DESIGN, SYNTHESIS, ANTIVIRAL EVALUATION AND METABOLISM OF NUCLEOSIDE ANALOGS

A Dissertation in Chemistry
by
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ABSTRACT

RNA viruses cause a plethora of diseases which are difficult to treat due to the high mutation rate of such pathogens. Current therapies for RNA virus infections are severely limited due to the development of antiviral drug resistance. Such viruses exist in nature as a quasispecies resulting from the high error frequency of replication. RNA viruses as thus highly adaptable and also exist on the edge of “error catastrophe.” Thus small increases in the mutation rate can cause a drastic decrease in viral viability. Based on this concept, a new approach to the design of antiviral agents termed “lethal mutagenesis” has emerged whereby drugs that increase the error rate of RNA viruses may be developed to combat viral diseases.

Chapter one provides an overall review of lethal mutagenesis as an antiviral strategy. Ribavirin, a clinically utilized nucleoside antiviral agent, has shown to be a lethal mutagen. Once transported across cellular membranes and phosphorylated to the 5’ triphosphate, the base-modified nucleoside triphosphate is a degenerate substrate for the viral RNA-dependent RNA polymerase (RdRP). In the viral genome, misincorporation is templated by the ribavirin pseudo base resulting in an increase in overall frequency of mutations and error catastrophe and decreased infectivity of the virus.

The work described herein explores the efforts to design and synthesize effective antiviral nucleosides and probe their mechanism of action as lethal mutagens. Chapter two describes the investigation of isocarbostyril nucleosides as antiviral agents. Three isocarbostyril ribonucleoside analogs, ICS-R, MICS-R,
and SICS-R, were synthesized by the Vorbrüggen procedure and evaluated as antivirals. ICS-R was discovered to be the most potent analog followed by SICS-R and MICS-R demonstrating antiviral activity greater than clinical agent Ribavirin against poliovirus. Additionally, 5´ and 2´ modified ICS-R control compounds demonstrated no antiviral effect. The corresponding ICS-R analog triphosphates displayed slow kinetics of incorporation and revealed inhibition of PV viral RdRP as the mode of action. ICS-R(TP) accumulated intracellularly to a greater degree than MICS-R and SICS-R corroborating antiviral and biochemical results.

Chapter three is divided into two sections exploring the mechanism of action of 5-nitroindole nucleoside and the development of 5-halo indole nucleosides. The mechanism of antiviral activity of 5-nitroindole ribonucleoside was investigated though the evaluation of a series of mechanistic probes and biochemical characterization of phosphorylated 5-nitroindole analogs. 5NINDN(DP) and 5NINDN(MP) were synthesized and evaluated as inhibitors of PV viral polymerase in comparison with 5NINDN(TP) and the parent nucleoside 5NINDN. Surprisingly, both triphosphate and diphosphate inhibited the viral polymerase, however reversed-phase HPLC analyses revealed detectable phosphorylation limited only to 5NINDN(MP). Further evaluation of mechanistic probes 5´OMe5NINDN, 5-nitroindole, and N-Eth-OH 5-nitroindole indicated that intracellular phosphorylation was not necessary for antiviral activity and revealed inhibition of cellular translation was proposed as a mediator of antiviral activity. Due to instability of the nitroaromatic nucleobase, stable 5-halo analogs 5ClINDN
and 5-BrINDN were synthesized and evaluated as agents more active than the parent 5NINDN.

Chapter four focuses on the application of ribosylation agents with modified protecting groups to optimize access to indole nucleosides. By altering the electronic substituents on ribose protecting groups, a series of ribosylation agents were designed to improve access to biologically active indole nucleosides. Additionally, there was a decrease in an undesired orthoamide byproduct and an increase in the overall yield of the nucleoside.

Chapter five demonstrates the detection of phosphorylation of nucleoside analogs as determinants of antiviral activity. Reversed phase HPLC methods to detect phosphorylation of nucleosides were optimized and applied to evaluation of hydrogen-bonding lethal mutagens. The lack of antiviral activity for nucleoside P is attributed to undetectable conversion to the 5’ triphosphate. Additionally, purine analog JA28, is a lethal mutagen which is efficiently phosphorylated to JA28 (TP). Structural comparisons between phosphorylated and non- phosphorylated nucleosides reveal insights into the future design of lethal mutagens.
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Lastly and firstly I dedicate this work to my love, Jesus Christ. I did this for You.
Chapter 1

Lethal Mutagenesis as an Antiviral Strategy

1.1 Overview

RNA viruses cause a multitude of human diseases ranging from the common cold to Hepatitis C and AIDS. Unfortunately, such diseases are often problematic to treat due to the high mutation rate of RNA viruses. Currently, there is a lack of therapeutics available to adequately combat such diseases. Additionally, due to drug resistance to known antivirals, there is an increasing unmet clinical need for new strategies in antiviral drug design. Therefore, lethal mutagenesis as an antiviral strategy is an innovative approach for the development of antivirals. Base-modified nucleoside analogs that can be phosphorylated may be substrates for the promiscuous RNA-dependent RNA polymerase and ambiguously template multiple nucleotides. These compounds will then act as chemical mutagens to alter the mutation rate of RNA viruses and surpass the “error threshold” thereby forcing the virus into error catastrophe and extinction. Ribavirin (1), a broad spectrum nucleoside utilized in the clinic, has been discovered to be a lethal mutagen to poliovirus and hepatitis C virus among
other RNA viruses. Using ribavirin as a model mutagen, one can begin to research the design elements of lethal mutagenesis in order to develop a more potent antiviral. The following description of known antivirals, current drug targets, and groundbreaking work into the mechanism of action of ribavirin and the resulting insights into the design of novel lethal mutagens is discussed.

1.2 RNA Viral Pathogens

RNA viruses cause significant morbidity and mortality worldwide. Since the emergence of HIV, approximately 25 million people globally have died and there are estimates that 46 million are currently infected.\(^1\) Every year, AIDS causes nearly 3 million deaths and the deaths of a half million children. Sub-Saharan Africa has a substantial infection rate and mortality, as well as decreased access to antiretrovirals, resulting in catastrophic loss of life and resources. Nearly 200 million are infected with Hepatitis C virus (HCV) around the world.\(^2\) Chronic HCV infection is associated with liver cirrhosis and hepatocellular carcinoma causing significant global burden. Additionally, co-infection of HIV and HCV is seen frequently and drastically complicates treatment plans.\(^3\) RNA viruses have caused widespread panic in recent years as the cause of emerging diseases such as SARS\(^4\) (severe acute respiratory distress syndrome), Ebola,\(^5\) and West Nile\(^6\) and H5N1 avain influenza.\(^4\) Additionally, the RNA viruses are frequently postulated as bioterrorism agents. Therefore, RNA
viruses have attracted worldwide attention as having disastrous potential and posing real threats to humans.

1.3 Life Cycle of RNA Viruses

Viruses are infectious agents and obligate intracellular pathogens. RNA viruses utilize ribonucleic acid (RNA) to store their genetic code. Classification criteria include single versus double strandedness, polarity of genome, capsid symmetry and enveloped or non-enveloped status. The viral growth cycle includes 1) attachment and penetration, 2) uncoating of the viral genome, 3) translation 4) replication, 5) assembly into virions and 8) release. Poliovirus (PV) is the simplest and most well characterized RNA virus, enabling facile study due to extensive availability of experimental systems.7-9 PV is classified as an enterovirus in the Picornavirus family. The life cycle of PV is dictated by its positive sense, single stranded genome. Namely, the virion binds to a cell surface receptor, RNA is released and translated producing a polyprotein that is cleaved to produce the required viral RNA replication proteins. The role of the PV RNA-dependent RNA polymerase (RdRP) or 3D<sup>pol</sup> is to replicate the positive sense genome via a negative stranded intermediate. The lack of 3´→ 5´ exonuclease proofreading mechanism explains some of the inherent error rate found in PV and other RNA viruses; however, the intrinsic error rate is also significantly higher than other known polymerases.
1.4 Current Drugs and Targets in the Viral Life Cycle

Although successful vaccine development is crucial for prevention of many viral infections, once infected, treatment options are severely limited. Current antiviral drug development has focused heavily on selective targeting of key aspects of the viral life cycle. Therefore, unique viral proteins have attracted the most significant interest as potential drug targets, especially viral polymerases and proteases. HIV therapy has been revolutionized by the HAART (highly active-antiretroviral therapy) which is characterized by the combination of antivirals that target both the HIV reverse transcriptase (RT) and the viral protease. Recently, more attention has been given to integrase and fusion inhibitors which have received FDA approval. As shown in Table 1-1, the majority of antivirals have been developed as antiretrovirals. The remainder consists of antivirals against herpes simplex virus (HSV), influenza, cytomegalovirus (CMV), hepatitis B and C viruses mostly aiming to inhibit viral proteins as the mode of action. The FDA approval rate for new antivirals has improved in the past few years accenting the importance of antiviral drug development to meet worldwide unmet clinical needs. However, traditional approaches against RNA viruses are problematic due to the high intrinsic mutation rates. HAART uses multiple antivirals to prevent the production of treatment-resistant strains. However, the ever-increasing number of drug resistant viruses is a growing threat. Therefore, there is a need for the development of non-traditional approaches to the development of antiviral agents.
Table 1-1: Current antivirals.

<table>
<thead>
<tr>
<th>Virus</th>
<th>FDA Approved Antiviral Drug</th>
<th>Drug Target</th>
</tr>
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<tbody>
<tr>
<td>HIV</td>
<td><strong>Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTI)</strong></td>
<td>HIV RT</td>
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<tr>
<td></td>
<td>Zidovudine, Didanosine, Zalcitabine,</td>
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<td>Stavudine, Lamivudine, Emtricitabine, Abacavir</td>
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<td><strong>Nucleotide Reverse Transcriptase Inhibitor (NtRTI)</strong></td>
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<td>Ganciclovir, Valganciclovir, Idoxuridine</td>
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<tr>
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<td>Lethal Mutagen</td>
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1.5 Lethal Mutagenesis as an Antiviral Strategy

1.5.1 Quasispecies and the Error Threshold

RNA viruses maintain a drastically higher mutation rate compared to DNA-based microbes. The mutation rate of RNA viruses is approximately $10^{-4}$ to $10^{-5}$ mutations per nucleotide incorporated compared to $10^{-8}$ to $10^{-11}$ for DNA-based organisms.\textsuperscript{14} Thus, the genomic error rate for RNA viruses is roughly 1 per genome per replication cycle compared to 0.003 for DNA life forms.\textsuperscript{15} The high error rate has been attributed to the lack of proofreading function of viral RNA-dependent RNA polymerases (RdRP).\textsuperscript{16} Therefore, RNA viruses exist as a quasispecies, an extremely heterogeneous population which hovers near a consensus sequence.\textsuperscript{14, 17-19} Consequently, RNA viruses have evolved to exist at a highly flexible state to quickly respond to environmental conditions. For small RNA viral genomes, each nucleotide of the sequence is much more important to the overall maintenance of genetic information than corresponding DNA counterparts. While conferring an advantage for adaptability, such inherent genomic variability has an inherent upper limit termed “error threshold”\textsuperscript{20} in the
Therefore, slight increase in mutation rate can have drastic implication for viral fitness.

Figure 1-1: Poliovirus exists at the edge of error catastrophe. LI50 indicates the point at which 50% of the genomes are lethally mutated. Modified from Crotty et al.21

1.5.2 Lethal Mutagenesis of HIV
Loeb and coworkers were able to mutate HIV-1 into extinction using 5-hydroxy-2’-deoxycytidine (2) thereby demonstrating the feasibility of this approach as an antiviral strategy. The approach was termed “lethal mutagenesis” to reflect the induction in mutation rate which radically decreases infectivity. Both deoxy and ribonucleosides were examined reflecting multiple pathways through which HIV mutagenesis may occur. Base-modified deoxynucleoside 2 was phosphorylated intracellularly, and incorporated by HIV reverse transcriptase (RT). The 5-hydroxy-cytosine base induced mutations into the viral genome. A ribonucleoside strategy is feasible due to HIV lifecycle utilizing the host enzyme RNA pol II. Such an approach avoids the theoretical threat to host genomic mutation that might occur with deoxynucleoside analogs, although the stringent human DNA polymerase and DNA repair machinery are highly effective counter measures. Nonetheless, ribonucleoside strategies are more attractive than deoxyribonucleosides as lethal mutagens due to potential toxicity issues.
More recently, Daifuku and coworkers, developed a deoxy-azacytidine derivative, KP1212 (3) and its prodrug derivative KP1461 (4). Koronis pharmaceuticals has brought these compounds to Phase 1a/IIb clinical trials marketing them as “viral decay accelerators” as an alternate term for “lethal mutagenesis” for the wider community. KP1461 (4) has entered Phase II clinical trials, however it was suspended recently. Notable is the inactivation of KP1212 by cellular cytosine deaminase converting the active cytosine analog to the corresponding modified uridine. To date, this is the first novel lethal mutagen to
enter clinical trials thereby further supporting lethal mutagenesis as a viable strategy for the development of nucleoside antivirals.

