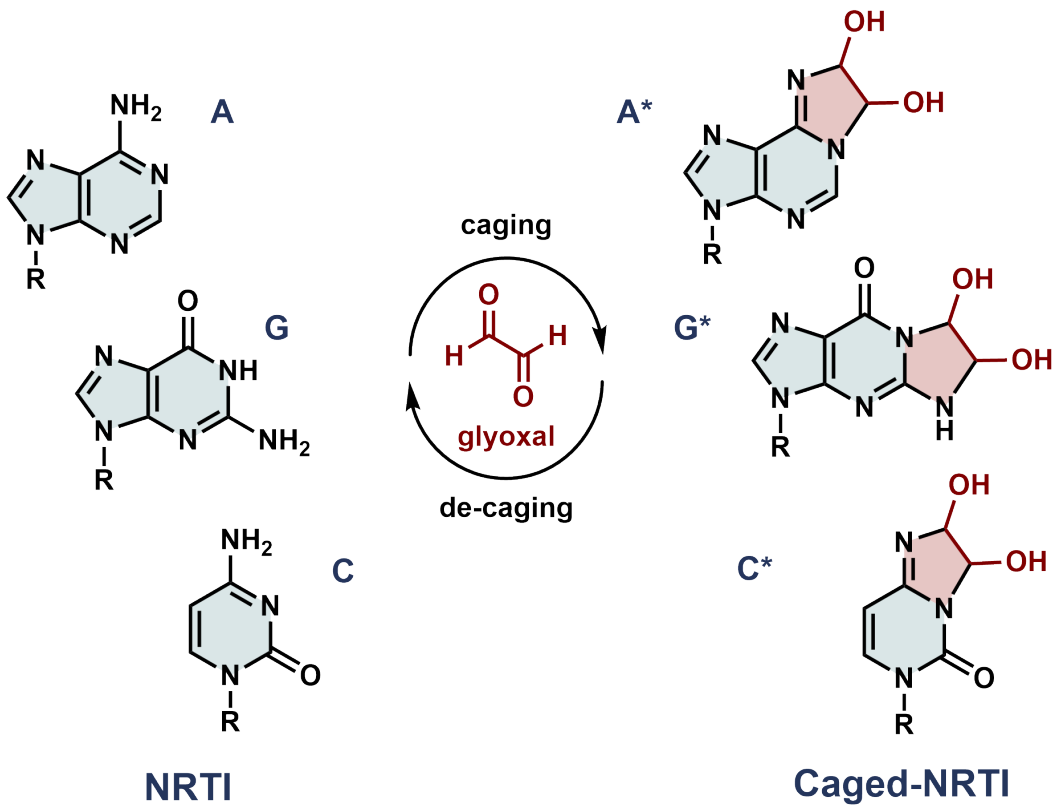

Figure 1.2: Glyoxal-based caging / de-caging of adenosine, guanosine, and cytidine NRTIs as a method to achieve time-released activation.

use of glyoxal caging to reversibly deactivate a wide range of oligonucleotides such as aptamers, guide RNAs (gRNAs), and antisense oligonucleotides (ASOs), including those having non-natural sugar structures, which is an important feature in NRTIs.[3] In the work presented here, this principle is applied to nucleoside-based small-molecule therapeutics (nucleoside reverse transcriptase inhibitors, NRTIs) as a method to reversibly control their activation / deactivation and mitigate shortcomings associated with the class of drugs.

1.2 Nucleoside Reverse Transcriptase Inhibitors

The prevalence of global health threats from viral infections, such as SARS-CoV-2 (COVID-19) and Human Immunodeficiency Virus (HIV), combined with the in-

creased risk of antiviral drug resistance, poses a significant biomedical challenge. Additionally, patient adherence levels to antiretroviral therapies can be low – adherence rates have been reported between 27-80%, far below the required level of 95% for effective management.[4] Moreover, it is currently estimated that of the 80 million individuals living with HIV/AIDS, fewer than half are receiving antiretroviral therapies. Despite these shortcomings, limited progress has been made towards combating the challenges that lead to low adherence.[5, 6] Simple modifications of existing antiretroviral drugs may unlock methods to promote an extended-release mechanism, which can increase patient adherence by prolonging the mean plasma half-life and thus decrease the frequency of required dosing. Current approaches have primarily focused on the development of novel pharmaceuticals with enhanced pharmacokinetic properties; however, these efforts require extensive time and resources, and discoveries are specific to each chemical entity. Alternatively, the identification of prodrug moieties acting upon the nucleobase-pairing face may provide a generalizable approach to creating extended-release versions of a broad range of existing nucleoside-based pharmaceuticals. We hypothesize that glyoxal-caging of antiviral nucleoside analogs may provide a mechanism for the extended release of existing FDA-approved antiviral therapeutics by reversibly blocking the Watson-Crick-Franklin base-pairing face. This method would enable faster development of extended-release therapies by taking advantage of existing motifs common within most nucleoside analogs, offering a broadly applicable approach to overcoming the pharmacokinetic challenges that lead to low patient adherence.

Nucleoside reverse transcriptase inhibitors (NRTIs) are a class of drugs that were first approved for clinical use in 1987 for the treatment of viral pathogens, and act by targeting viral reverse transcriptase. Specifically, NRTIs are metabolically activated (phosphorylated) to their triphosphate derivatives, which can be incorporated into complementary DNA by viral reverse transcriptase (Figure 1.1). NRTIs typi-

cally lack a 3'-hydroxyl group, and thus cause chain termination to inhibit reverse transcription of viral RNA into DNA (Figure 1.1).[7] Together, NRTIs comprise over 40% of antiretroviral therapies approved for clinical use by the Food and Drug Administration (FDA), and are active components of multiple anti-HIV combinatorial therapies prescribed as a first-line standard of care. [7] Despite their prevalence in antiretroviral therapy, it has been reported that patient adherence to NRTIs is often inadequate, [4, 8] as NRTIs suffer from a short mean plasma half-life and commonly require daily multi-dosing.

Purine-based NRTIs can have particularly challenging pharmacokinetic properties. As an example, the guanosine analog acyclovir is an NRTI having potent therapeutic activity against several viruses, including herpes simplex virus (HSV) and Varicella-Zoster virus (HZV), both of which are opportunistic infections that can disproportionately impact HIV patients.[9] However, the poor solubility, permeability, and bioavailability of acyclovir limit its therapeutic potential, especially for the treatment of viruses requiring higher therapeutic doses, such as Epstein-Barr virus (EBV) and cytomegalovirus (CMV).[9, 10] An additional consequence of its poor pharmacokinetics is that intravenous (IV) administration of acyclovir is required for patients receiving high-doses (up to 1.2 g/day).[11, 12, 13] Acyclovir has a maximum solubility of 2.5 mg/mL at pH 7, and has been reported to induce nephropathy in 5-10% of patients receiving IV-administered treatment due to the precipitation of needle-shaped crystals.[14, 1] In addition to poor solubility and permeability, acyclovir also suffers from a short mean plasma half-life (2.5 h). Thus, multiple doses may be required daily, which contributes to decreased levels of adherence.[13]

In an attempt to overcome these unfavorable pharmacokinetic properties, several ester-based prodrugs of acyclovir have been reported, with one currently approved for clinical use (Valacyclovir).[15, 16, 17] Valacyclovir, the L-valyl ester of acyclovir, benefits from increased oral bioavailability compared to acyclovir (54% verses 20%)

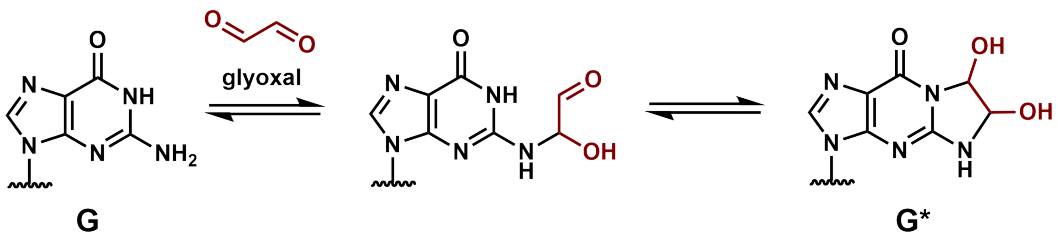

Figure 1.3: Mechanism for the glyoxal-based caging of guanosine as a method to achieve time-released activation.

and is enzymatically hydrolyzed to acyclovir during first-pass hepatic metabolism.[10, 17, 18] However, despite the improved pharmacokinetic properties of valacyclovir, this prodrug approach does not provide a mechanism for extended release *in vivo*. We hypothesized that glyoxal caging of NRTIs would generate prodrugs having both increased solubility and permeability characteristics in addition to a mechanism for their extended release via spontaneous activation.

1.3 Glyoxal Caging of Nucleobases

It has previously been demonstrated by the Heemstra Lab that the reaction of glyoxal with purine nucleobases can effectively cage nucleic acid oligomers by disrupting the Watson-Crick-Franklin base-paring face[19, 3, 20, 21].(Figure 1.2) Glyoxal, the simplest di-aldehyde, is highly electron deficient and is susceptible to nucleophilic addition with amines of purine and pyrimidine nucleobases, forming a stable bis-hemiaminal product (Figures 1.2 & 1.3).[21] This caging reaction proceeds in high yield under mildly acidic aqueous conditions (pH 6) and does not require the use of toxic reagents or catalysts, consistent with green chemistry practices.[3, 21, 22] Interestingly, the reverse (de-caging) reaction is spontaneous and proceeds under physiological conditions (37 °C and pH 7.4) to restore function over time.[3, 23]

Although cytotoxic at higher concentrations (IC50 = 310 ± 83 uM), small quantities of glyoxal are transformed to glycolate by glyoxalase I II enzymes.[3, 24, 25]

Knutson et al. previously reported the delivery of glyoxal-caged antisense oligonucleotides (ASOs) in HEK293T cells with "no differences in morphology or growth rate between any cell groups receiving differentially caged ASOs".[3] Therefore, the activation of glyoxal-caged NRTIs (invloving the release of glyoxal) is likely compatible with an *in vivo* model.