1.6 Ribavirin as a Lethal Mutagen

1.6.1. Initial Mechanism of Action and Alternative Theories

Ribavirin currently is the only broad spectrum nucleoside in clinical use. Marketed as Virazole® and in its aerosolized form as Rebetol® among others, Ribavirin is a unique antiviral for its utility and mechanism of action. Ribavirin was first synthesized by Witkowski and Robins25 and evaluated as an antiviral by ICN Pharmaceuticals by Sidwell and coworkers26 while evaluating a series of triazole nucleoside analogs. Ribavirin was found to exhibit broad spectrum

Figure 1-3: Lethal mutagens developed by Loeb and coworkers.24
antiviral activity against a series of both RNA and DNA viruses. It was shown that ribavirin monophosphate (5, RMP) inhibited inosine monophosphate dehydrogenase (IMPDH), a crucial enzyme in GTP biosynthesis\textsuperscript{27}. IMPDH catalyzes the conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP) which is subsequently converted to GMP. Production of XMP is the rate limiting step of guanine nucleosides. Therefore, since altering GTP pools greatly hinders viral replication, a mechanism of action was assigned. For nearly 30 years, IMPDH inhibition has remained as the most widely accepted mechanism of action for ribavirin. However, alternate mechanistic theories against distinct viruses have been proposed such as immunomodulation,\textsuperscript{28} inhibition of viral capping,\textsuperscript{29} and inhibition of polymerase.\textsuperscript{30} Invoking IMPDH inhibition as the major mechanism of action of ribavirin is problematic due to the fact that not all IMPDH inhibitors are antiviral agents.\textsuperscript{21, 31} Additionally, IMPDH inhibition results in only a 2-fold reduction in GTP which

![Figure 1-4: Ribavirin and its 5'-phosphates.](image)

Invoking IMPDH inhibition as the major mechanism of action of ribavirin is problematic due to the fact that not all IMPDH inhibitors are antiviral agents.\textsuperscript{21, 31} Additionally, IMPDH inhibition results in only a 2-fold reduction in GTP which
does not correlate with antiviral activity. In fact, RBV (1) continues to demonstrate dose-dependent antiviral activity beyond the level of saturated inhibition of GTP production. Additionally, ribavirin triphosphate (RTP, 7) accumulates to 20-100 fold higher than RMP. Therefore, it was proposed that RTP (7) may be a substrate for viral RNA-dependent RNA polymerase.

1.6.2. Development of *In Vitro* Methods to Measure Rates of Incorporation

To study the mechanism of action of ribavirin, a primer-extension assay for PV RNA-dependent RNA polymerase (RdRP) was developed by the Cameron laboratory that employs end labeled primers that are extended *in vitro* by RdRP from poliovirus. Ribavirin was incorporated in RNA by PV RdRP and is templated by both cytidine and uridine with rates of incorporation nearing mispairing by natural substrates. Upon incorporation into RNA, ribavirin templates both CTP and UTP equally via isoenergetic carboxamide rotation. This increases G → A and A → G transition mutations (Figure 1-5).
1.6.3. Guanidine Resistance Assay for Lethal Mutagenesis

A guanidine resistance assay\textsuperscript{35} was utilized to further elucidate the mechanism of action. The assay takes advantage of the fact that wild type poliovirus will not grow in the presence of 2 mM guanidine. This compound exhibits its growth inhibitory effect by inhibition of the viral 2C ATPase that is essential for replication. However, a single C→U transition mutation in this 2C gene is sufficient to confer resistance to guanidine. Poliovirus grown in the presence of ribavirin was shown to induce a higher frequency of guanidine resistant variants than untreated virus. Further evidence was obtained by the sequencing of capsid (VP1)-coding sequence of the virus grown in 1000 μM ribavirin. Sequencing revealed an average of 15 mutations per genome compared with 2 mutations per genome in the absence of ribavirin. Poliovirus
has been shown to exist on the threshold of “error catastrophe” in that a 3-fold increase in mutation frequency can decrease the specific infectivity of viral RNA by 20-fold. These results demonstrate that the antiviral activity of ribavirin is based on lethal mutagenesis. It is important to note that lethal mutagenesis has been demonstrated against VSV\textsuperscript{36} HCV\textsuperscript{37} GV virus B,\textsuperscript{33} HIV-1,\textsuperscript{22} FMDV,\textsuperscript{38} LCMV\textsuperscript{39} and Hantaan virus.\textsuperscript{40}

![Figure 1-6: RBV (1) is a mutagen to PV. Virus stocks grown in increasing concentrations of RBV were analyzed for gua’ genetic marker. gua = guanidine. Adapted from Crotty et. al.\textsuperscript{41}](image-url)
1.7. Design Elements of a Lethal Mutagen

In order to design more effective lethal mutagens, a few strict criteria must be met. First, the base-modified nucleoside must accumulate in the cell. Second, cellular kinases should be able to phosphorylate the given nucleoside intracellularly to the nucleoside triphosphate. A third criterion is that the base modified nucleoside triphosphate be recognized and incorporated by the RNA-dependent RNA polymerase. Last, the base-modified nucleoside should ambiguously template multiple nucleotides either by hydrogen bonding or utilizing a hydrophobic nucleobase scaffold resulting in an increased number of mutations.

Figure 1-7: Design elements of a lethal mutagen.
in the viral genome and loss of infectivity. In addition to these minimal requirements, host-cell toxicity should be minimized.

1.7.1 Cellular Transport of Nucleosides

Cellular transport of nucleoside analogs may occur via multiple pathways. Certain artificial nucleosides may enter by passive diffusion across the cellular plasma membrane. However, alternate pathways exist including concentrative and equilibrative nucleoside transporters (CNTs and ENTs). Among the sodium dependent concentrative nucleoside transporters, there are a variety of subtypes that show substrate specificity (N1-N5). N1 is purine preferring, N2 is for pyrimidine transport and N3 and N4 are broad spectrum enzymes. Known nucleoside antivirals such as AZT and ddC do not inhibit transport whereas acyclovir, ddl and ddA can inhibit cellular uptake by CNTs. Therefore, the mode of transport of nucleoside analogs and intracellular accumulation plays an important role in lethal mutagenesis.

1.7.2 Intracellular Phosphorylation of Nucleoside Analogs

Intracellular phosphorylation is an essential criterion for nucleoside analogs to function as lethal mutagens. Ribavirin is converted to ribavirin monophosphate (RMP, 5) by adenosine kinase. After conversion to the monophosphate, cellular kinases are able to phosphorylate RMP to ribavirin
diphosphate (RDP, 6) and then to ribavirin triphosphate (RTP, 7) sequentially. In the case of ribavirin, RTP accumulates in cells 20-100 fold greater than RMP.\textsuperscript{47}

Nucleoside analogs with drastically altered nucleobase scaffolds may suffer from lack of recognition and phosphorylation by intracellular kinases. Recognition elements for cellular kinases may include steric shape and hydrogen bonding donor and acceptor patterns.\textsuperscript{48} Subtle modifications in natural and non-natural nucleobase scaffolds can radically alter the phosphorylation efficiency of nucleoside kinases.\textsuperscript{49} In the future, to address the issue of cellular kinase recognition, a pro-drug strategy may be pivotal. Therefore, the critical importance of enzymatic and intracellular phosphorylation should be appreciated and evaluated.

1.7.3 Polymerase Recognition of Nucleoside Analogs

Polymerase recognition of unnatural nucleosides has been studied extensively, resulting in a new understanding of promiscuous substrate utilization. In the context of the development of lethal mutagens, designed nucleosides may increase the mutation rate by ambiguous templating of natural nucleotides. Two approaches can be utilized for the design of base-modified nucleosides as lethal mutagens, that is, hydrogen bonding and non-hydrogen bonding universal bases. Nucleosides that rely on tautomerization or rotation of a hydrogen bonding moiety can alter mutation rate and benefit from often being derived from a more natural scaffold. Therefore, the advantages of this approach
include more facile flow though the required cellular machinery including transport, phosphorylation efficiency and polymerase recognition. However, they are limited by multiple factors as well. By mimicking natural scaffolds, the nucleoside may hit multiple targets as nucleoside binding pockets are ubiquitous in the cell. Therefore, the resulting cellular toxicity may be rate limiting. Additionally, nucleosides that rely on tautomerization or rotation exhibit a templating preference for only two of the natural nucleotides. Conceptually, a nucleoside that is incorporated across all natural nucleotides, revealing pure universal base characteristics, may be a more potent mutagen by drastically increasing the mutation rate. The ground breaking work of Benner, Bergstrom, Kool, Romesburg and Shultz among others has laid a foundation for the study of hydrophobic nucleoside analogs. Studies in this area have revealed that contrary to the earlier dogma of hydrogen bonding patterns as determinants of polymerase selectivity, a greater importance has been given to size and shape pattern recognition. Additionally, even the traditional shape patterns can be broadened to include truly non-traditional scaffolds as polymerase substrates. Inspired by their work, much of the studies herein reflect a universal base inspired approach to the design of lethal mutagens as antiviral agents.
1.8 Incentives for Development of Lethal Mutagens

The importance of the development of new antivirals for treating and preventing of human illness cannot be overestimated. Nucleoside analogs and other antivirals have revolutionized retroviral therapy transforming the lives of HIV patients. Additionally, ribavirin is a main agent in the therapeutic regimen for current standard of care for hepatitis C. Therefore, although many pharmaceutical companies have exhibited concern with nucleoside analogs due to perceived potential off-target effects, the impact of modified nucleosides for the management of infectious disease has been substantial. Therefore, the development of nucleosides as antiviral agents continues to garner significant attention and support. Needle stick injuries have plagued the health care worker and are a significant clinical risk. The current threat is no longer accidental HIV-1 infection but HCV due to the effective prophylaxis of HAART (highly active anti-retroviral therapy) post injury. Additionally, emerging viruses, such as SARS, West Nile and avian influenza have caused worldwide trepidation. Another remaining threat includes poliovirus which is endemic in certain areas of the world making the eradication of poliovirus challenging. The development of vaccines for RNA viruses has been problematic due to the high mutation rate. Furthermore, the highly adaptable nature of RNA viruses can result in the development of drug resistant strains relatively quickly. Additionally, contrary to making a “super virus” by utilizing the lethal mutagenesis approach, most mutations would accrue and produce nonviable virus. Accordingly, there is a
growing need for novel therapeutics for the containment of emerging diseases and to contribute to the arsenal of antiviral drugs. Therefore, lethal mutagenesis offers a unique approach to the development of new drugs. By taking advantage of the high error rate of RNA viruses, one can capitalize of this deficiency by designing base- modified nucleoside analogs with promiscuous bases to further increase the mutation rate thereby decreasing overall fitness. Universal base ribonucleosides may be less toxic due to selective viral NTP utilization over cellular utilization due to unnatural universal base scaffolds. Therefore, the development of lethal mutagens may offer a solution to the ever increasing prevalence of treatment- resistant viral strains.
1.9 References

1. UNAIDS, 2006 report on global aids epidemic.


polymerase (3d<sup>pol</sup>) by ubiquitin-protease-mediated cleavage in Escherichia coli.


Chapter 2

Isocarbostyril Nucleosides as Antiviral Agents

2.1 Overview

In an effort to develop lethal mutagens, base-modified ribonucleoside analogs bearing hydrophobic, universal bases were designed, synthesized and evaluated as antiviral agents. Hydrophobic, universal base analogs were modeled after a unique isocarbostyril scaffold first reported\(^1\) as a deoxyribonucleotide\(^2\) that displayed remarkable unbiased pairing properties when incorporated by DNA polymerase in duplex DNA. Therefore, three isocarbostyril (ICS) ribonucleoside analogs, ICS-R (8), MICS-R (9) and SICS-R (10) were synthesized and evaluated as antivirals. ICS-R (8) was discovered to be the most potent analog with antiviral activity greater than clinical agent ribavirin (1) against poliovirus. The corresponding ICS-R analog triphosphates displayed slow kinetics of incorporation and revealed inhibition of viral RdRP as the mode of action. Correlation of phosphorylation efficiencies of ICS-R analogs further corroborated antiviral and biochemical results.
2.2 Hydrophobic Bases

Hydrophobic universal bases have been explored in recent years as nucleosides which display degenerate base pairing across multiple templating nucleotides when incorporated by polymerases.\textsuperscript{1, 3, 4} The field developed with the discovery that the hydrogen bonding demonstrated by canonical Watson-Crick base pairing was not required for polymerase recognition of a given nucleotide as a substrate.\textsuperscript{5} Rather, aromatic $\pi$-stacking interactions were invoked as stabilizing thermodynamic forces. By eliminating restrictive hydrogen bonds, non-selectivity can be achieved to produce a universal base.\textsuperscript{6} Many universal nucleotides have been developed since then for a variety of applications including degenerate PCR primers, hybridization probes, detection of SNPs, and incorporation into DNA hairpins.\textsuperscript{7} Much work has been done to evaluate the kinetics of incorporation of base modified deoxyribonucleotides for such applications. Since RNA polymerases in general and especially RNA-dependent RNA polymerases (RdRPs) are vastly less discriminatory that DNA polymerases, we proposed to design promiscuous ribonucleoside analogs that should be recognized by RdRP. In an ongoing effort to design lethal mutagens, the use of hydrophobic universal ribonucleosides was attractive as a novel approach to antiviral drug design.
2.3 Design of an Isocarbostyril Lethal Mutagen

Previous studies in our research group revealed the importance of not only the thermodynamic stability of universal base analogs, but also rates of polymerase incorporation play a crucial role in the ability of a nucleoside analog to function as a lethal mutagen. 3-Nitropyrrrole ribonucleoside triphosphate (3NPNTP, 10) was incorporated into RNA at a rate of 100-fold less that RTP making lethal mutagenesis non-viable.\(^8\) Polioivirus RNA-dependent RNA polymerase incorporates RTP at approximately the rate of incorrect nucleotide incorporation, which is about 1-2 molecules per genome.\(^{11}\) Therefore, a suitable analog exhibiting significant incorporation rates of incorporation with DNA polymerase was sought. Previously reported isocarbostyril deoxyribonucleotides\(^2\), \(^9\), \(^{10}\) displayed the desired kinetic incorporation profile. As shown in Table 2-1, isocarbostyril deoxyribonucleoside triphosphates dICSTP

![Chemical structures of previously studied 3NPNTP (11)\(^8\) and deoxy-ICSTP analogs (12-14).\(^2\), \(^9\), \(^{10}\)](image)

Figure 2-1: Chemical structures of previously studied 3NPNTP (11)\(^8\) and deoxy-ICSTP analogs (12-14).\(^2\), \(^9\), \(^{10}\)
(12), dMICSTP (13), dSICSTP (14)\textsuperscript{12} evaluated against exonuclease free Klenow fragment from DNA pol I of \textit{E. coli} exhibit impressive rates of incorporation. Based on the rates of incorporation of the \textit{deoxy} ICS analogs, three ribonucleoside analogs, ICS-R (8), MICS-R (9) and SICS-R (10) were designed and chosen to be synthesized and evaluated as antiviral nucleosides.

Table 2-1: The efficiency of incorporation of unnatural and natural triphosphates opposite natural and unnatural bases in the template.\textsuperscript{2,9,10}

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<th>Template</th>
<th>TP</th>
<th>kcat (min(^{-1}))</th>
<th>Km ((\mu\text{M}))</th>
<th>kcat(/Km) (M(^{-1}) min(^{-1}))</th>
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<tr>
<td>A</td>
<td>T</td>
<td>163 ± 7</td>
<td>3.5 ± 1.0</td>
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<tr>
<td>dICS</td>
<td>A</td>
<td>0.04 ± 0.01</td>
<td>7.7 ± 2.7</td>
<td>5.70E+03</td>
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<td></td>
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<td>n.d.</td>
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<td></td>
<td>G</td>
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<td>95 ± 32</td>
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<td></td>
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<td>147 ± 39</td>
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</tr>
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<td>83 ± 27</td>
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2.4 Chemical Synthesis of ICS-R Analogs

2.4.1 Chemical Synthesis of MICS-R (9)

Figure 2-3: Synthesis of MICS nucleobase (19).
The synthesis of ribonucleoside 9 utilizes a previously reported synthesis of the MICS nucleobase (Scheme 1).\textsuperscript{13} Pomeranz-Fritsch methodology was utilized to form the isoquinoline ring system. This approach involves an acid catalyzed cyclization. To begin the synthesis of MICS, o-tolualdehyde 15 was combined with aminoacetaldehyde dimethyl acetaldehyde to produce the condensed imine 16 in 99% yield. A successful cyclization was achieved from the imine 16. Subjecting imine 16 to ethyl chloroformate and trimethylphosphite afforded carbamate-phosphonate intermediate and was cyclized using TiCl\textsubscript{4} as a Lewis acid catalyst to afford 8-methyl-isoquinoline 17 in 70% yield over three steps. With isoquinoline 17 in hand, hydrogen peroxide in acetic acid oxidized 8-methyl-isoquinoline to the N-oxide 18 in 78% yield. Rearrangement of 18 was achieved with acetic anhydride at reflux and subjecting to subsequent basic conditions at high temperature to produce the desired nucleobase 8-methyl-2H-isoquinolin-1-one 19 (also known as methylisocarbostyril).

A variety of methods exist for the coupling of nucleobases to ribose moieties.\textsuperscript{14} For the synthesis of MICS-R, three approaches were explored. The sodium salt glycosylation method involves protection of the 2’ and 3’ alcohols of D-ribose (20) as the cyclic ketal under acidic conditions with acetone.\textsuperscript{15} Subsequently, the 5’ alcohol was protected as a t-butyldimethylsilyl ether. Stereoselective chlorination of 21 with CCl\textsubscript{4} and hexamethylphosphorus triamide afforded α-chlorosugar 22,\textsuperscript{16,17} which was used in situ for the alkylation reaction. Nucleobase 19 was deprotonated with sodium hydride with subsequent addition of 22 to the reaction mixture. Isolation of the coupled product 23 afforded only
10% yield, and purification of the desired β-product from the α diastereomer proved to be difficult.

An alternate sodium salt methodology was employed invoking neighboring group participation to achieve stereoselectivity. Commercially available β-1-O-acetyl-2,3,5-O-benzoyl ribofuranose (24) was chlorinated with TiCl₄ to afford a mixture of α and β chlorosugars 25.¹⁸ Again, nucleobase 19 was deprotonated with sodium hydride and added to a solution of 25. Unfortunately, this reaction also yielded less than 10% of the desired product.

Figure 2-4: Coupling strategy using sodium salt glycosylation.
Another coupling strategy that was attempted was the Vorbrüggen reaction. Many nucleosides with lactam moieties are synthesized via this route. Nucleobase 19 was converted to the trimethylsilyl ether with a catalytic amount of trimethylsilyl chloride in hexamethyldisilazane. The resulting 27 was coupled to β-1-O-acetyl-2,3,5-O-benzoyl ribofuranose 24 in the presence of Tin (IV) chloride. This reaction was more stereoselective and a 54% yield of 26 was obtained. Deprotection in sodium methoxide in methanol afforded desired MICS-R product 9 in 79% yield.

Figure 2-5: Alternate sodium salt glycosylation strategy.
2.4.2 Chemical Synthesis of ICS-R(8) and SICS-R(10)

Once the coupling methodology was optimized utilizing the MICS nucleobase, the chemical syntheses of analogs ICS-R (8) and SICS-R (10) were achieved through similar coupling strategies. Isocarbostyril (28) was commercially available and used directly in the Vorbrüggen procedure. 20, 21 Isocarbostyril (28) was silylated with trimethylsilyl chloride in hexamethyldisilazane. The resulting 29 was coupled to β-1-O-acetyl-2,3,5-O-benzoyl ribofuranose 24 in the presence of Tin (IV) chloride providing 30 in 50% yield. Deprotection with sodium methoxide in methanol afforded desired ICS-R product 8 in 68% yield.

Figure 2-6: Vorbrüggen coupling strategy to access MICS-R (9).
Initial attempts to synthesize SICS-R (10) by coupling a thio-ICS nucleobase were unsuccessful, therefore the lactam moiety of protected ICS-R intermediate was transformed using Lawesson’s reagent\textsuperscript{22} affording the protected thio-lactam 31 selectively in the presence of benzoyl ester protecting groups. Deprotection in methanolic ammonia afforded SICS-R (10) in 85% yield.
2.4.3 Synthesis of 5’OMeICS-R (34)

In order to probe the role of 5’ phosphorylation in ICS nucleosides, a 5’-methoxy- analog was synthesized. ICS-R (8) was protected as the 2’, 3’ isopropylidene ketal using standard conditions, namely, 2,2-dimethoxy-propane and p-toluenesulfonic acid hydrate as a catalyst in acetone which provided nucleoside 32 in 59% yield. The NMR shift difference between the methyl groups was 0.22 ppm, thus further confirming the isolation of the β-anomer by Imbach’s rule.23 Deprotonation of the 5’ hydroxyl was achieved with sodium hydride, followed by methylation with methyl iodide afforded protected 5’O-methyl intermediate 33 in nearly quantitative yield, which was deprotected with aqueous trifluoroacetic acid to afford 5’OMeICS-R 34 in 72% yield.

Figure 2-8: Synthesis of 5’OMe ICS-R (34).
2.4.4 Synthesis of dICS(40)

To compare the ribo-ICS-R analog with the previously reported 2´-deoxy ICS analog, dICS-R (40) was synthesized.² Briefly, standard 2´-deoxy ribosylating agent 35 was synthesized by the method of Hashmi et. al.²⁴ converting 2´-deoxy-D-ribose to 1-O-methyl-2-deoxy-D-ribose under acid catalyzed conditions. 2´-Deoxy-methyl-ribose (36) was protected as the p-toluyl diester (37) by treatment with p-toluyl chloride in dry pyridine. Conversion to the glycosyl chloride (38) was achieved by dissolution with hydrochloric acid in acetic acid where 38 precipitated as a pure solid. Alkylation with deprotonated ICS base 28 afforded a mixture of N, and O- linked glycosides 39, which were deprotected in methanolic ammonia and carefully purified to afford β anomer 40.²
2.5. Evaluation of Cellular Toxicity of ICS-R analogs

The cellular toxicity of the ICS-R analogs 8-10, 34, and 40 toward cultured HeLa S3 cells was examined at varying concentrations from 100 μM to 2 mM after a 7 hour treatment and compared to known antiviral ribavirin. Following 24 hour recovery, a trypan blue exclusion assay was utilized to evaluate toxicity by manual counting of viable cells, providing the data in Figure 2-9. ICS-R (8) and MICS-R (9) were more toxic than RBV (1), followed by SICS-R (10) and the mechanistic probes 5´OMeICS-R (35) and dICS (40).
2.6 Antiviral Evaluation of ICS-R Analogs

The antiviral activity of the ICS-R analogs was measured in poliovirus infected HeLa S3 cells grown with various concentrations of ICS-R analogs (8-10, 35, 40) and compared with RBV. The most potent compound of the series is ICS-R (8), which is able to reduce viral titers by 1 log at 100 μM. ICS-R (8) continued to exhibit antiviral activity demonstrating a substantial 3.5 log reduction in virus titer at a clinically relevant concentration of 2 mM, surpassing the known antiviral RBV by 100-fold. MICS-R (9) and SICS-R (10) also displayed antiviral
activity superseding RBV (1) at higher concentrations. Mechanistic probes 5´OMeICS-R (35) and dICS (40) display no antiviral activity up to 2 mM highlighting the importance of the 5´ and 2´ hydroxyls for activity and suggesting that phosphorylation is involved in the mode of action.