Chapter 2

Glyoxal-caging of Nucleoside Reverse Transciptase Inhibitors

2.1 Glyoxal-Caging Optimization

While glyoxal has been utilized for several decades in electrophoretic analysis and structural probing of large ribonucleic acids (RNAs), [26, 27] the Heemstra lab recently recognized its potential utility for the reversible caging of nucleic acids to control their structure and function.[3] Previous reports from the Heemstra Lab observed full caging of deoxyribonucleic acid (DNA) oligomers in 40 minutes when heated at 50 °C in the presence of an aqueous glyoxal solution (1.3 M). Reaction progress was monitored using 20% denaturing polyacrylamide (PAGE) gel, and the glyoxylated oligonucleotides were subsequently isolated by ethanol precipitation and stored at reduced temperature (∼4 °C) without significant glyoxal decaging observed.[3] Knutson et al then utilized this glyoxal-caging strategy to modulate the function of a structurally diverse set of nucleic acid oligomers, including aptamers, xenonucleoic acids (XNAs), and catalytic DNAs. When this system was applied to CRISPR-Cas9 via caging of a guide RNA (gRNA), the glyoxal caging strategy was found to thermore-

versibly control the catalytic function for cleavage of the target DNA sequence, with full activity restored upon decaging between 2-4 hours at 37 °C.[3] Additionally, the Heemstra Lab previously generated an antisense oligonucleotide to target Green Fluorescent Protein (GFP) and quantified gene expression in cells over 7 days for caged and non-caged ASOs. It was found that compared to the normal ASO, the glyoxal-caged ASO provided timed-release inhibition of gene expression over the full time period. For the work reported here, which has the goal of providing timed release of NRTIs via glyoxal caging, we would ideally want the decaging reaction to occur on the timescale of hours to a few days. Fortuitously, this aligns well with the observations from previous Heemstra Lab studies on nucleic acids, and we have also begun to demonstrate that analogues of glyoxal may provide routes to tunable decaging.

As a first step toward exploring the glyoxylation of NRTIs, 1 mM solutions of the four canonical ribonucleosides (adenosine, guanosine, cytidine, and uridine) were heated to 50 °C in an aqueous, pH 6 ammonium acetate buffered solution in the presence of glyoxal (10 equiv.) for up to 24 hours. The individual crude reaction mixtures were analyzed by liquid chromotrography coupled to mass spectrometry (LC/MS) (by diluting 1:10) and compared to authentic standards of the canonical nucleobases in an ammonium acetate buffered solution. For the reactions of purine bases (adenosine and guanosine) with glyoxal, high-yields of new species with a mass / charge (m/z) ratio (309.11 for adenosine and 325.10 for guanosine) consistent with the caged structures were observed after 2 hours (Figure A.1). For the reactions of pyrimidine bases with glyoxal (uridine and cytidine), only the reaction with cytidine yielded a species consistent with an m/z corresponding to the glyoxal-caged adduct. The reaction of uridine with glyoxal yielded no new species after heating for up to 24 hours likely due to the absence of an exocyclic amine, further confirming our hypothesis of the mechanism of glyoxal caging (Figure 1.2).

Subsequently, we then reacted the guanosine analog acyclovir with glyoxal as a

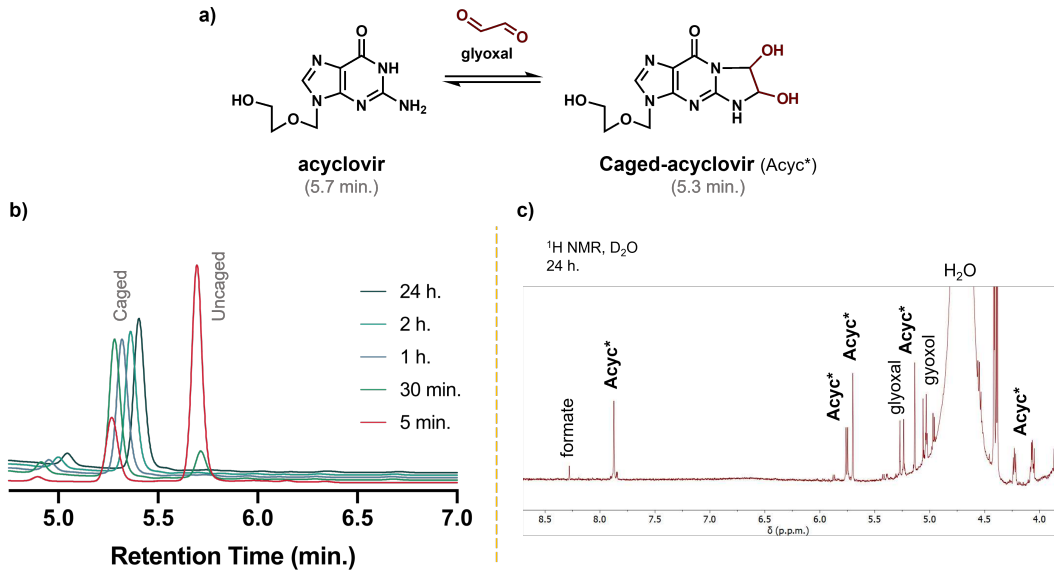

Figure 2.1: (A) Glyoxal-caging of acyclovir monitored by NMR and HPLC, (B) an HPLC-UV (254 nm) time course study over 24 hours, and (C) ^{1}H NMR in D_2O of an aliquot of the reaction between 100 mM acyclovir and 10 equiv. glyoxal in a pH 6 ammonium acetate buffered solution.

proof of principle experiment to observe the caging reaction with a non-canonical nucleoside. Similar to what was observed with guanosine and what has preciously been reported with oligonucleotides, the caging reaction for acyclovir was achieved in a near quantitative yield within 2 hours when heated at 50 °C in a pH 6 PBS solution, as monitored and characterized by liquid chromotrography - quantitative time of flight mass spectrometry (LC/QTOF-MS), and ^{13}C- and ^{1}H-NMR (nuclear magnetic resonance) spectroscopy (Figure 2.1). The caged species could be identified by a downfield shift in the aromatic proton and anomeric protons, as well as the appearance of two new signals consistent with hemiaminal protons. The splitting of the two hemiaminal protons are both singlets, consistent with a 90° dihedral angle in accordance with the Karplus plot. Upon using control reactions of acyclovir with varying equivalents of glyoxal (1.2 equiv., 1.0 equiv., and 0.8 equiv.), it was determined by ^{1}H NMR that the caging reaction was quantitative with no observable glyoxal remaining in solution when heated at 50 °C for two hours in a phosphate buffered

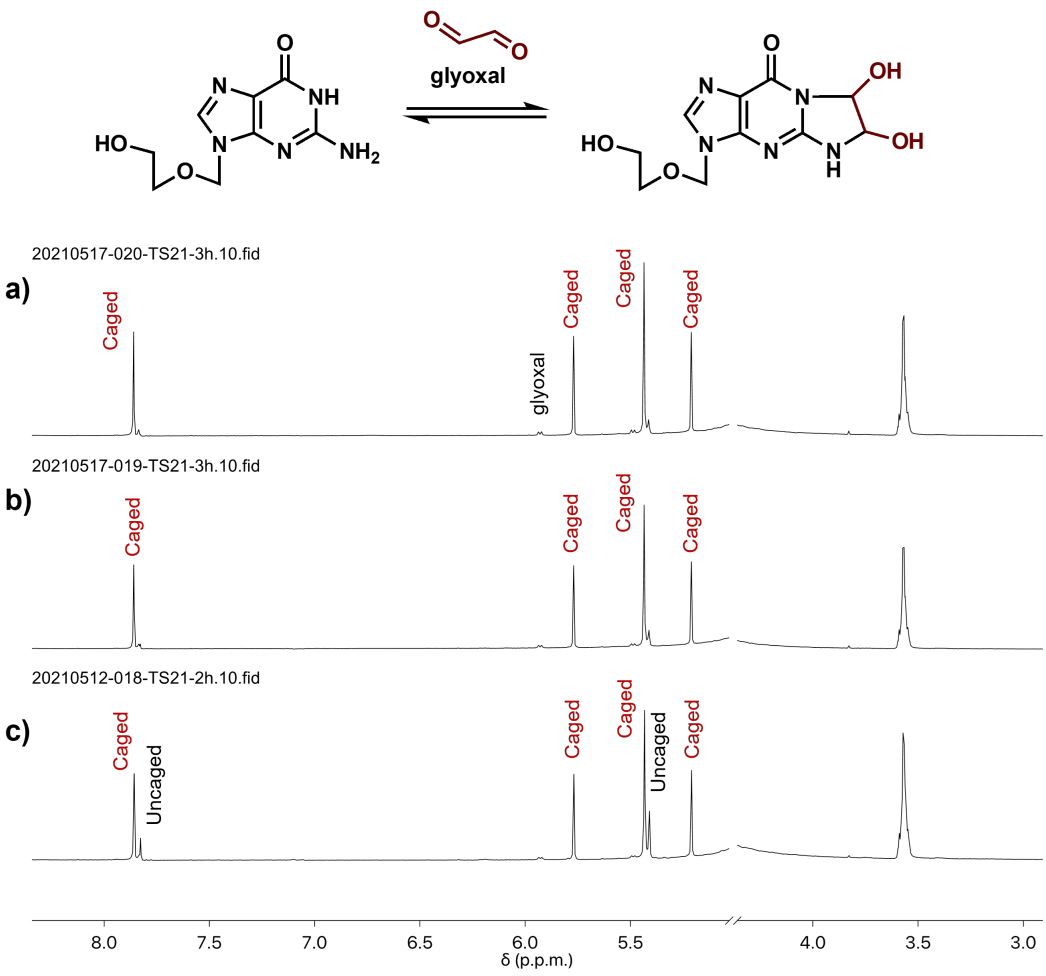

Figure 2.2: ^{1}H NMR, in D_2O, of the reaction between 100 mM acyclovir in a PBS (phosphate buffered saline) solution heated to 50 °C for 2 hours with (A) 1.2 equiv. glyoxal, (B) 1.0 equiv. glyoxal, and (C) 0.8 equiv. glyoxal.

saline (PBS) solution (Figure 2.2).

2.2 Substrate Scope

A central innovation of the efforts disclosed here is the strategy's potential broad compatibility with any nucleobase having an amidine or guanidine functional group, thus creating a highly-generalizable strategy for imparting extended-release properties and improving pharmacokinetic characteristics of existing NRTIs. Thus, it was a primary focus of the experimental efforts to establish the substrate scope for reversible glyoxal caging. Using a library of structurally diverse NRTI pharmaceuticals, we identified compounds susceptible to glyoxal-caging at the base-pairing face, and characterized the caging adducts by LC/MS and NMR spectroscopy.

While our previous studies focused on the thermoreversible control of glyoxal-caged nucleic acid oligomers, glyoxal-based caging of small molecules has yet to be explored. We hypothesized that nucleoside analogs containing an amidine or guanidine functional group (guanosine, adenosine, and cytidine) would be susceptible to nucleophilic addition with glyoxal under mild, aqueous conditions. We validated this hypothesis by exploring the caging reactivity of canonical nucleosides with glyoxal under conditions similar to those previously reported for oligonucleotides to establish optimal caging conditions for each NRTI. Control reactions run in the absence of glyoxal validated that the products were not generated by competing side reactions occurring on the nucleoside (ex. transamination).[20] Subsequently, we explored the glyoxal caging of acyclovir and six other NRTIs currently approved for use by the FDA (Figure 2.3).

Similar to the experiments used to evaluate the caging of canonical nucleobases and acyclovir, the caging reaction between six structurally-diverse NRTIs (entecavir, tenofovir disoproxil, vidarabine, emtricitabine, zalcitabine, and lamivudine) was in-

vestigated by LC/MS and/or NMR spectroscopy. These NRTIs were selected based on their prevalence in medicine (many are listed on the WHOs list of essential medicines) and for their diversity of nucleobase and "sugar" structures. Similar to results observed form the reaction of canonical nucleobases with glyoxal, NRTIs containing purine bases were most efficiently caged with glyoxal. Specifically, NRTI solutions (10 mM) were combined with glyoxal (1 M, 1000 equiv.) in a pH 6 buffered ammonium acetate solution and heated to 50 °C for up to 24 hours. Reaction progression was monitored by LC/MS for each reaction aliquot and the reaction was determined to be successful if the NRTI caged in a yield $\geq$70%. Based on that criteria, the caging reactions between 4 of the 7 total NRTIs screened were successfully caged with glyoxal: acyclovir, entecavir, vidarabine, and lamivudine.

Optimization of glyoxal caging was accomplished by screening the reaction of acyclovir at varying glyoxal concentrations, pH values, temperatures, and reaction times. Glyoxal concentrations were set to maximize the caging reaction yield, while minimizing unwanted side products in order to simplify purification. Previously reported work by our lab showed that a slightly acidic reaction pH was ideal for glyoxal caging of nucleic acid oligomers, and neutral to slightly basic pH was required for decaging. However, this may vary based on nucleoside structure and functional groups. Our work began with the the optimization of reaction parameters at pH 6, and subsequently explored the reaction efficiency at higher or lower pH values. Reactions were monitored by LC/MS, and an high-performance liquid chromatography (HPLC) method was successfully developed to provide baseline resolution between caged- and uncaged-species (Figure 2.1). To ensure rigor and reproducibility ^{1}H NMR and ^{13}C NMR was used when applicable to validate the caged products. Future studies using commercially available ^{15}N-labeled nucleosides may be used in the caging studies to validate product regioselectivity through ^{15}N and ^{13}C NMR spectroscopy.

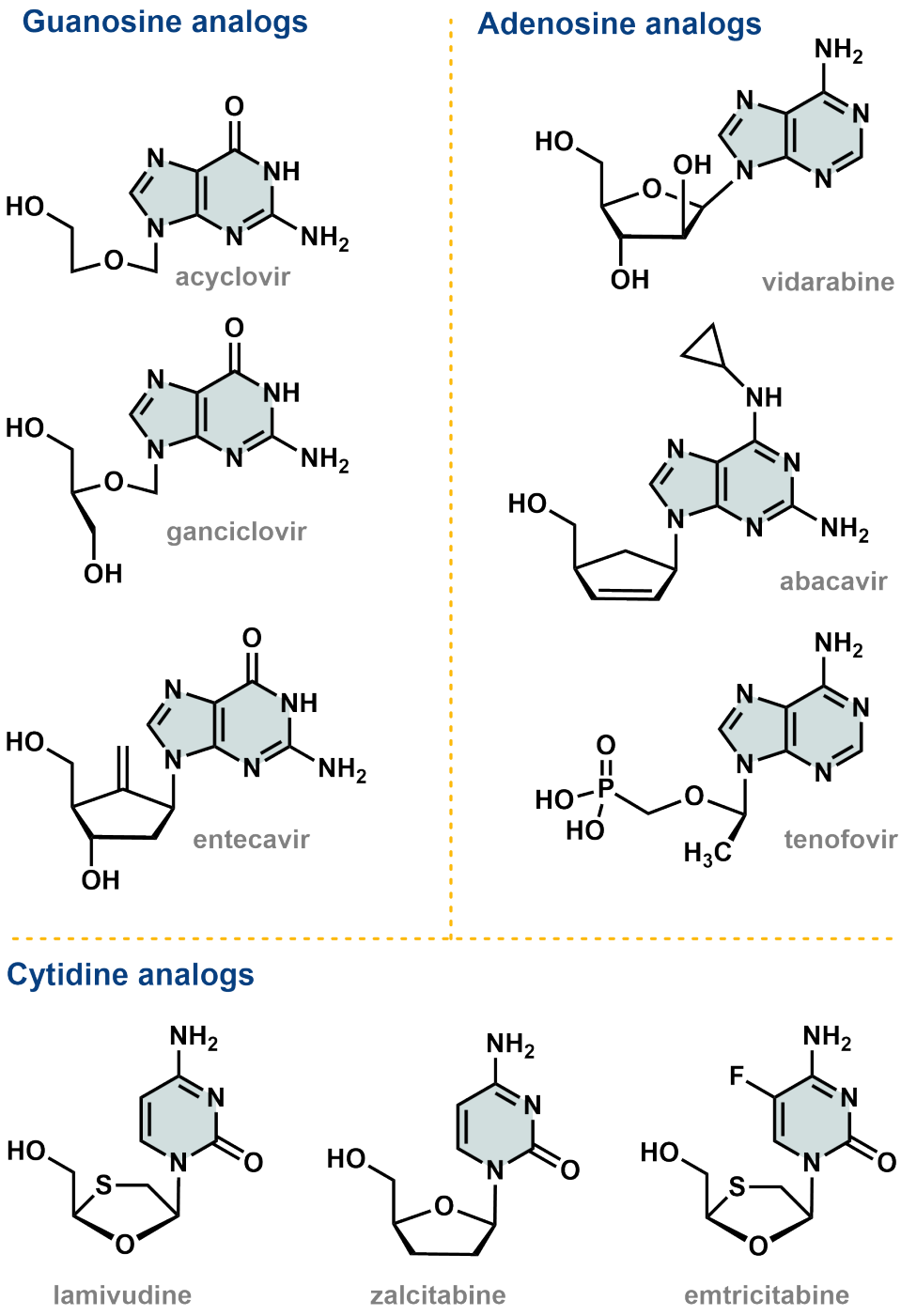

Figure 2.3: Guanosine-, adenosine-, and cytidine-based nucleoside reverse transcriptase inhibitors (NRTIs) approved for clinical use in the treatment of viral infections.