2.7 Evaluation of Mutagenicity of ICS-R Analogs by a Guanidine Resistance Assay

To investigate the ability of ICS-R analogs to function as lethal mutagens, a previously described guanidine resistance assay was performed to examine
the increase in mutation frequency. PV replication is blocked in the presence of 3 mM guanidine hydrochloride due to inhibition of the 2C protease; however, resistance is conferred by a single mutation,\textsuperscript{26} which occurs in PV at a rate of $10^{-5}$. As seen in Figure 2-12, in the presence of 1 mM ICS-R (8), the mutation rate went up approximately 16-fold compared to untreated control. Additionally, slight increases in mutation rate were observed for PV grown in MICS-R (9) and SICS-R (10). However, RBV (1) increases the frequency of guanidine resistant mutants 74-fold at 2 mM nucleoside.\textsuperscript{25}

![Figure 2-12: Guanidine resistance assay. Cells treated with 0.5 or 1 mM analog and infected with PV and incubated in presence of guanidine. Data obtained by Jason D. Graci.](image-url)
2.8 Synthesis of ICS-R analog Triphosphates (41-43)

In order to further examine the mechanism of action of the ICS-R analogs, the respective triphosphates were synthesized and purified utilizing the three-step, one-pot method\(^\text{21}\) for preparation of nucleoside triphosphates. Analogues ICS-R (8) MICS-R (9) and SICS-R (10) were subjected to these conditions. Namely, nucleoside was converted to phosphodichloridate by reaction with POCl\(_3\). This unstable intermediate was reacted with tributylammonium pyrophosphate and quenched with aqueous base. Purification of the triphosphates of the ICS-R analogs was achieved by reversed-phase HPLC\(^\text{27}\) to yield desired pure triphosphates (41-43) in modest yields.

Figure 2-13: Synthesis of ICS-R triphosphates (41-43).

\[ \text{ICS-R} \quad (X=O, \ R=H) \quad (8) \Rightarrow \text{ICS-R(TP)} \quad (X=O, \ R=H) \quad (41) \ 18\% \]
\[ \text{MICS-R} \quad (X=O, \ R=\text{CH}_3) \quad (9) \Rightarrow \text{MICS-R(TP)} \quad (X=O, \ R=\text{CH}_3) \quad (42) \ 15\% \]
\[ \text{SICS-R} \quad (X=S, \ R=H) \quad (10) \Rightarrow \text{SICS-R(TP)} \quad (X=S, \ R=H) \quad (43) \ 14\% \]
2.9 Incorporation of ICS-R(TP) and MICS-R(TP) by PV RdRP across natural nucleotides

The ICS-R analog triphosphates (41-43) were analyzed in a series of biochemical experiments with poliovirus RNA-dependent RNA polymerase (3D pol) utilizing the primer extension assay previously described.28 The incorporation of ICS-R(TP) 41 and MICS-R (TP) 42 were studied opposite all natural ribonucleotides revealing that both ICS-R(TP) and MICS-R(TP) are universal bases (Figure 2-14).
The preliminary rate of incorporation of MICS-R(TP) 42 was measured to be 0.0039 s\(^{-1}\) against s/s-U by PV 3D\(^{pol}\), validating that MICS-R(TP) 42 displays faster kinetics of incorporation than previously studied analogs (Table 2-2).

Figure 2-14: Incorporation of monophosphates derived from ICS-R(TP) 41 and MICS-R(TP) 42 opposite nucleotides in symmetrical substrates (90 s). Data obtained by Jason D. Graci.
Table 2-2: Kinetics of incorporation of MICS-R(TP) (42) into the s/s-U mediated by PV 3Dpol compared to RTP (7). Data obtained by Jason D. Graci.

<table>
<thead>
<tr>
<th>Nucleotide TP</th>
<th>kpol (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MICS-R(TP) (42)</td>
<td>0.0039</td>
</tr>
<tr>
<td>RTP(7)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

2.10 ICS-R(TP) analogs are not chain terminators

To further evaluate the ability of MICS-R(TP) (42) to be incorporated into s/s–U by PV 3Dpol, a time course was conducted, allowing MICS-R(TP) (42) to be utilized as a substrate at longer intervals. As seen in Figure 2-15, MICS-R(TP) (42) was multiply incorporated at longer time periods, indicating chain termination was not occurring (Figure 2-15).
To further probe the mechanism of action of ICS-R(TP) analogs, a chain termination assay was conducted evaluating the ability of PV 3D\(^{\text{pol}}\) to extend beyond the incorporated ICS-R (MP) lesion. In the presence of 1 mM GTP and increasing concentrations of ICS-R(TP) analogs, extension is seen past all lesions. ICS-R(TP) (41) was incorporated less than MICS-R(TP) (42) or SICS-R(TP) (43). Surprising was the resolving of two 11-mer products, that is, the MICS-R (MP) and misincorporated GMP products. Thus, the ICS-R analogs may rival GTP misincorporation rates. UTP was added in the last lane as a control to clearly demonstrate that a 13-mer product was able to be formed due to extension past the ICS-analog-MP lesion (Figure 2-16).

Figure 2-15: MICS-R(TP) (42) is incorporated robustly at longer time points (1 mM MICSRTP), 15 to 300 s). Data obtained by Victoria Korneeva.
Intrigued by the apparent delay in ICS-R(TP) incorporation and decrease in GTP misincorporation, ICS-R(TP) analogs 41-43 were evaluated for the ability to inhibit 11-mer formation. Another surprising result was seen as ICS-R(TP) (41) was less potent than the more hydrophobic and less natural nucleotides MICS-R(TP) (42) and SICS-R(TP) (43).
2.12 Lack of phosphorylation of ICS-R analogs explains decreased antiviral activity

Since the biochemical inhibition did not correlate with the antiviral results, phosphorylation of base-modified nucleosides was examined by reversed-phase HPLC. We developed an optimized extraction-HPLC detection screening protocol for more facile detection of intracellular phosphorylation based on a known extraction method and utilizing our HPLC method with a narrow-bore Atlantis column. Briefly, HeLa cells were treated with nucleosides ICS-R (8), MICS-R (9) and SICS-R (10) for 3 hours, lysed, and nucleotides were extracted. The cell-extracts were analyzed carefully by HPLC to display phosphorylation of ICS-R to the nucleoside triphosphate. However, triphosphates of MICS-R (9) and SICS-R (10) were not detected (Figure 2-17). This correlates biochemical and antiviral results. Interestingly, the corresponding deoxy-ICS analogs are known to be poorly phosphorylated in vitro by known pyrimidine kinases TK1(cytosolic) and TK2 (mitochondrial) and deoxy cytidine kinase, dCK. Broad spectrum Dm-
dnK from *D. melangogaster* also showed decreased activity toward deoxy-ICS analogs.\textsuperscript{33}
Figure 2-17: ICS-R is phosphorylated in HeLa cells. (A) Analysis of pure ICSR-TP(41) including the full UV spectrum of the peak 41 at 7.0 min (inlay). mAU, milli-absorbance units. (B) Analysis of extracts of untreated HeLa S3 cells (C) Analysis of extracts of HeLa S3 cells treated with ICS-R 8 (2.0 mM) including the full UV spectrum of the eluent at 3.6 min (inlay). D) Analysis of extracts of HeLa S3 cells treated with MICS-R 9 (2.0 mM). E) Analysis of extracts of HeLa S3 cells treated with SICS-R 10 (2.0 mM). The long-wavelength absorption characteristic of ICSR-TP is illustrated by the arrows to the right. Peaks were detected by $A_{325}$. 
2.13 Conclusion and Future Directions

The design of novel chemical mutagens of RNA viruses as antiviral nucleosides is a lofty goal that requires fulfilling a number of strict criteria, namely, intracellular transport, phosphorylation, and efficient rates of incorporation by the viral polymerase to exert a mutagenic effect. Aspiring to this goal, the design of hydrophobic universal base ribonucleoside analogs was undertaken. ICS-R (8), MICS-R (9) and SICS-R (10) ribonucleoside analogs were synthesized and discovered to be antiviral agents more active than the clinically utilized drug RBV (1). Additional biochemical analysis of the corresponding triphosphates revealed ICS-R(TP) analogs 41-43 are slow substrates of PV 3Dpol. Furthermore, the guanidine resistance assay results did not strongly corroborate a lethal mutagenesis mechanism of antiviral activity. Indeed, experimental evidence points toward inhibition of 3Dpol as main mediator of the antiviral effect, The selective intracellular phosphorylation of unsubstituted ICS-R analog 8 as a pyrimidine mimic over the methyl and thio-ICR analogs 9 and 10 explains the order of the observed antiviral effects.

A few key observations from this study can be applied to the future design of lethal mutagens. First, hydrophobic base-modification provides both theoretical advantages and realistic disadvantages. Whereas more potent induction of mutagensis may be achieved though this strategy, the level of “unnatural”-ness of the nucleoside of interest will alter the ability of the drug to maneuver though the cellular machinery efficiently. Therefore, cellular kinase
recognition will continue to be as important as viral polymerase function and prodrug strategies may need to be developed. Although MICS-R(TP) (42) inhibited the viral polymerase, and MICS-R (9) was not efficiently phosphorylated, it did indeed exhibit faster kinetics of incorporation than previous studies.\textsuperscript{34} Therefore, future studies may focus on developing heteroatom substitutions on the ICS scaffold to more closely mimic a pyrimidine nucleoside as well as exploring a pro-nucleotide strategy.

2.14 Experimental Section

2.14.1 Cytoxicity Assays

HeLa S3 cells were maintained in DMEM/F-12 media supplemented with 2% dialyzed fetal bovine serum and penicillin/streptomycin (1X, Invitrogen). Nucleosides were freshly suspended in 100% DMSO (200 mM) immediately prior to use. Ribavirin was suspended in deionized water. For cytotoxicity studies, HeLa S3 cells (1 X 10\textsuperscript{5}) were plated the day before in 24-well plates. Cells were incubated with ribonucleosides at various concentrations for 7 h at 37 °C. All wells were adjusted to a final concentration of 1% DMSO. Media was removed and cells were washed with PBS (0.5 mL). Cells were allowed to grow for an additional 24 h in the absence of either compound. Cell monolayers were washed in PBS (0.5 mL), dissociated by treatment with trypsin (1X, Invitrogen), and viable cells were counted by trypan blue exclusion using a hemacytometer.
2.14.2 Antiviral Evaluation

Infection with PV employed HeLa S3 host cells (1 X 10^5) plated 1 day prior to treatment in 24-well plates. Cells were pretreated by addition of nucleoside at the specified concentration in fresh media adjusted to a final concentration of 1% DMSO. After a 1-hour incubation at 37 °C, media was removed and cells were infected with PV (1 X 10^6 PFU) in PBS (0.1 mL). Plates were incubated for 15 min. at 23 °C, PBS was removed by aspiration, and fresh, prewarmed (37 °C) media containing the specified amount of nucleoside was added. The infection was allowed to proceed at 37 °C for 6 h. Cells were washed with PBS and collected after treatment with trypsin. Cells were pelleted by centrifugation, resuspended in PBS (0.5 mL), and subjected to 3 freeze-thaw cycles. Cell debris was removed by centrifugation and the supernatant containing the cell-associated virus was saved. Titer was determined by applying serial dilutions of supernatant to HeLa S3 monolayers (plated in 6-well plates 1 day before at 5 X 10^5 cell/well) and overlaying with growth media containing low melting point agarose (1%). Plates were incubated for 2 days at 37 °C, at which time the agar was removed and plaques were visualized by staining with crystal violet (1%) in aqueous ethanol (20%).

Resistance to 2 mM guanidine was conferred by a single specific mutation: C to U at position 4,605 of the poliovirus genome (C4605U), in protein 2C (Pro to Ser at amino acid 161). HeLa S3 cells were plated 1 d before the experiment at 25% confluence in 10-cm dishes, and were infected with 50 PFU or 1 x 10^6 PFU poliovirus from the appropriate viral stock of full-length genome RNA transcribed using T7 RNA polymerase from a plasmid containing the viral cDNA (pMoRA or pMoRA-G64S) as previously described\(^{35}\) (previously grown in the presence or absence of nucleoside).\(^{36}\) Cells were covered with a 20-mL overlay of 1% agar and DMEM/F12 plus 10% FBS supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (plus 2 mM guanidine hydrochloride (Sigma) in gua' plaque assays). Recovered virus was passaged a minimum of 5 times in HeLa cells to allow for diversification of the virus population from the initial cDNA sequence.

2.14.4 Nucleotide Extraction

Nucleotide extraction was modified from a previously published procedure.\(^{29}\) A total of 7.5 x 10^6 HeLa S3 cells were plated in a 100-mm dish 15 to 18 h before treatment. Cells were treated with 2.5 \(\mu\)g/mL actinomycin D (Sigma) for 15 min at 37 °C, and nucleosides were added to the medium to a final concentration of 2 mM. Cells were incubated for 3 h at 37 °C. After incubation, the medium was aspirated, plates were washed with 5 mL PBS, and 1 mL prewarmed trypsin-EDTA solution (Invitrogen) was added to each plate. Cells were incubated for 5 min at 37 °C to facilitate detachment, after which cells were
collected, pelleted, and resuspended in 0.05 mL ice-cold 0.6 M trichloroacetic acid (Sigma). The cell suspension was incubated on ice for 10 min and then centrifuged at 14,000 x g for 2 min at 4 °C. The supernatant was collected and extracted with an equal volume of ice-cold 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane (Sigma). Samples were then vortexed for 30 s and centrifuged for 30 s at 14,000 x g and 4 °C. The upper (aqueous) layer was removed and analyzed on a Hewlett Packard 1100 series instrument equipped with an Atlantis column (2.3 by 150 mm, 5 µm; Waters corporation) running the following mobile phase (flow rate, 0.3 ml/min): gradient of 1% to 50% CH₃CN in 100 mM potassium phosphate buffer (KH₂PO₄, pH 6.0) over 20 min, followed by 50 to 80% CH₃CN in 100 mM KH₂PO₄ buffer over 10 min.

2.14.5. Nucleotide Incorporation by PV 3Dpol in Vitro

Incorporation of NTPs (41-43) opposite each of the four templating bases (A, C, G and U) was examined using pre-assembled 3Dpol-primer/template complexes. As previously reported, symmetrical substrates (sym/subs) served as the primer and template RNA for 3Dpol nucleotide incorporation assays. Annealing of ³²P-end-labeled and unlabeled sym/sub oligos to form the primer/template duplex was preformed as previously described. 3Dpol was allowed to preassemble with sym/sub duplex for 90 s at 30 °C. Incorporation assays with A, C and U as templating bases were performed at 30 °C in HEPES
buffer (pH 7.5, 50 mM) containing 2-mercaptoethanol (10 mM), MgCl$_2$ (5 mM), $3D_{pol}$ (1 μM), sym/sub duplex RNA (0.5 μM) and nucleotide (varying concentrations and time points). All incorporation assays were initiated by addition of nucleotide, reaction products were separated by denaturing PAGE, gels were visualized using a phosphorimager, and radioactivity was quantified using ImageQuant software (Molecular Dynamics). For the time course of 42, 1mM 42 was used in the presence of sym/sub-U. Inhibition constants were calculated by first quantifying reduction of 11-mer formation with sym/sub-U and 1mM GTP (misincorporation) in presence of 41, 42, and 43 (0, 50, 100, 500, 1000 and 2000 μM). IC$_{50}$ was calculated and fit to the Chung-Prusoff equation.

$K_i = \text{IC}_{50} / (1 + [\text{G}] / K_d)\]

where [GTP]= 1000 μM, and the $K_d= 210$ μM for GTP. To validate this approach, kinetic simulation was conducted using the scheme shown below.

![Kinetic simulation diagram](image)

Kinetic simulations were performed by using KinTekSim Version 2.03 (KinTek Corp., Austin, TX). The experimental data was fitted to the competitive inhibition mechanism shown. The values of $K_d$, app and $k_{pol}$ for the uninhibited reaction to
be used in the simulation were determined experimentally and are 210 µM for $K_d$, app and 0.01 s⁻¹ for $k_{pol}$. The agreement between the experimental data and kinetic simulations was determined by visual inspection.

### 2.14.6 General Synthesis Information.

Reagents and solvents were obtained from Aldrich, Acros, TCI, and Fisher and were used without further purification unless otherwise noted. Reactions were performed under an atmosphere of dry nitrogen. Methanol was dried using a solvent purification system and used immediately. Thin-layer chromatography was performed on glass-backed silica gel-coated plates with silica gel 60 F$_{254}$ (EM Science, 0.25 mm thick). ICN SiliTech Silica Gel (32-63 µm) was employed in column chromatography. TLC plates were visualized by UV irradiation (254 nm) or stained with a solution of methanol, sulfuric acid, and $p$-anisaldehyde (18:2:1). Infrared spectra were obtained with a Mattson GL-4020 Series FTIR. Bruker DRX 400, DPX 300, CDPX 300, and AMX 360 spectrometers were employed in nuclear magnetic resonance (NMR) spectroscopy. NMR peaks are reported as parts per million (ppm) and are referenced to internal CHCl$_3$ or DMSO. Mass spectral data was obtained from The Pennsylvania State University Mass Spectrometry Facility (ESI). Melting points were determined on a Thomas Hoover apparatus and are reported uncorrected. Purification by preparative reverse phase HPLC employed an Agilent 1100 analytical pump / gradient extension instrument equipped with a Hamilton PRP-1 (polystyrene-
divinylbenzene) reverse phase column (7 μM particle size, 21.5 mm x 25 cm). The HPLC gradient for purity analysis for compounds 8-10, 34, 40 was 1 to 99% CH₃CN (containing 0.1% trifluoroacetic acid [TFA]) in double distilled water (ddH₂O, 0.1% TFA, 0 to 30 min) then held at 99% from 30-40 min using a Hamilton PRP-1 (polystyrene-divinylbenzene) reversed-phase column (7 μM particle size, 250 mm x 4.1 cm) with a flow rate of 0.8 mL/min. The HPLC gradient for purity analysis for compounds 41-43 was 99 to 50% 100 mM KH₂PO₄ in acetonitrile over 30 min. An Aquasil column (4.6 by 250 mm, 5 μm; Thermoelectron corporation) was used with a flow rate of 1.0 mL/min.

2-(β-D-ribofuranosyl)isoquinolin-1(2H)-one (8).

2-(2´,3´,5´-tri-O-benzoyl-β-D-ribofuranosyl)isoquinolin-1(2H)-one (30, 370 mg, 0.63 mmol) was dissolved in distilled MeOH (10.0 mL), NaOMe (25 wt% in MeOH, 3.7 mL) was added dropwise, and the solution was allowed to stir for 2 h. The solution clarified and was quenched by addition of activated Dowex-H⁺ to pH 7.0, filtered, and the solution was concentrated in vacuo. The crude product was preadsorbed onto silica, purified by flash column chromatography (3 to 10%
MeOH/ CH$_2$Cl$_2$) and recrystallized from EtOH to afford 8 (120 mg, 69%). m.p. 169-170 °C. $^1$H NMR (400.1 MHz, DMSO-$d_6$): $\delta$ 8.26 (d, $J = 8.0$ Hz, 1H), 7.73 (dd, $J = 7.5$, $J = 5.2$, 2H), 7.57, (d, $J= 8.0$, 1H), 7.47 (app t, $J= 7.3$, 1H), 6.64, (d, $J = 7.6$, 1H), 6.31 (d, $J = 4.5$, 1H), 5.51 (d, $J = 5.4$ Hz, 1H), 5.30 (m, 1H), 5.25 (d, $J = 4.5$, 1H), 4.15 (m, 2H), 4.01, (m, 1H), 3.84 (m, 1H), 3.73, (m, 1H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 161.1, 136.6, 132.7, 127.9, 127.3, 126.8, 126.1, 125.2, 105.3, 87.7, 84.6, 74.5, 69.9, 60.9. IR (KBr): 3331, 1588 cm$^{-1}$. HRMS(ESI+) calcd. for C$_{14}$H$_{16}$NO$_5$ [M+H]$^+$ 278.1023. Found 278.1028. UV $\lambda_{max}$ (MeOH): 207 nm ($\lambda = 34,600$ M$^{-1}$ cm$^{-1}$).

Analysis of 8 revealed > 99% purity:

![Absorbance wavelength = 254 nm](image)

Analytical reversed-phase HPLC profile of ICS-R (8) after purification (retention time = 12.1 min).
8-methyl-2-((β-D-ribofuranosyl)isoquinolin-1(2H)-one (9).

8-methyl-2-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)isoquinolin-1(2H)-one (19, 1.12 g, 1.86 mmol) was dissolved in distilled MeOH (100 mL), NaOMe in MeOH (0.5 M, 100 mL) was added. The reaction mixture was stirred for 4 h, quenched by pouring over activated Dowex- H⁺ resin, and concentrated in vacuo. The crude reaction mixture was preadsorbed onto silica and purified by column chromatography (2 to 10% MeOH in CH₂Cl₂) and recrystallized from EtOH to afford 9 (430 mg, 79%). m.p. 168-169 °C. ¹H NMR (400.1 MHz, DMSO-d₆): δ 7.71 (d, J = 7.5 Hz, 1H), 7.52 (app t, J = 7.6, 1H), 7.44, (d, J = 5.5, 1H), 7.23 (d, J = 7.2, 1H), 6.56 (d, J = 7.5, 1H), 6.19 (d, J = 3.3 Hz), 4.02 (m, 2H), 3.91 (m, 1H), 3.62 (m, 1H), 3.44 (m, 1H), 2.82 (s, 3H). ¹³C NMR (75.1 MHz, DMSO-d₆): δ 162.0, 140.8, 138.3, 131.9, 129.7, 129.6, 127.6, 127.5, 123.5, 105.6, 87.9, 84.2, 74.6, 69.6, 60.7, 23.7. IR (KBr): 3331, 1588 cm⁻¹. HRMS(ESI+) calcd. for C₁₅H₁₈NO₅ [M+H]⁺ 292.1180. Found 292.1185. UV λ max (MeOH): 211 nm (λ= 31,000 M⁻¹ cm⁻¹).

Analysis of 9 revealed > 99% purity:
Analytical reversed-phase HPLC profile of MICS-R (9) after purification (retention time = 13.9 min).

2-(β-D-ribofuranosyl)isoquinolin-1(2H)-thione (10).

2-(2,3,5-tri-O-benzoyl β-D-ribofuranosyl)isoquinoline-1(2H)-thione (31, 250 mg, 0.40 mmol) was added to a pressure tube and dissolved in NH₃ in MeOH (15 mL, ca. 7 N). The sealed tube was heated to 60 °C for 24 h, cooled, concentrated in vacuo, and purified by column chromatography (3 to 10% MeOH in CH₂Cl₂) to afford 10 (103 mg, 85%). m.p. 152-155 °C. ¹H NMR (400.1 MHz, DMSO-d₆): δ 8.96 (d, J = 8.3 Hz, 1H), 8.45 (d, 7.5 Hz, 1H), 7.75 (m, 2H), 7.60 (m, 1H), 7.17 (d, J = 7.2 Hz, 1H), 7.0 (s, 1H), 5.5 (m, 1H), 5.32 (m, 1H), 5.0 (m, 1H), 4.0 (m, 3H), 3.90 (m, 1H), 3.70 (m, 1H). ¹³C NMR (100.6 MHz, DMSO-d₆): δ 182.2, 132.9,
132.8, 132.3, 132.2, 129.3, 128.2, 127.0, 111.4, 94.8, 84.0, 75.4, 68.0, 59.3. IR (KBr): 3322, 1625 cm$^{-1}$. HRMS(ESI+) calcd. for C$_{14}$H$_{16}$NO$_{5}$ [M+H]$^+$ 294.0795. Found 292.0080. UV $\lambda_{\text{max}}$ (MeOH): 241 nm ($\lambda$ = 19,832 M$^{-1}$ cm$^{-1}$).

Analysis of 10 revealed > 96% purity:

Analytical reversed-phase HPLC profile of SICS-R (10) after purification (retention time = 15.2 min).

(2,2-Dimethoxy-ethyl)-(2-methyl-benzylidene)-amine (16).
A solution of o-tolualdehyde (26.0 mL, 233 mmol) in benzene (100.0 mL) was added to aminoacetaldehyde dimethyl acetal (23.0 mL, 213 mmol) and heated to reflux for 15 h under a Dean Stark apparatus. The solution turned from clear to dark red-orange. Solution was concentrated *in vacuo* to afford 16 (44.0 g, 99%). $^1$H NMR (400.1 MHz, DMSO-$d_6$): $\delta$ 8.51 (s, 1H), 7.81 (d, $J = 7.7$ Hz, 1H), 7.19 (t, $J = 7.4$ Hz, 1H), 7.15 (t, $J = 7.4$ Hz, 1H), 7.08 (d, $J = 7.4$ Hz, 1H), 4.63 (t, $J = 2.4$ Hz, 1H), 3.73 (t, $J = 5.3$ Hz, 1H), 3.35 (s, 6H), 2.42 (s, 3H). $^{13}$C NMR (100.6 MHz, DMSO-$d_6$): $\delta$ 162.3, 137.8, 134.3, 130.9, 130.5, 127.7, 126.4, 104.2, 64.3, 54.34, 54.33, 19.5. LRMS (ESI+) calcd. For C$_{12}$H$_{17}$NO$_2$ [M+H]$^+$ 207.3 Found 207.8.