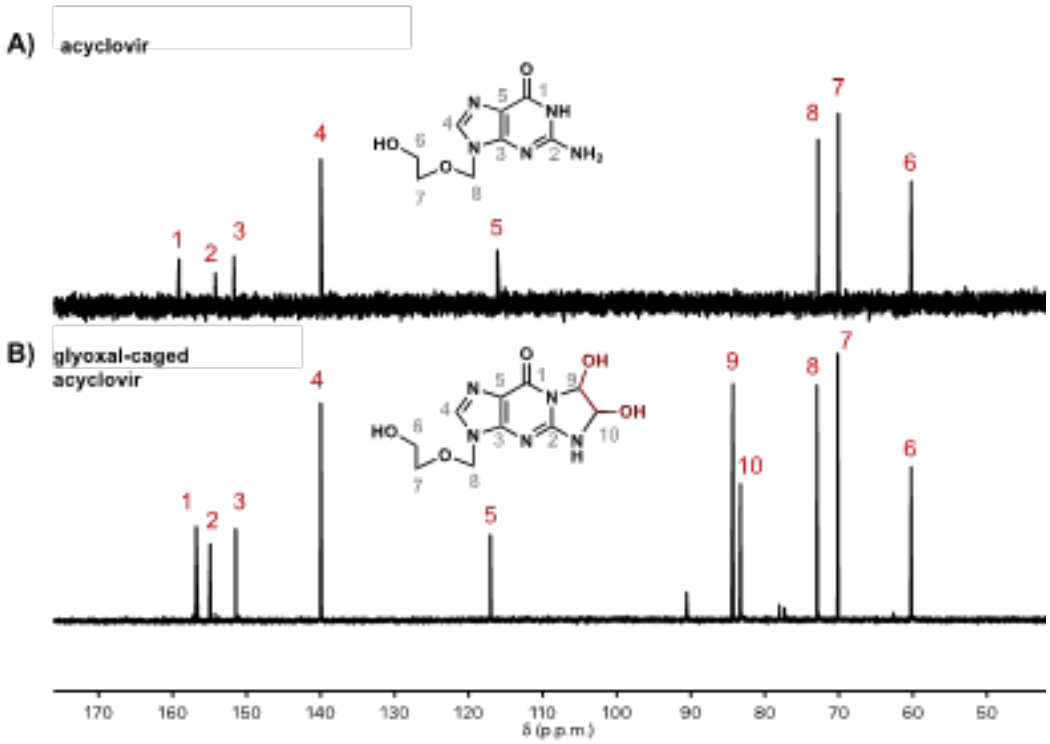

Figure 2.4: ^{13}C NMR, in D_2O, of (A) an authentic standard of acyclovir, and (B) the reaction between 100 mM acyclovir and 1 eq. glyoxal in a pH 6 PBS solution heated to 50 °C for 2 hours.

2.3 Caged-NRTI Purification

Once glyoxal-caged NRTIs were synthesized, we first began purification of the species by reversed phase prep-HPLC. To limit undesired decaging of the purified derivatives, the HPLC method was run at room temperature under mildly acidic conditions (previously shown to slow decaging) and HPLC fractions were collected over ice. However, this method proved to be inefficient and did not allow for the collection of sufficient material needed for subsequent studies. To resolve this challenge, caging reaction conditions were further optimized by varying reaction pH, time, and glyoxal equivalents to limit impurities (mainly unreacted glyoxal / acyclovir). After optimization (Figure 2.2), the caging reaction for acyclovir was found to proceed in a near quantitative yield, and the crude reaction mixture could be lyophilized to yield solid glyoxal-caged acyclovir to be collected (this sample will be reconstituted in H_2O

for future studies since remaining buffer salts will remain). This material was then characterized by ^{1}H and ^{13}C NMR (Figure 2.4). Consistent with the regioselectivity of our mechanistic hypothesis for caging at the Watson-Crick-Franklin base-paring face, we observed a downfield shift for carbon 2 (Figure 2.4) as compared to an authentic acyclovir standard, with little to no changes in chemical shift for other carbons. Additionally, the ^{13}C-NMR spectrum revealed two new signals with a chemical shift consistent with hemiaminal formation, rather than imine formation (signals 9 & 10, Figure 2.4).

2.4 In-vitro De-caging Kinetic Study

A critical goal of any extended-release system is to achieve drug concentrations within the therapeutic index for as long as possible. To increase the likelihood of identifying these desirable properties, future studies comprising all glyoxal-caged NRTI conjugates characterized and purified will be carried forward for analysis of decaging kinetics. Initial studies were conducted under physiological conditions and at physiologically relevant concentrations (both 1 mM and 1 μM) determined to be within standard HPLC limits of detection. The reaction solution was monitored at regular intervals for up to 5 days using HPLC and the free NRTI was quantified by comparison to the integration of a standard solution. These decaging half-life values should ideally be between 1.5 hours to 1 day, and thus are consistent with other modified release systems under development.[5, 2] For the acyclovir decaging reaction at 1 mM, 79% of the caged species remained after 24 hours, and 70% remained after 5 days (Figure 2.5). These results likely suggest that an equilibrium of caged-vs-uncaged was reached. For the acyclovir decaging reaction conducted at 1 μM, 62% of the caged species remained after exposure to physiological conditions for 5 days (Figure 2.6).

Future efforts will expand upon these results by establishing a full kinetic profile

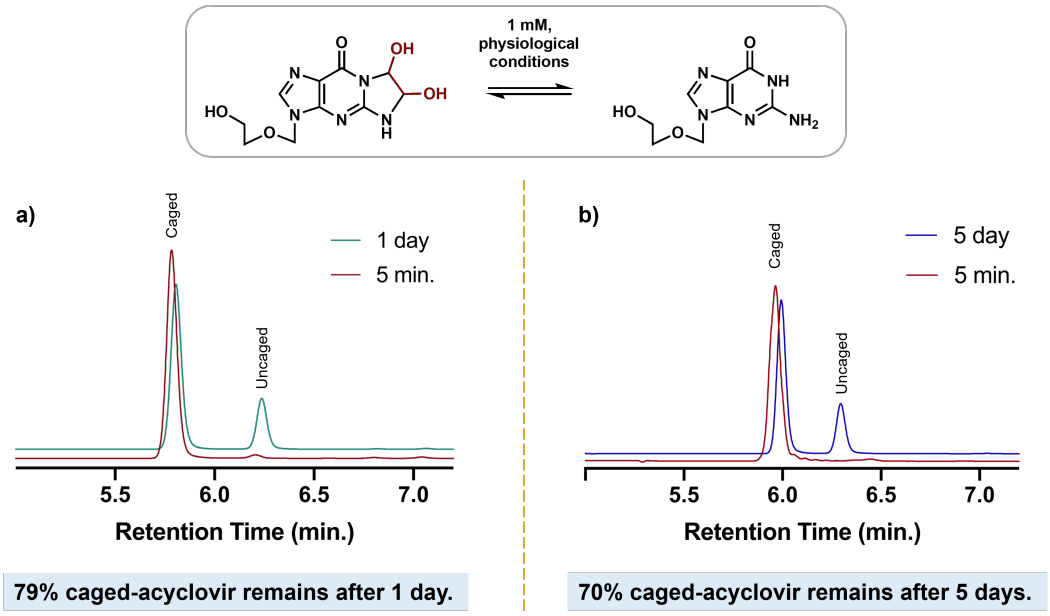

Figure 2.5: The decaging reaction of 1 mM caged-acyclovir in a pH 7.4 PBS solution heated to 37 °C (physiological conditions) for (A) up to 1 day, (B) up to 5 days, as measured by HPLC-UV at 254 nm.

for the *in vitro* decaging reaction under physiological conditions. While we do not anticipate major limitations in the decaging kinetics for activation, a key challenge of developing any extended-release drug is to remain within the therapeutic index for as long as possible. Future approaches utilizing a broad substrate scope of dielectrophilic species will increase the likelihood of identifying caged-NRTIs capable of decaging on a physiologically relevant timescale.

An even more appropriate model for evaluating decaging kinetics is one in which the glyoxal is consumed after decaging, as this better represents an *in vivo* setting where glyoxal can be diluted by diffusion or consumed by enzymes. Therefore, future studies in the Heemstra Lab will monitor NRTI-decaging kinetics in the presence of the endogenous enzyme, glyoxalase. Glyoxalase transforms glyoxal into glycolic acid, a nonelectrophilic byproduct that is no longer reactive with nucleosides. Decaging reactions will be carried out and monitored by HPLC with rate constants quantified. Half-life values determined from these data will provide an initial approximation for

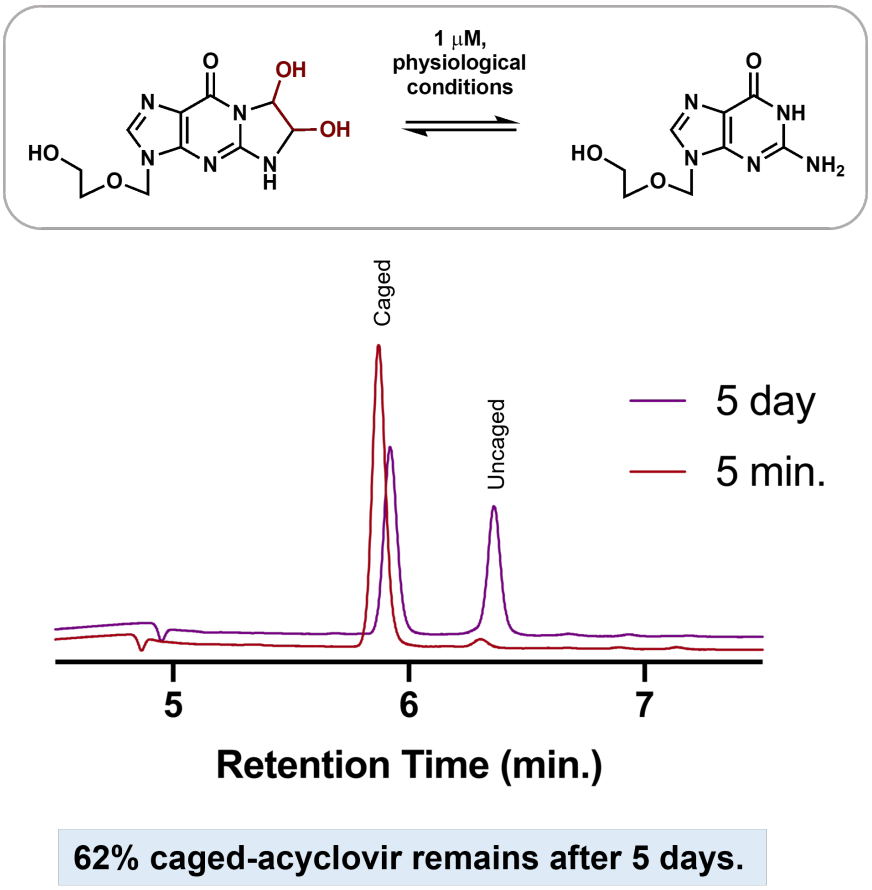

Figure 2.6: The decaging reaction of 1 μM caged-acyclovir in a pH 7.4 PBS solution heated to 37 °C (physiological conditions) for 5 days.

the rate of activation of caged NRTIs *in vivo* and will inform subsequent metabolic (plasma and hepatocyte) stability studies and potentially *in vivo* decaging assays. All of these decaging kinetic studies will be run in triplicate with standard error reported; authentic standards of uncaged NRTIs will be used as chromatographic/spectroscopic standards.