8-Methyl-isoquinoline (17).

(2,2-Dimethoxy-ethyl)-(2-methyl-benzylidene)-amine (16, 25.0 g, 120.6 mmol) was dissolved in THF (100 mL) and cooled to 4 °C. Ethyl chloroformate was added (11.0 mL, 89.7 mmol) and stirred for 5 min. To this stirring solution, trimethylphosphite (15.5 mL, 75.6 mmol) was added dropwise. The resulting solution was warmed to 22 °C, stirred for 24 h, and concentrated *in vacuo*. The carbamate-phosphonate intermediate was dissolved in CH$_2$Cl$_2$ (100 mL), TiCl$_4$ (58 mL in 150 mL CH$_2$Cl$_2$) was added via cannula over 25 min, and the solution was refluxed carefully over 2 d. The reaction was quenched by slowly pouring the
solution into ice (600 mL) and extracted with CH₂Cl₂. To the aqueous layer, saturated aq. Rochelle’s salt was added to chelate the excess TiCl₄ and basified with aq. NH₃ (10% in dH₂O, 300 mL) to pH 9. Care was taken to add sufficient Rochelle’s salt to prevent white precipitation of the titanium complex. The solution was extracted with CH₂Cl₂ (200 mL, 4X). The organic layer was dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo to afford isoquinoline 17 (12.15 g, 70% yield). 

1H NMR (400.1 MHz, DMSO-d₆): δ 9.32 (s, 1H), 8.42 (d, J = 5.7 Hz, 1H), 7.48 (m, 2H), 7.41 (d, J = 7.3 Hz, 1H), 7.21 (d, J = 6.9 Hz, 1H), 2.62 (s, 3H). 13C NMR (100.6 MHz, DMSO-d₆): δ 148.8, 142.3, 135.7, 134.9, 129.8, 127.6, 124.4, 120.4, 17.8. LRMS(ESI+) calcd. for C₁₀H₉N [M] 143.1 Found 143.8.

8-Methyl-isoquinoline 2-oxide (18).

8-Methyl-isoquinoline (17, 12.15 g, 52.9 mmol) was dissolved in HOAc (50.0 mL), H₂O₂ (30%, 27.0 mL) was added and the solution was heated to 60 °C for 27 h. The reaction mixture was concentrated in vacuo, redissolved in CH₂Cl₂, washed with sat. aq. NaHCO₃, dried over anhydrous Na₂SO₄, and concentrated in vacuo to afford pure N-oxide 18 (7.7 g, 57% yield). 

1H NMR (400.1 MHz, DMSO-d₆): δ 8.93 (s, 1H), 8.15 (dd, J = 7.1 Hz, J = 1.6 Hz, 1H), 7.64 (t, J = 7.3 Hz, 2H), 7.47 (t, J = 7.3 Hz, 1H), 7.41 (d, J = 7.1 Hz, 1H), 2.62 (s, 3H). 13C NMR
8-methylisoquinolin-1(2H)-one (19).

8-Methyl-isoquinoline 2-oxide (18, 3.07 g, 19.3 mmol) was partially dissolved in acetic anhydride (40 mL), heated to 140 °C for 5 h, and concentrated in vacuo. The resulting residue was redissolved in aqueous NaOH (1 M, 100 mL), heated to 100 °C for 40 min, and stirred at 22 °C for 20 h. The crude reaction mixture was extracted with CH₂Cl₂, dried over anhydrous Na₂SO₄, concentrated in vacuo, and recrystallized from MeOH to afford isocarbostyril 19 (1.64 g, 53% yield) ¹H NMR (400.1 MHz, DMSO-d₆): δ 11.4, (broad singlet, 1H), 7.48 (t, J = 7.5 Hz, 1H), 7.35 (d, J = 7.8 Hz, 1H), 7.23 (d, J = 7.2 Hz, 1H), 7.09 (d, J = 7.8 Hz, 1H), 6.56 (d, J = 7.0 Hz, 1H), 2.98 (s, 3H). ¹³C NMR (100.6 MHz, DMSO-d₆): δ 165.9, 142.1, 140.3, 132.2, 130.0, 128.0, 125.1, 124.9, 107.4, 24.2. LRMS(ESI+) calcd. for C₁₀H₉NNaO [M+Na]⁺ 181.1. Found 181.8.
8-methyl-2-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)isoquinolin-1(2H)-one (26)

8-Methyl-2H-isoquinolin-1-one (19, 1.14 g, 7.16 mmol) was added to a flame-dried round bottom and partially dissolved in HMDS (5.0 mL, 23.5 mmol). To this stirring solution under nitrogen was added TMSCl (0.12 mL, 0.94 mmol, 0.13 eq). The solution was refluxed for 22 h and concentrated \textit{in vacuo} under high vacuum. The crude silylated product was dissolved in MeCN (10.0 mL) and transferred to a solution of commercially available β-D-ribofuranose-1-acetate-2,3,5 tribenzoate (24, 3.6 g, 7.14 mmol) dissolved in MeCN (10.0 mL). The resulting solution was cooled to 4 °C and SnCl\(_4\) (8.0 mL of 1 M solution in CH\(_2\)Cl\(_2\)) was added. The solution turned clear and then was stirred at 22 °C for 26 h. The resulting solution turned green. The reaction was quenched by pouring the reaction mixture into saturated aq. NaHCO\(_3\) and extracted with CH\(_2\)Cl\(_2\) (40 mL, 5x). The organic layer was dried over MgSO\(_4\), filtered over Celite, and concentrated \textit{in vacuo}. The crude material was purified by column chromatography (5 to 30% EtOAc in Hexanes) to afford 26 (2.43 g, 54% yield) as a white foam. \(^1\)H NMR (400.1 MHz, CDCl\(_3\)): \(\delta\) 8.18 (d, \(J = 7.4\) Hz, 2H), 8.00 (dd, \(J = 7.6\) Hz, \(J = 7.6\) Hz, 4H), 7.63-7.22 (m, 13H), 6.80 (d, \(J = 4.9\) Hz, 1H), 6.35 (d, \(J = 7.6\) Hz, 1H), 6.03 (app t, \(J = 5.6\) Hz, 1H), 5.92 (app t, \(J = 5.4\) Hz, 1H), 4.92 (dd, \(J = 12.1\) Hz, \(J = 2.7\) Hz, 1H), 4.79 (m, 1H), 4.73 (dd, \(J = 12.0\) Hz, \(J = 3.8\) Hz, 1H), 2.92 (s, 3H). \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta\) 166.4, 165.6, 165.5, 162.9, 142.5, 138.5, 133.8, 133.7, 133.6, 132.2, 130.5, 130.1, 130.0, 1 129.95, 129.7, 129.04, 128.98, 128.8, 128.63, 128.60, 126.29, 124.6, 107.6, 88.0, 80.1, 74.6,

2-(2′,3′,5′-tri-O-benzoyl-β-D-ribofuranosyl)isoquinolin-1(2H)-one (30).

Isocarbostyril (1.15 g, 7.92 mmol) was added to a flame dried round bottom and partially dissolved in HMDS (5.0 mL, 23.5 mmol). To this stirring solution under nitrogen TMSCI (0.10 mL, 0.78 mmol, 0.10 eq) was added and refluxed for 12 h and concentrated in vacuo. The crude silylated product was dissolved in MeCN (10.0 mL) and transferred to commercially available β-D-ribofuranose-1-acetate-2,3-5 tribenzoate (30, 4.0 g, 7.92 mmol) dissolved in MeCN (10.0 mL) and cooled to 4 °C and then SnCl₄ (8.0 mL of 1M solution in CH₂Cl₂) was added. The solution turned clear and then was stirred at 22 °C for 26 h. The resulting solution turned green. The reaction was quenched by pouring the reaction mixture into saturated aq. NaHCO₃ and extracted with CH₂Cl₂ (50 mL, 4x) and washed with saturated aq. NaCl. The organic layer was dried over MgSO₄ and concentrated in vacuo. The crude material was purified by column chromatography (100% CH₂Cl₂) to afford 30 (2.35 g, 50% yield) as a white foam. ¹H NMR (400.1 MHz, CDCl₃): δ 8.40 (d, J = 8.0 Hz, 1H), 8.17 (d, J = 7.5 Hz, 2H), 7.98 (dd, J = 4.7 Hz, J = 2.4 Hz, 4H), 7.63-
7.30 (m, 14H), 6.81 (d, J = 5.0 Hz, 1H), 6.42 (d, J = 7.3 Hz, 1H), 6.02 (m, 1H), 5.92 (m, 1H), 4.88 (m, 1H), 4.74 (m, 1H), 4.75 (m, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.4, 165.5, 165.4, 162.1, 130.09, 130.00, 129.9, 129.6, 129.0, 128.9, 128.8, 128.63, 128.61, 128.3, 128.3, 127.3, 126.6, 126.2, 126.20, 126.17, 107.2, 88.3, 80.2, 74.7, 71.4, 64.0. IR (film): 1725, 1663 cm$^{-1}$. HRMS(ESI+) calcd. for C$_{35}$H$_{27}$ClNO$_8$ [M+H]$^+$ 590.1737. Found 590.1815.

2-(2,3,5-tri-O-benzoyl $\beta$-D-ribofuranosyl)isoquinoline-1(2H)-thione (31)

2-(2‘,3’,5’-tri-O-benzoyl-$\beta$-D-ribofuranosyl)isoquinolin-1(2H)-one (30, 600 mg, 1 mmol) was added to distilled toluene (40 mL) and then Lawesson’s reagent (1.65 g, 4.0 mmol) was added. The resulting solution was refluxed for 12 h and quenched by allowing the solution to cool, filtered the insoluble material and concentrated remaining solution in vacuo. The crude solution was puriﬁed by column chromatography (20% EtOAc in Hexanes) to afford 31 (313 mg, 51% yield) as a yellow gel-like foam. $^1$H NMR (400.1 MHz, CDCl$_3$): δ 9.05 (d, J = 8.3 Hz, 1H), 8.16 (J = 7.3 Hz, 2H), 8.08 (J = 7.2 Hz, 2H), 7.96 (J = 7.5 Hz, 1H), 7.89 (J = 7.3 Hz, 2H), 7.81 (d, 2.8 Hz, 1H), 7.69-7.44 (m, 11H), 7.32 (t, J = 7.6 Hz, 2H), 6.73 (d, J = 7.5 Hz, 1H), 5.99 (dd, J = 5.3 Hz, J = 2.9 Hz, 1H), 5.86 (dd, J = 7.3 Hz, J = 5.5 Hz, 1H), 4.98 (dd, J = 12.5 Hz, J = 2.5 Hz, 1H), 4.90 (m, 1H), 4.72
(dd, \( J = 12.6 \) Hz, \( J = 3.3 \) Hz, 1H) \(^{13}\)C NMR (75 MHz, DMSO-\( d_6 \)): \( \delta \) 184.5, 166.0, 165.1, 164.9, 134.0, 133.5, 133.4, 132.9, 132.2, 132.0, 130.0, 129.8, 129.7, 129.4, 128.7, 128.6, 128.5, 128.4, 128.3, 126.9, 126.7, 112.3, 92.2, 79.9, 65.4, 69.6, 62.7. IR(film): 1728, 1286 cm\(^{-1}\)    HRMS(ESI+) calcd. for C\(_{35}\)H\(_{27}\)NO\(_7\)S [M+H]\(^+\) 606.1581. Found 606.1586.

2-[2',3'-O-Isopropylidene-\( \beta \)-d-ribofuranosyl]isoquinolin-1(2H)-one (32).

2-(\( \beta \)-d-ribofuranosyl)isoquinolin-1(2H)-one (8, 214 mg, 0.77 mmol) was dissolved in dry acetone (6.0 mL) and 2,2- dimethoxypropane (0.6 mL, 48.7 mmol) and \( p \)-TsOH (20 mg, 0.11 mmol) was added and let stir 30 min. Reaction mixture was poured into saturated aq. NaHCO\(_3\) and extracted with CH\(_2\)Cl\(_2\). The organic layers were combined and washed with dH\(_2\)O and saturated aq. NaCl, dried over MgSO\(_4\), filtered and concentrated in vacuo. Purification by column chromatography in 100% CH\(_2\)Cl\(_2\) afforded 32 (144 mg, 59% yield) as an off-yellow foam. \(^1\)H NMR (300.1 MHz, DMSO-\( d_6 \)): \( \delta \) 8.22 (d, \( J = 7.9 \) Hz, 1H), 7.74-7.51 (m, 4 H), 6.68, (d, \( J = 7.5 \) Hz, 1H), 6.24 (d, \( J = 2.6 \) Hz, 1H), 5.09 (t, \( J = 5.3 \) Hz, 1H), 4.91 (dd, \( J = 2.6 \) Hz, \( J = 6.3 \) Hz, 1H), 4.83 (dd, \( J = 6.2 \) Hz, \( J = 3.7 \) Hz, 1H), 4.11 (m, 1H), 3.63, (m, 2H). 1.52 (s, 3H), 1.30 (s, 1H) \(^{13}\)C NMR (75 MHz, DMSO-\( d_6 \)): \( \delta \)
2-[5-O-methyl-2,3-O-(1-methylethylidene) β-D-ribofuranosyl]isoquinolin-1(2H)-one (33).

2-[2',3'-O-Isopropylidene-β-D-ribofuranosyl]isoquinolin-1(2H)-one (32, 46 mg, 0.14 mmol) was dissolved in dry THF (2.0 mL) and cooled to 4 °C. To the cooled solution, NaH (8.0 mg, 0.33 mmol, 2 eq) was added and let stir at 4 °C. After 15 min, iodomethane (35 µL, 0.57 mmol, 4.0 eq) was added and let stir at 22 °C for 16 h. Reaction mixture was poured into dH$_2$O and extracted with CH$_2$Cl$_2$, washed with dH$_2$O, dried over MgSO$_4$, filtered, concentrated in vacuo and purified by column chromatography (100% CH$_2$Cl$_2$) to afford 33. (46 mg, 96 % yield). $^1$H NMR (400.1 MHz, CDCl$_3$): δ 8.38 (d, $J$ = 8.0 Hz, 1H), 7.62 (t, $J$ = 8.0 Hz, 1H), 7.45 (m, 2H), 7.28 (d, $J$ = 5.0 Hz, 1H), 6.48 (d, $J$ = 7.5 Hz, 1H), 6.10 (d, $J$ = 2.1 Hz, 1H), 4.97 (dd, $J$ = 6.3 Hz, $J$ = 2.1 Hz, 1H), 4.87 (dd, $J$ = 6.3 Hz, $J$ = 3.7 Hz, 1H), 4.38 (m, 1H), 3.67 (t, $J$ = 4.0 Hz, 2H), 3.39 (s, 3H), 1.61 (s, 3H), 1.35 (s, 3H). $^{13}$C NMR (100.6 MHz, CDCl$_3$): 136.8, 132.4, 128.4, 127.6, 126.7, 126.1,
125.8, 113.8, 105.9, 94.2, 85.4, 81.2, 72.8, 59.1, 27.2, 25.3. IR (KBr): 1647 cm\(^{-1}\).

HRMS(ESI+) calcd. for C\(_{18}\)H\(_{21}\)NO\(_5\) [M+H]\(^+\) 332.1493 Found 332.1492.

2-(5’-O-methyl-\(\beta\)-D-ribofuranosyl)isoquinolin-1(2H)-one(34)

2-[5-O-methyl-2,3-O-(1-methylethylidene)-\(\beta\)-D-ribofuranosyl]isoquinolin-1(2H)-one (33, 46 mg, 0.13 mmol) was dissolved in dH\(_2\)O (5 mL) and trifluoroacetic acid (5.0 mL) was added dropwise into the stirring solution. After 15 min, the solution was concentrated in vacuo and purified by column chromatography in 5% MeOH/CH\(_2\)Cl\(_2\) to afford 34 (29.2 mg, 72% yield) of an off-yellow film. Further purification was performed by HPLC. Gradient 1 to 99% CH\(_3\)CN (containing 0.1% trifluoroacetic acid [TFA]) in double distilled water (ddH\(_2\)O, 0.1% TFA, 0 to 30 min) (99 to 1% over 30 min) with a Hamilton PRP-1 (polystyrene-divinylbenzene) reverse phase column (7 \(\mu\)M particle size, 21.5 mm x 25 cm). with a flow rate of 20 ml/min. \(t_R=\) 19.2 min. m.p. 100-101 °C. \(^1\)H NMR (400.1 MHz, DMSO-\(d_6\)): \(\delta\) 8.22, (d, \(J = 8.0\) Hz, 1H), 7.71 (m, 1H), 7.65, (d, \(J = 8.0\) Hz, 1H), 6.68, (d, \(J = 4.5\) Hz, 1H), 4.05 (m, 1H), 4.00, (m, 2H), 3.62 (m, 1H), 3.55 (m, 1H), 3.35 (s, 3H). \(^{13}\)C NMR (100.6 MHz,
DMSO-$d_6$: $\delta$ 161.0, 136.5, 132.7, 127.6, 127.2, 126.8, 126.1, 125.2, 105.5, 88.1, 82.5, 74.2, 72.0, 70.0, 58.7. IR (film): 3406, 1655 cm$^{-1}$. HRMS(ESI+) calcd. for C$_{15}$H$_{18}$NO$_5$ [M+H]$^+$ 292.1180. Found 292.1185.

Analysis of 34 revealed > 97% purity:

![Analytical reversed-phase HPLC profile of 5'OMeICS-R (34) after purification (retention time = 15.2 min).](image)

Analytical reversed-phase HPLC profile of 5'OMeICS-R (34) after purification (retention time = 15.2 min).

*Methyl 3,5-Di-O-(p-toluyl)-2-deoxy-D-ribofuranoside(37).*

2-deoxy-D-Ribose (25.0 g, 0.19 moles) was partially dissolved in MeOH (425 mL), cooled to -10 °C and concentrated sulfuric acid was added dropwise
(2.75 mL) and let stir overnight. Reaction was quenched with addition of activated polymer-bound piperdine resin to pH 7, filtered, concentrated in vacuo to afford crude 1-O-Methyl-2-deoxy-D-ribose which was dissolved in dry pyridine (270 mL) and cooled to 4 °C. To this stirring solution, p-Toluoyl chloride (50 mL, 42.8 mmol) was added via addition funnel over 1 h with the solution turning light orange. After 36 h, reaction mixture was concentrated in vacuo, redissolved in CH₂Cl₂, washed with sat aqueous NaHCO₃, dried over MgSO₄, filtered, and concentrated in vacuo and purified by column chromatography (5 to 15% EtOAc in Hexanes) to afford 37 (8.0 g, 13% over 2 steps). ¹³C NMR (100.6 MHz, CDCl₃): δ 166.8, 166.7, 166.6, 166.5, 144.4, 144.3, 144.2, 144.0, 130.2, 130.10, 130.08, 129.5, 129.4, 127.7, 127.6, 127.5, 127.3, 106.0, 105.5, 82.3, 81.4, 75.8, 65.0, 65.6, 55.5, 39.7, 22. LRMS(ESI+) calcd. for C₂₂H₂₄NaO₆ [M+Na]⁺ 407.1. Found 407.3.

3,5-Di-O-(p-toluyl)-2-deoxy-D-ribofuranosyl chloride (39).

Methyl 3,5-Di-O-(p-toluyl)-2-deoxy-D-ribofuranoside (37, 3.0 g, 7.7 mmol) was dissolved in hydrochloric acid in acetic acid (1M, 25 mL) and cooled to 4 °C. The solution turned cloudy white and a thick white precipitate came out of
solution after 1 h. The white solid was collected by filtration and washed with cold ether to afford 39 (2.0 g, 67% yield). \(^1\)H NMR (300.1 MHz, CDCl\(_3\)): \(\delta\) 7.93 (d, \(J = 8.1\) Hz, 1H), 7.89 (d, \(J = 8.0\) Hz, 2H), 7.25 (m, 4H), 6.47 (d, \(J = 4.9\) Hz, 1H), 5.56 (m, 1H), 4.86 (m, 1H), 4.71-4.56 (m, 2H), 2.86 - 2.72 (m, 1H), 2.42 (m, 6H). \(^1^3\)C NMR (75.5 MHz, CDCl\(_3\)): \(\delta\) 166.9, 166.6, 144.8, 144.4, 130.4, 130.2, 129.7, 129.6, 127.3, 127.1, 95.8, 85.2, 74.0, 64.0, 45.0, 22.2 LRMS(ESI+) calcd. for C\(_{21}\)H\(_{21}\)ClNaO\(_5\) [M+Na] \(^+\) 411.1 Found 412.1.

![Structural formula of 2-(2-deoxy-β-D-ribofuranosyl)isoquinolin-1(2H)-one (40).](image)

2-(2-deoxy-β-D-ribofuranosyl)isoquinolin-1(2H)-one (40).

Isocarbostyril (300 mg, 2.07 mmol) was added to a precooled solution of NaH (100 mg, 4.2 mmol) in MeCN (80 mL) at 4 °C. After stirring for 15 min, 3,5-Di-O-(p-tolyl)-2-deoxy-D-ribofuranosyl chloride was added (38, 1.0 g, 2.6 mmol) was added to the solution and let stir at 22 °C for 22 h. Reaction mixture was filtered through Celite and rinsed with cold ether and concentrated in vacuo. The crude mixture was a mixture of \(N\)- and \(O\)- linked products (1.4 g) and was transferred to a pressure tube and dissolved in NH\(_3\) in MeOH (8.0 mL, ca. 7N), sealed and heated to 55 °C for 24 h. The reaction mixture was cooled, vented and concentrated in vacuo. Purification by column chromatography in 5% MeOH in CH\(_2\)Cl\(_2\) afforded 40 (94 mg, 17% over 2 steps). \(^1\)H NMR (400.1 MHz, DMSO-
$d_6$: $\delta$ 8.21 (d, $J = 7.9$, 1H), 7.69 (m, 2H), 7.65 (m, 1H), 7.52 (app t, $J = 7.0$, 1H), 6.67 (d, $J = 7.5$, 1H), 6.59 (pseudo-t, $J = 6.0$, 1H), 5.26 (d, $J = 4.0$ Hz, 1H), 5.03 (m, 1H), 4.29 (m, 1H), 3.86, (m,1H), 3.61 (m, 2H), 2.22 (m, 1H), 2.0 (m, 1H). $^{13}$C NMR (100.6 MHz, DMSO-$d_6$): $\delta$ 160.6, 136.6, 132.6, 127.4, 127.4, 127.1 126.7, 126.1, 125.0, 105.5, 87.5, 84.0, 70.7, 61.5. LRMS(ESI+) calcd. for C$_{14}$H$_{15}$NO$_4$ [M+H]$^+$ 262.1 Found 262.0.

Analysis of 40 revealed > 97% purity:

![Absorbance wavelength = 254 nm](image)

Analytical reversed-phase HPLC profile of dICS (40) after purification (retention time = 12.7 min).