2.5 Enhancement of Solubility and Permeability

As previously stated, several ester-based prodrugs of acyclovir have been reported in an attempt to combat these unfavorable pharmacokinetic properties, with one currently approved for clinical use (Valacyclovir).[17] Valacyclovir, the L-valyl ester

of acyclovir, possesses increased oral bioavailability compared to acyclovir (54% verses 20%) and is enzymatically hydrolyzed to acyclovir during first-pass metabolism.[17] Despite the improved pharmacokinetics of valacyclovir, this prodrug system still lacks an extended-release mechanism that addresses poor patient adherence.

To visualize the enhanced solubility of glyoxal caged-acyclovir versus uncaged, acyclovir (34 mg, 0.15 mmol, 100 mM) was added to 1.48 mL of a PBS buffered solution containing 17 μL (9 mg, 1 equiv.) of a 40 wt% glyoxal solution. Initially, the majority of acyclovir remained undissolved (maximum solubility of 2.5 mg/mL), but as the caging reaction progressed over time, the precipitate was solubilized (Figure 2.7). These results indicate a $\geq$10-fold increase in solubility. Future studies in the Heemstra lab will further quantify these results (maximum solubility), via a six-point, UV, calibration curve at pH 7.4.

An additional consequence of poor pharmacokinetics is that intravenous (IV) administration of acyclovir is required for patients receiving high-doses (up to 1.2 g/day).[11, 12, 13] Acyclovir has a maximum solubility of 2.5 mg/mL at pH 7, and has been reported to induce nephropathy in 5-10% of patients receiving IV-administered treatment due to the precipitation of needle-shaped crystals. [14, 1] We hypothesize that acyclovir-prodrug systems could possibly be improved upon by incorporation of a prodrug moiety with increased solubility/permeability that simultaneously provides a route to extended-release.

2.6 Future Development

2.6.1 Nucleobase Caging with Glyoxal Derivatives

If decaging half-life values determined in future studies are too short resulting in early release of the free drug and excess serum concentrations, additional caging-motifs expected to form more stable adducts in comparison to a bis-hemiaminal can

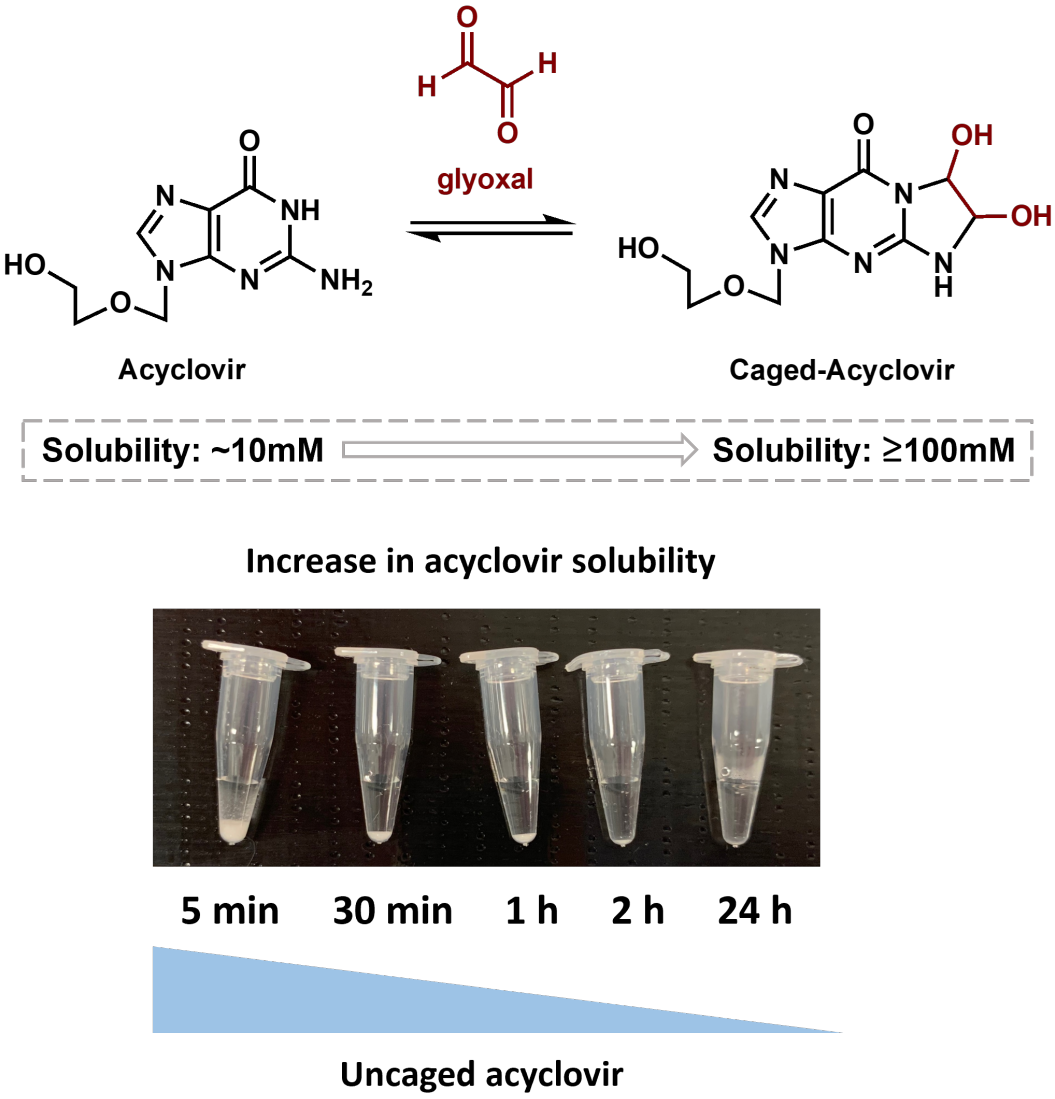

Figure 2.7: Glyoxal caging as a method to increase solubility of NRTIs.

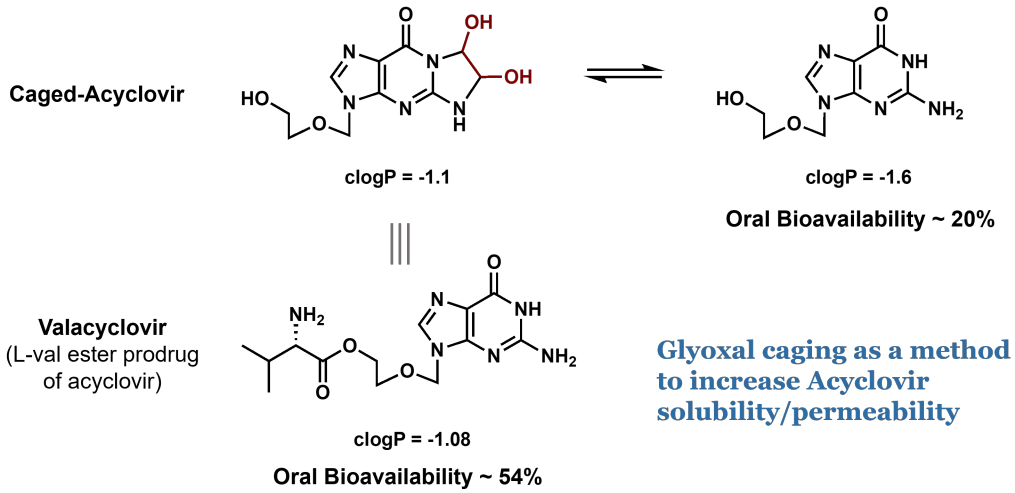

Figure 2.8: Glyoxal caging as a method to increase permeability of NRTIs.

be tested. This alternative caging will take advantage of stable amide functional groups at the base-pairing face generated from methyl glyoxylate and oxalyl chloride (Figure 2.9). Too short of a decaging half-life will be defined as a time that may still necessitate multi-dosing per day. If decaging half-life values exceed metabolic half-life (circulation lifetime), we will investigate additional caging-motifs less stable in comparison to a bis-hemiaminal. Caging reagents that may possess faster decaging kinetics than glyoxal will be tested and include both di-keto species such as diacetyl, whose caged-structure may be destabilized by steric interactions, and mono-aldehydic species such as glycolaldehyde, which will form a mono-dentate (rather than bi-dentate) cage.

Using strategies similar to those reported here, we hypothesize that it may be possible to cage nucleoside analog therapeutics (either antiretroviral or antineoplasia compounds) with dielectrophilic species containing moieties that may target their delivery and activation to specific tissues. As an initial attempt to investigate this strategy, dehydroascorbic acid (Figure B.2, a metabolic presourcer to ascorbic acid) was used as a structure that may preferentially favor a decaging equilibrium in the presence of the reductive enviroment of a cancerous cell caused by the Warburg ef-

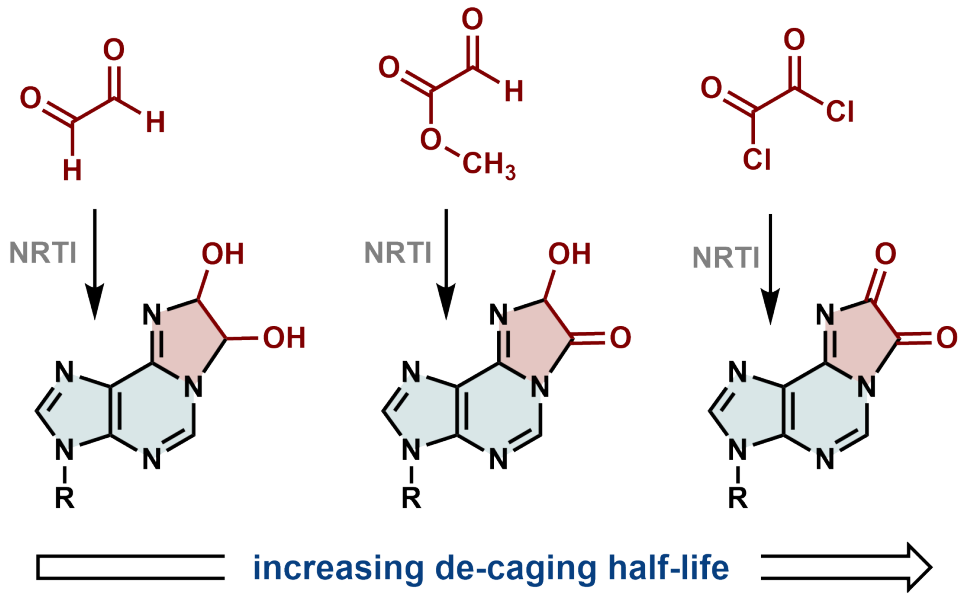

Figure 2.9: Proposed alternative structures that may decrease decaging half-life to generate ideal drug release times. A) glyoxal, B) methyl glyoxylate, and C) oxalyl chloride.

fect. Upon decaging, we hypothesized that dehydroascorbic acid would irreversibly be reduced to ascorbic acid, preventing the cage from reattaching. To test the initial reactivity of dehydroascorbic acid with different nucleosides, cytidine (50 mM, 24 mg) was reacted with 5.0 equiv. dehydroascorbic acid (87 mg) in 2.0 mL of an aqueous, pH 6, sodium acetate buffered solution (100 mM) heated to 50 °C in a thermocycler for 18 hours. The crude reaction mixture was analyzed by both ^{1}H NMR and LC/MS, however no species consistent with dehydroascorbate-caged bases were observed.

Additional alternative di-electrophilic species containing properties with specific targeting mechanisms will be investigated in future studies. As an example, kethoxal (Figure B.2) is an alpha-keto aldehyde that also possesses antiviral properties. Additional efforts in the Heemstra Lab are focused on the caging oligonucleotides with 4-azidophenyl glyoxal, an alpha-keto aldehyde amendable to attach groups with targeting functionality via Cu-free azide-alkyne or strain-promoted click chemistry reactions.

2.6.2 Pharmacokinetic Studies

Given the significant structural change induced by glyoxylation, it is important that future efforts in the Heemstra Lab reassess ADME pharmacokinetic properties for these NRTI-caged analogues. In addition to providing timed-release activation, we also hypothesize that glyoxal caging of NRTIs will generate prodrugs having increased solubility and permeability characteristics that may also significantly improve pharmacokinetic properties previously described as limitations within this class of drugs. Future studies may be accomplished in collaboration with support from the Emory Institute for Drug Development (EIDD) and will employ standard assays to evaluate the pharmacokinetic properties (hepatic / plasma stability, cellular toxicity, and membrane permeability) of each caged NRTI structure relative to its parent (uncaged) NRTI, and utilize extended time points up to 72 hours for testing, given our ultimate goal of extended release formulation.

To visualize the enhanced solubility of caged-acyclovir verses uncaged, acyclovir (34 mg, 0.15 mmol, 100 mM) was added to 1.48 mL of a PBS buffered solution containing 17 μL (9 mg, 1 equiv.) of a 40 wt% glyoxal solution. Initially, the majority of acyclovir remained undissolved (maximum solubility of 2.5 mg/mL) but as the caging reaction progressed over time, the precipitate was solubilized representing a $\geq$10-fold increase in solubility compared to uncaged-acyclovir. Additionally, computational LogP (cLogP) values of acyclovir and caged-acyclovir have been calculated to equal -1.6 and -1.1 respectively. It should be noted that Valacyclovir, the L-valyl ester of acyclovir, which has a cLogP value equal to -1.08, demonstrates significantly increased oral bioavailability compared to acyclovir (54% verses 20%). These preliminary calculations also support our hypothesis that glyoxal caging of NRTIs may increase the bioavilabililty of the pharmaceutical by increasing membrane permeability. The stability of caged NRTIs in human plasma at pH 7.4 will be determined at regular time intervals (1, 4, 12, 24, 48, and 72 hours) and measured by LC/MS after

methanol quench.[28] Membrane permeability of uncaged- and caged-NRTI analogs will be assessed using a precoated PAMPA membrane permeability plate assay and the data compared to assess the impact of glyoxylation on cellular uptake. Finally, a hepatic microsome stability assay may be employed to assess the impact of glyoxalation on xenobiotic metabolism. A 3 μM solution of each uncaged- and caged-NRTI will be incubated with pooled human liver microsomes, and the rate of metabolism quantified at 1, 4, 12, 24, 48, and 72 hours by LC/MS, using verapamil (rapid clearance) and diazepam (low clearance) as positive controls. Each of these experiments will be performed in triplicate with standard error reported.

2.6.3 Cellular Toxicity

Using a caging motif that possesses desirable properties, but also lacks toxicity is an important aspect of our drug design, since the cage will be released *in vivo*. The lack of glyoxal cytotoxicity has previously been reported (IC50 = 310 $\pm$ 83 mM). Future studies in the Heemstra lab aiming to assess the cytotoxicity of glyoxal-caged NRTIs will be studied using an MTS tetrazolium reduction cytotoxicity assay performed with HEK293T cells. Glyoxal caged NRTIs will be incubated with cells at concentrations varying from 1 uM to 1 mM, and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay will be performed on separate aliquots of cells at 1, 4, 12, 24, 48, and 72 hours. In parallel, we will test the parent (uncaged) NRTIs across the same concentration range and include a no-drug control. All experiments will be performed in triplicate with standard error reported.

2.6.4 Anti-Viral Activity

Utilizing standard enzymatic assay protocols that have been previously employed in the Heemstra lab, future studies will assess the ability of glyoxal-caged NRTIs to

induce chain termination during reverse transcription. We anticipate that caged NRTIs will not serve as substrates for reverse transcriptase, but that chain termination activity will be re-stored as the caged molecules undergo spontaneous reactivation.[29] A key technical challenge is that NRTIs typically undergo phosphorylation by cellular kinases to provide their putative nucleoside triphosphate as the active substrate for reverse transcriptases. Thus, for this assay, glyoxal caged-NRTI structures will be synthetically phosphorylated to their triphosphate analogs via a one-pot reaction previously reported by our lab.[29] A primer extension assay using HIV reverse transcriptase will be used to analyze the extent of chain termination both for the caged NRTI and at progressive stages of decaging. Briefly, a fluorescently labeled DNA primer will be annealed to an RNA template and incubated in the presence of the reverse transcriptase and the NRTI. Subsequent analysis via denaturing PAGE will enable quantification of chain termination. Decaging of the NRTIs will be performed at 37 °C in the presence and absence of glyoxalase to mimic physiological conditions, and aliquots will be removed at 1, 4, 12, 24, 48, and 72 hours for analysis in the chain termination assay.

If these antiviral activity assays described above are unsuccessful, future collaborations with the Emory Institute for Drug Discovery may be established to perform both *in cellulo* and *in vivo* experiments to validate the glyoxal-caged system. Specifically, EIDD offers assays in live bio-safety level 2 (BSL-2) viral-infected cells and assays using viral replicons to evaluate the extended-release antiviral activity.

2.7 Materials and Methods

2.7.1 Experimental Overview

All reactions were run under an ambient atmosphere. All reagents were used as purchased from their chemical manufacturer. ^{1}H-NMR ^{13}C-NMR spectra were col-

lected using a Varian INOVA 400, Bruker AVANCE III HD 600, or Bruker NANO HD III 400 spectrometer with chemical shifts (δ) reported in ppm. ^{1}H-NMR samples were prepared by adding 100 μL of the crude reaction mixture to 600 μL of D_2O, and ^{13}C-NMR samples were prepared by adding 650 μL of the crude reaction mixture to 50 μL of D_2O. HPLC chromatograms were collected using an Agilent 1290 Flexible Pump HPLC (with 1260 vial sampler) coupled to an Agilent 1260 Multi-wavelength Detector. HPLC samples were prepared by adding 10 μL of the crude reaction mixture to 90 μL of 0.1% formic acid in water. Samples were eluted with a gradient mobile phase of a) 0.1% formic acid in water, 0 minutes b) 90% of a 0.1% formic acid in water and 10% acetonitrile, 5 minute c) 80% of a 0.1% formic acid in water and 20% acetonitrile, 10 minute at a flow rate of 0.600 mL/min for 10 min on a Waters Atlantis HPLC Column heated to 30 °C with an injection volume of 2 μL (200 uL/min draw speed, 400 uL/min eject speed) and visualized at by UV absorbance at 256 nm. Mass spectrum analysis of samples was performed using an Aglient Accurate Mass TOF LC/MS. pH Values of solutions were measured using pH paper and a Fisherbrand pH meter. Computational clogP calculations were performed using the ChemDraw Professional software package.

2.7.2 General Method to Generate Caged NRTIs

10-mM stock solutions of each nucleoside reverse transcriptase inhibiotr (NRTI) were prepared in high-purity H_2O. To a microcentrifuge tube, 25 μL of the 10 mM stock NRTI solution was combined with 28.5 μL of a 40% glyoxal solution (8.8 M) and 25 μL of a 100 mM, pH 6, ammonium acetate buffer. The solution was diluted with 171.5 μL of high-purity H_2O.

Chapter 3

Conclusions

3.1 Summary

The work presented here aims to combat low antiviral drug adherence through the glyoxal-based caging of antiviral therapeutics, which may provide a mechanism for their extended release *in vivo* by temporarily blocking the Watson-Crick-Franklin base pairing face. This strategy is highly generalizeable to a number of diverse NRTIs with varying nucleobase and "sugar" structures. Additionally, we found that the glyoxal-caging may enhance pharmacokinetic properties, such as solubility and permeability, which are accepted as limitations within this class of drug. In this work, a broad class of nucleoside reverse transcriptase inhibitors (seven existing FDA-approved NRTIs) were reacted with glyoxal to evaluate the generation of their respective caged species. The caging reactions were monitored over time by LC/QTOF-MS, ^{1}H, and ^{13}C NMR. Control reactions were run in the absence of glyoxal to validate that products were not the result of competing side reactions occurring on the nucleoside (ex. hydrolysis or transamination). From these experiments, we determined that most purine-based NRTIs (acyclovir, entecavir, and vidarabine), in addition to the cytidine analog lamivudine, could successfully be caged with glyoxal in high yields.

Caged-NRTI structures were then characterized by LC/QTOF-MS, ^{1}H, and/or ^{13}C NMR.

Subsequently, we explored the kinetics of glyoxal-decaging of caged acyclovir as a mechanism to prolong its delivery. We found that *in vitro* decaging half-life values measured to approximately equal 3-7 days under physiological conditions. While this half-life value exceeds likely the circulatory lifetime of the drug (based on NRTI circulatory half lifes), these *in vitro* decaging studies likely do not accurately represent kinetic de-caging measurements from *in vivo* models. Future studies will be preformed in collaboration with the Emory Institute for Drug Development (EIDD) and the Emory Yerkes National Primate Research Center to evaluate the extended release mechanism of caged-acyclovir in a small rodent model by measuring drug concentration over time.

In addition, computational clogP and preliminary solubility studies revealed that glyoxal-based caging of NRTIs may enhance (absorption, distribution, metabolism, and excretion) ADME characteristics of the compounds. Future studies preformed in collaboration with EIDD will aim to evaluate the pharmacokinetic and toxicokinetic parameters (including solubility, permeability, and toxicity) of the glyoxal-caged NRTIs presented here.

To summarize, the central innovative feature of this research is the design and implementation of a glyoxal-based caging system for NRTIs. This novel extended-release prodrug system provides time-controlled activation, and may also have benefits of increasing solubility and permeability pharmacokinetic properties, leading to decreased dosing frequency and greater patient adherence. Because glyoxal caging functions by reversibly altering the Watson-Crick-Franklin base-pairing face, this method is highly compatible with a broad range of existing FDA-approved NRTIs and may enable the rapid development of a novel class of antiretroviral therapies. Furthermore, the reverse decaging reaction is spontaneous and does not require exogenous reagents

for activation. Thus, this design is innovative in the use of glyoxal as a simple and generalizable approach for the production of extended-release antiretrovirals.

Future efforts based on the glyoxal-caging technology reported here will focus on the development of additional caging moieties capable of targeting the delivery to specific tissues. A prominent area of future efforts will be devoted to investigating the targeted delivery of antineoplasia agents built upon a nucleobase structure (similar to NRTIs). These discoveries have implications that may greatly advance both the efficacy and levels of adherence to medicines used in the treatment of a variety of disease states.

Appendix A

Omitted Data from Chapter 1

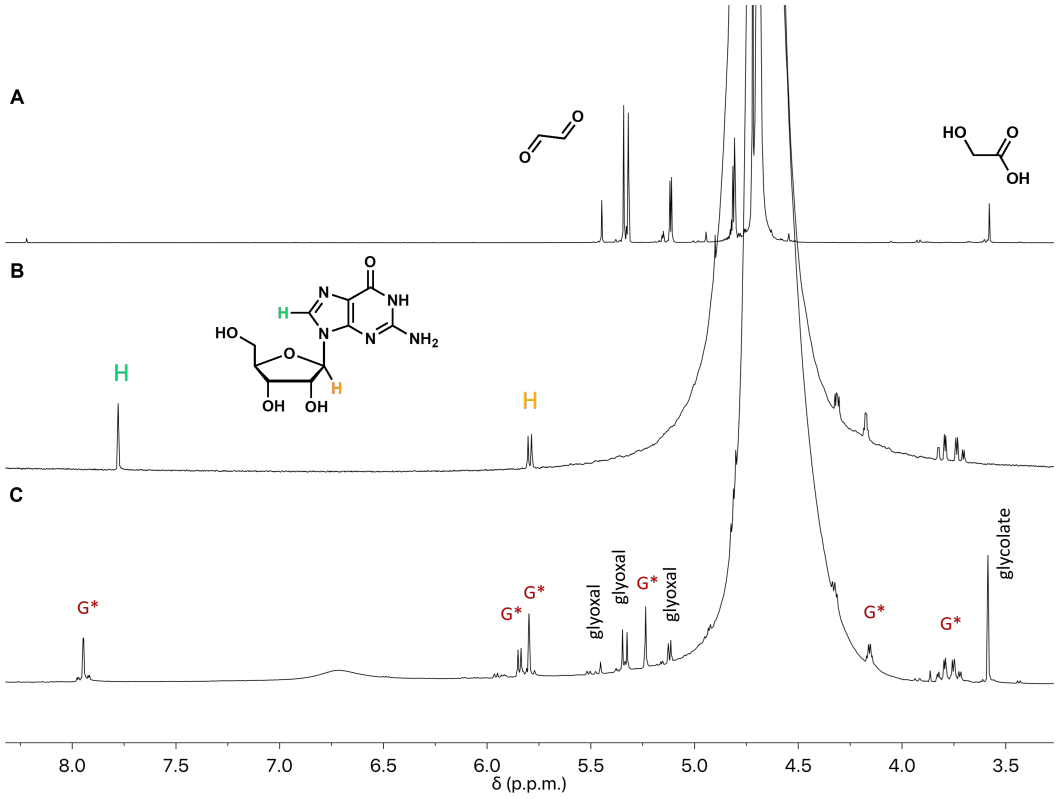

Figure A.1: ^{1}H NMR, in D_2O, of (A) an authentic standard of glyoxal, (B) an authentic standard of guanosine, and (C) the reaction between guanosine and glyoxal in a pH 6 ammonium acetate buffered solution heated to 50 °C for 24 hours.

Appendix B

Omitted Data from Chapter 2

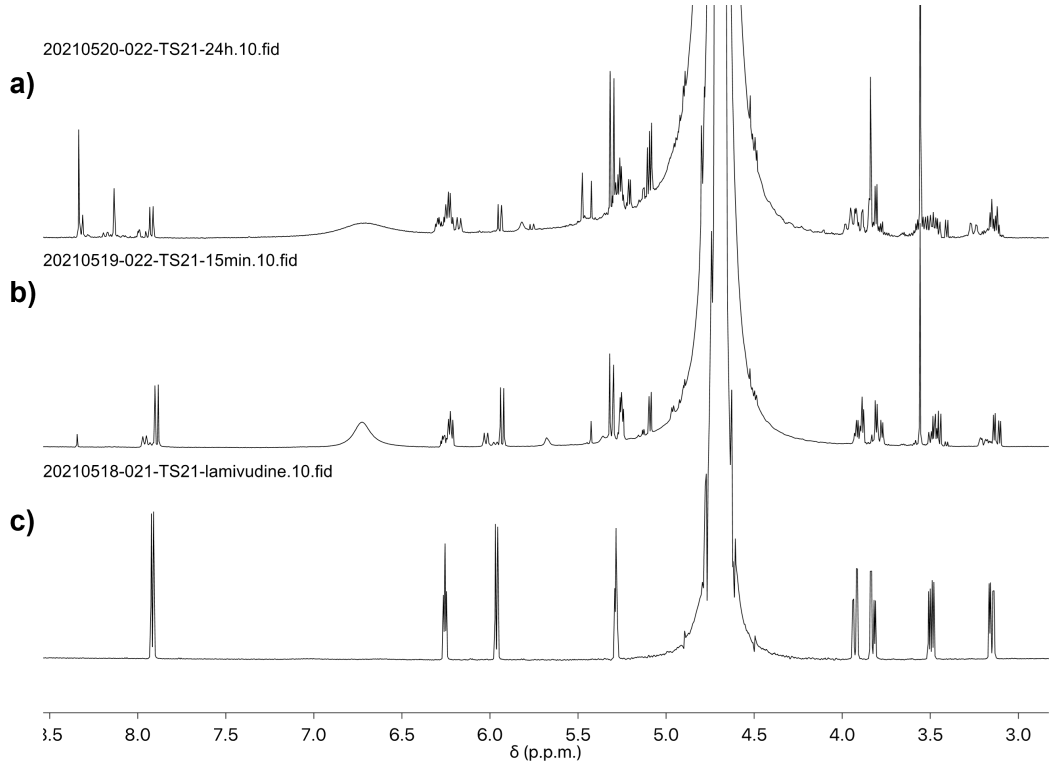

Figure B.1: ^{1}H NMR, in D_2O, of the reaction between lamivudine and glyoxal in a pH 6 ammonium acetate buffered solution heated to 50 °C for (A) 24 hours, and (B) 15 minutes. (C) An authentic standard of lamivudine.

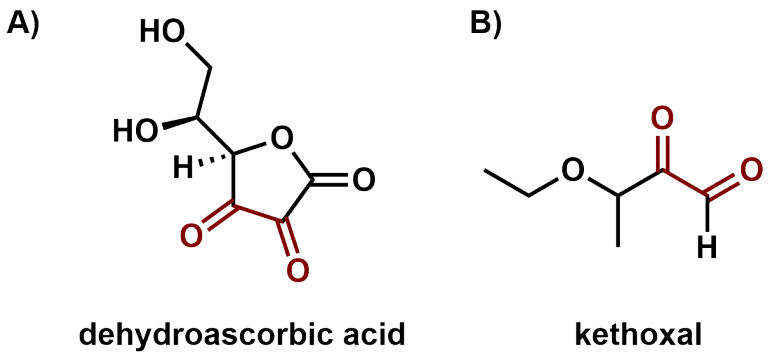

Figure B.2: Structures of the glyoxal derivatives, (A) dehydroascorbic acid and (B) kethoxal

Bibliography

[1] Mark H. Sawyer, David E. Webb, James E. Balow, and Stephen E. Straus. Acyclovir-induced renal failure. *The American Journal of Medicine*, 84:1067–1071, 1988. ISSN 00029343. doi: 10.1016/0002-9343(88)90313-0.

[2] Long-acting antiretroviral therapy. *Nature Materials*, 19:823–824, 2020. ISSN 14764660. doi: 10.1038/s41563-020-0690-z.

[3] Steve D. Knutson, Aimee A. Sanford, Colin S. Swenson, Megan M. Korn, Brea A. Manuel, and Jennifer M. Heemstra. Thermoreversible control of nucleic acid structure and function with glyoxal caging. *Journal of the American Chemical Society*, 142:17766–17781, 2020. ISSN 15205126. doi: 10.1021/jacs.0c08996.

[4] Simona A. Iacob, Diana G. Iacob, and Gheorghita Jugulete. Improving the adherence to antiretroviral therapy, a difficult but essential task for a successful hiv treatment-clinical points of view and practical considerations. *Frontiers in Pharmacology*, 8, 2017.

[5] Ramya Devi Durai. Drug delivery approaches of an antiviral drug: A comprehensive review. *Asian Journal of Pharmaceutics*, 9:1–12, 2015.

[6] Matthew Barnhart. Long-acting hiv treatment and prevention: Closer to the threshold. *Global Health Science and Practice*, 5:182–187, 2017.

[7] Nucleotide reverse transcriptase inhibitors: A thorough review, present status and future perspective as hiv therapeutics. *Current HIV Research*, 15, 2017.

[8] Saverio V. Luccarelli, Stefano Lucentini, Cecilia Acuti Martellucci, Luca Marelli, Matteo Sacchi, and Paolo Nucci. Impact of adherence (compliance) to oral acyclovir prophylaxis in the recurrence of herpetic keratitis. *Cornea*, Publish Ah: 1–6, 2020. ISSN 0277-3740. doi: 10.1097/ico.0000000000002578.

[9] Antona J. Wagstaff, Faulds Diana, and Karen L. Goa. Aciclovir a reappraisal of its antiviral activity, pharmacokinetic properties and therapeutic efficacy. *Drugs*, 47:153–205, 1994. ISSN 11791950. doi: 10.1007/BF03259119.

[10] Stephen Weller, M Robert Blum, Mama Doucette, Thimysta Burnette, Donna M Cederberg, Paulo De Miranda, and M Lynn Smiley. Pharmacolunetics of the acvclovir pro- drug valaciclovir after iscalati and multiple-dose adrninis tration normal volunteers. *Clinical Pharmacology Therapeutics*, pages 595–605, 2000.

[11] David Lembo, Shankar Swaminathan, Manuela Donalisio, Andrea Civra, Linda Pastero, Dino Aquilano, Pradeep Vavia, Francesco Trotta, and Roberta Cavalli. Encapsulation of acyclovir in new carboxylated cyclodextrin-based nanosponges improves the agent's antiviral efficacy. *International Journal of Pharmaceutics*, 443:262–272, 2013. ISSN 03785173. doi: 10.1016/j.ijpharm.2012.12.031. URL `http://dx.doi.org/10.1016/j.ijpharm.2012.12.031`.

[12] Sougata Jana, Rashmi Sharma, Sabyasachi Maiti, and Kalyan Kumar Sen. Interpenetrating hydrogels of o-carboxymethyl tamarind gum and alginate for monitoring delivery of acyclovir. *International Journal of Biological Macromolecules*, 92:1034–1039, 2016. ISSN 18790003. doi: 10.1016/j.ijbiomac.2016.08.017. URL `http://dx.doi.org/10.1016/j.ijbiomac.2016.08.017`.

[13] James Regun Karmoker, Ikramul Hasan, Nusrat Ahmed, Mohammad Saifuddin,

and Md Selim Reza. Development and optimization of acyclovir loaded mucoadhesive microspheres by box – behnken design. *Dhaka University Journal of Pharmaceutical Sciences*, 18:1–12, 2019. doi: 10.3329/dujps.v18i1.41421.

[14] Raymund R. Razonable. Antiviral drugs for viruses other than human immunodeficiency virus. *Mayo Clinic Proceedings*, 86:1009–1026, 2011. ISSN 00256196. doi: 10.4065/mcp.2011.0309. URL `http://dx.doi.org/10.4065/mcp.2011.0309`.

[15] L. M. Beauchamp, G. F. Orr, P. De Miranda, T. Burnette, and T. A. Krenitsky. Amino acid ester prodrugs of acyclovir. *Antiviral Chemistry and Chemotherapy*, 3:157–164, 1992. ISSN 09563202. doi: 10.1177/095632029200300305.

[16] Synthesis and antiviral activity of water-soluble esters of acyclovir [9-[(2-hydroxyethoxy)methyl]guanine]. *Journal of Medicinal Chemistry*, 26:602–604, 1983. ISSN 15204804. doi: 10.1021/jm00358a029.

[17] HENRY BALFOUR. Valacyclovir, the. *The New England Journal of Medicine*, 340:1255–1268, 1999.

[18] Karl R. Beutner. Valacyclovir: A review of its antiviral activity, pharmacokinetic properties, and clinical efficacy. *Antiviral Research*, 28:101–111, 1995. ISSN 01663542. doi: 10.1016/0166-3542(95)00066-6.

[19] Matthys Staehelin. Inactivation of virus nucleic acid with glyoxal derivatives. *BBA - Biochimica et Biophysica Acta*, 31:448–454, 1959. ISSN 00063002. doi: 10.1016/0006-3002(59)90019-8.

[20] Hiroshi Kasai, Naoko Iwamoto-Tanaka, and Shoko Fukada. Dna modifications by the mutagen glyoxal: Adduction to g and c, deamination of c and gc and ga cross-linking. *Carcinogenesis*, 19:1459–1465, 1998. ISSN 01433334. doi: 10.1093/carcin/19.8.1459.

[21] Robert Shapiro, Bertram I. Cohen, Shian-Jan Shiuey, and Hans Maure. On the reaction of guanine with glyoxal, pyruvaldehyde, and kethoxal, and the structure of the acylguanines. a new synthesis of n2-alkylguanines. *Biochemistry*, 8:238–245, 1969. doi: 10.1021/bi00829a034.

[22] Paul Anastas and Nicolas Eghbali. Green chemistry: Principles and practice. *Chemical Society Reviews*, 39:301–312, 2010. ISSN 14604744. doi: 10.1039/b918763b.

[23] William V. Burnett. Northern blotting of rna denatured in glyoxal without buffer recirculation. *BioTechniques*, 22:668–671, 1997. ISSN 07366205. doi: 10.2144/97224st01.

[24] Nandita Shangari and Peter J. O'Brien. The cytotoxic mechanism of glyoxal involves oxidative stress. *Biochemical Pharmacology*, 68:1433–1442, 2004. ISSN 00062952. doi: 10.1016/j.bcp.2004.06.013.

[25] Jessica N. Lange, Kyle D. Wood, John Knight, Dean G. Assimos, and Ross P. Holmes. Glyoxal formation and its role in endogenous oxalate synthesis. *Advances in Urology*, 2012:5–10, 2012. ISSN 16876369. doi: 10.1155/2012/819202.

[26] G. K. McMaster and G. G. Carmichael. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proceedings of the National Academy of Sciences of the United States of America*, 74:4835–4838, 1977. ISSN 00278424. doi: 10.1073/pnas.74.11.4835.

[27] A. F. Murant, M. Taylor, G. H. Duncan, and J. H. Raschke. Improved estimates of molecular weight of plant virus rna by agarose gel electrophoresis and electron microscopy after denaturation with glyoxal. *Journal of General Virology*, 53: 321–332, 1981. ISSN 0022-1317. doi: 10.1099/0022-1317-53-2-321.

[28] Srividya Kailasam and Kerry Frost. Rapid method development to study plasma stability of diverse pharmaceutical compounds using rapid resolution lc and triple quadrupole ms, application note. pages 1–7. 2009, Agilent Technologies and Cyprotex Discovery Limited, https://www.agilent.com/cs/library/applications/5990-4603EN.pdf.

[29] Zhe Chen, Kirsten N. Meek, Alexandra E. Rangel, and Jennifer M. Heemstra. Synthesis and polymerase incorporation of ,-modified -l-threofuranosyl thymine triphosphate mimics. *Bioorganic and Medicinal Chemistry Letters*, 26:3958–3962, 2016. ISSN 14643405. doi: 10.1016/j.bmcl.2016.07.008.