$2-(\beta$-D-ribofuranosyl)lisoquinolin-1(2H)-one-5'-triphosphate (41).
Ribonucleotide 41 was prepared by the widely-utilized “one-pot, three-step” methodology for nucleotide synthesis. Accordingly, a solution of 2-(β-D-ribofuranosyl)isoquinolin-1(2H)-one (8, 50 mg, 0.18 mmol), Proton-Sponge (1,8-bis(dimethylamino)naphthalene, 65.0 mg, 0.31 mmol), and trimethylphosphate (1.8 mL) was cooled to 2 °C. POCl₃ (35 µL, 0.38 mmol) was added dropwise, and the solution was stirred for 2 h. Anhydrous Bu₃N (190 µL, 1.30 mmol) was injected followed by a solution of tributylammonium pyrophosphate (450 mg) in DMF (1.5 mL). The reaction was stirred for 2 min then hydrolyzed by addition of triethylammonium bicarbonate (TEAB, 1.0 M, 15 mL). The crude reaction products were purified by preparative HPLC (Aquasil C-18 preparative column) to yield triphosphate 41 as the triethylammonium salt (tᵣ = 6-7.5 min). The HPLC flow rate was 20 mL/min and the mobile phase comprised 10% - 20% CH₃CN in triethylammonium acetate buffer (TEAA) (1-20 min, 20 mM TEAA, pH=6.0) followed by 20 to 50% CH₃CN in TEAA buffer (20 to 30 min, 20 mM TEAA, pH= 6.0). This material was concentrated in vacuo, redissolved in TEAB (100 mM, 10 mL) and lyophilized to dryness. The material was redissolved in deionized H₂O (3 mL) and lyophilized again to dryness. Triphosphate 41 was isolated as a white solid (27 mg, 18 % yield). ¹H NMR (360.1 MHz, D₂O): δ 8.20 (d, J = 8.2 Hz, 1H), 7.70 (t, J = 4.8 Hz, 1H), 7.65 (m, 2H), 652 (d, J = 7.4 Hz, 1H), 6.88 (d, J = 7.3 Hz, 1H), 6.39 (d, J = 4.8 Hz, 1H), 4.35 (m, 2H), 4.22 (m, 3H), 3.00 (q, J = 7.3 Hz, 18H, TEAA salt), 1.12 (t, J = 7.3 Hz, 27H, TEAA salt). ¹³C NMR (90.0 MHz, D₂O): δ 163.8, 137.2, 133.6, 127.5, 127.0, 126.7, 126.1, 124.4, 108.9, 88.0, 83.1, 74.5, 69.6, 64.9, 46.6 (TEAA salt), 8.2 (TEAA salt). ³¹P NMR
(145.8 MHz, D$_2$O): $\delta$ -10.29 (m), -10.90 (m), -22.52. HRMS (ESI-) calcd for C$_{14}$H$_{17}$NO$_{14}$P$_3$ [M-H]$^-$ 515.9867. Found: 515.9862.

Analysis of 41 revealed > 96% purity:

![HPLC profile](image)

Analytical reversed-phase HPLC profile of ICS-R(TP) (41) after purification (retention time = 7.0 min).

8-methyl -2-(β-D-ribofuranosyl)isoquinolin-1(2H)-one-5′-triphosphate (42)

Ribonucleotide 42 was prepared by the widely-utilized “one-pot, three-step” methodology for nucleotide synthesis. Accordingly, a solution of 8-methyl-2-(β-D-ribofuranosyl)isoquinolin-1(2H)-one (9, 75 mg, 0.27 mmol), Proton-Sponge
(1,8-bis(dimethylamino)naphthalene, 100 mg, 0.47 mmol), and trimethylphosphate (2.5 mL) was cooled to 2 °C. POCl₃ (50 µL, 0.54 mmol) was added dropwise, and the solution was stirred for 2 h. Anhydrous Bu₃N (250 mL, 1.05 mmol) was injected followed by a solution of tributylammonium pyrophosphate (625 mg) in DMF (2.1 mL). The reaction was stirred for 2 min then hydrolyzed by addition of triethylammonium bicarbonate (TEAB, 0.2 M, 15 mL). The crude reaction products were purified by preparative HPLC) Aquasil C-18 preparative column) to yield triphosphate 42 as the triethylammonium salt ($t_R = 9.0 -10.6$ min). The HPLC flow rate was 20 mL/min and the mobile phase comprised 10% - 20% CH₃CN in triethylammonium acetate buffer (TEAA) (1-20 min, 20 mM TEAA, pH=6.0) followed by 20 to 50% % CH₃CN in TEAA buffer ( 20 to 30 min, 20 mM TEA, pH= 6.0). This material was concentrated in vacuo, redissolved in TEAB (100 mM, 10 mL) and lyophilized to dryness. The material was redissolved in deionized H₂O (3 mL) and lyophilized again to dryness. Triphosphate 42 was isolated as a white solid (40 mg, 15 % yield).. $^1$H NMR (360.1 MHz, D₂O): δ 7.77 (d, $J = 7.5$ Hz, 1H), 7.55 (t, $J = 7.7$ Hz, 1H), 7.46 (d, $J = 7.5$ Hz, 1H), 7.26 (d, $J = 7.1$ Hz, 1H), 6.58 (d, $J = 7.7$ Hz, 1H), 6.19 (d, $J = 3.7$ Hz, 1H), 5.36 (d, $J = 5.2$ Hz, 1H), 5.12 (t, $J = 5.1$ Hz, 1H), 5.05 (d, $J = 5.2$ Hz, 1H), 4.02 (m, 2H), 3.91 (m,1H), 3.72 (m, 1H), 3.60 (m, 1H), 3.11 (q, 27H), 2.82 (m, 3H), 1.23 (t, 42H) $^{13}$C NMR (90.6 MHz, D₂O): δ 180.0, 164.0, 141.2, 138.6, 132.7, 120.5, 125.8, 124.7, 122.9, 106.9, 87.9, 82.5, 74.3, 69.6, 65.0, 46.6 (TEAA salt), 8.2 (TEAA salt). $^{31}$P NMR (145.8 MHz, D₂O): δ -9.00 (m), -10.51 (m) -21.68. HRMS (ESI-) calcd for C$_{15}$H$_{19}$NO$_{14}$P$_{3}$ [M-H]$^-$ 530.0024. Found: 530.0018.
Analysis of 42 revealed > 98% purity:

Analytical reversed-phase HPLC profile of MICS-R( TP) (42) after purification (retention time = 9.1 min).

2-(β-D-ribofuranosyl)isoquinolin-1(2H)- thione-5´-triphosphate (43)

Ribonucleotide 43 was prepared by the widely-utilized “one -pot, three-step” methodology for nucleotide synthesis. Accordingly, a solution of 2-(β-D-ribofuranosyl)isoquinolin-1(2H)-thione. (10, 30.0 mg, 0.10 mmol), Proton-Sponge (1,8-bis(dimethylamino)naphthalene, 20.0 mg, 0.09 mmol), and trimethylphosphate (1.0 mL) was cooled to 2 °C. POCl₃ (20 µL, 0.21 mmol) was
added dropwise, and the solution was stirred for 2 h. Anhydrous Bu₃N (110 µL, 0.46 mmol) was injected followed by a solution of tributylammonium pyrophosphate (261 mg) in DMF (0.9 mL). The reaction was stirred for 2 min then hydrolyzed by addition of triethylammonium bicarbonate (TEAB, 1.0 M, 5 mL). The crude reaction products were purified by preparative HPLC using an Aquasil C-18 preparative column. The HPLC flow rate was 20 mL/min and the mobile phase comprised 10% - 20% CH₃CN in triethylammonium acetate buffer (TEAA) (1-20 min, 20 mM TEAA, pH=6.0) followed by 20 to 50% % CH₃CN in TEAA buffer (20 to 30 min, 20 mM TEA, pH=6.0) to yield triphosphate 42 as the triethylammonium salt. This material was concentrated in vacuo, redissolved in TEAB (100 mM, 10 mL) and lyophilized to dryness. (tᵣ = 13-14.2 min.). Triphosphate 43 was isolated as a bright yellow solid (13 mg, 14% yield). ¹H NMR (360.1 MHz, D₂O): δ 8.95 (d, J = 8.1 Hz, 1H), 8.33 (d, J = 7.6 Hz, 1H), 7.82 (m, 2H), 7.69 (m, 1H), 7.42 (d, J = 7.3 Hz, 1H), 7.22 (s, 1H), 4.45 (m, 3H), 4.40 (m, 1H), 3.16 (q, 23H), 1.24 (t, 35H). ¹³C NMR (90.0 MHz, D₂O): δ 179.7, 133.7, 133.2, 132.8, 130.8, 128.8, 128.6, 127.5, 114.7, 95.0, 82.5, 75.6, 67.7, 46.7 (TEAA salt), 8.2 (TEAA salt). ³¹P NMR (145.8 MHz, D₂O): δ -9.41 (m), -11.36 (m), -22.85. HRMS (ESI-) calcd for C₁₄H₁₈NO₁₃P₃S [M-H]⁻ 531.9639. Found: 531.9634.

Analysis of 43 revealed > 99% purity:
Analytical reversed-phase HPLC profile of SICS-R(TP) (43) after purification (retention time = 9.1 min).
2.15 References


30. Harki, D. A.; Graci, J. D.; Galarraga, J. E.; Chain, W. J.; Cameron, C. E.; Peterson, B. R., Synthesis and antiviral activity of 5-substituted cytidine


Chapter 3
Mechanism of Action Studies of 5-Nitroindole Ribonucleoside and Development of Novel Indole Antiviral Agents

3.1 Overview

The mechanism of antiviral activity of 5-nitroindole ribonucleoside (44) was investigated through the evaluation of a series of mechanistic probes and biochemical characterization of phosphorylated 5-nitroindole analogs. 5NINDN(DP) (45) and 5NINDN(MP) (46) were synthesized and evaluated as inhibitors of PV RdRP(3D\text{\text{pol}}) in comparison with 5NINDN(TP) (47) and the parent nucleoside 5NINDN (44). Surprisingly, both triphosphate and diphosphate inhibited the viral polymerase, however reversed-phase HPLC analyses revealed detectable phosphorylation limited only to 5NINDN(MP) (46). Further evaluation of mechanistic probes 5′OMe5NINDN (48), 5-nitroindole (49), and N-Eth-OH 5-nitroindole (50) indicated that intracellular phosphorylation was not necessary for antiviral activity and revealed inhibition of cellular translation was proposed as a mediator of antiviral activity. Due to instability of the nitroaromatic nucleobase, stable 5-halo analogs 5ClINDN (51) and 5BrINDN (52) were synthesized and evaluated as agents more active than the parent 5NINDN (44).
3.2 Mechanistic Studies with 5NINDN (44)

3.2.1 5NINDN (44) as an Antiviral Agent

5-nitroindole ribonucleoside (5NINDN, 44) was previously synthesized and evaluated in our laboratory in an effort to develop hydrophobic, universal ribonucleosides as lethal mutagens.\(^1\) The 5-nitroindole scaffold has attracted significant attention in deoxyribonucleosides since strong stabilization via π-stacking interactions with adjacent DNA bases is observed.\(^2\)\(^-\)\(^4\) Additionally, the large, aromatic system devoid of hydrogen bonding donors and acceptors shows unbiased incorporation across all natural nucleotides. Due to these properties, d5NINDN(TP) (53) has been used in primers for PCR and DNA sequencing,\(^5\)\(^-\)\(^7\) incorporation into hairpin DNA,\(^8\) incorporation into PNAs,\(^7\) and for the detection of single nucleotide polymorphorphisms.\(^9\),\(^10\) Therefore, 5NINDN (44) was designed as a ribonucleoside that may accelerate viral mutagenesis via universal base pairing properties.
5NINDN (44) was shown to be a weak antiviral whose antiviral activity was potentiated by cytochrome P450 inhibitor, sulconazole (54), presumably due to prevention of the metabolism of the unstable nitroaromatic ring of 5NINDN (44). Therefore, we embarked upon an investigation of the mechanism of action of 5NINDN (44) by biochemical evaluation of the phosphorylated intermediates of 5NINDN (44) as well probing the structural requirements for antiviral activity.

### 3.2.2 Synthesis of 5NINDN(DP) (45) and 5NINDN(MP) (46)

Phosphorylated analogues of 5-nitroindole ribonucleoside 45 and 46 were synthesized as shown in Figure 3-2. Monophosphate 46 was synthesized by modification of the conditions used to prepare 5NINDN(TP) (47) except the initially formed phosphodichloridate (step i) was immediately hydrolyzed with aqueous triethylammonium bicarbonate (TEAB). Diphosphate 45 was elaborated from monophosphate 46 by CDI activation of the α-phosphate to yield a
phosphoimidazolidate intermediate,\textsuperscript{13} which was subsequently displaced with phosphate to install the $\beta$-phosphate.

\textbf{3.2.3 Inhibition of PV RdRP by phosphorylated analogs}

As shown in Table 3-1, 5NINDN(TP) (47) and 5NINDN(DP) (45) inhibited PV 3D\textsubscript{pol} ($K_i = 30, 50$ $\mu$M, respectively), whereas 5NINDN (MP) (46) and parent nucleoside 5NINDN (44) demonstrated no detectable inhibition of the polymerase demonstrating the importance of the $\beta$-phosphate for inhibition.

\textbf{Table 3-1: Inhibition of PV 3D\textsubscript{pol} by 5NINDN 5$'$ phosphates. Data collected by Christian Castro.}

\begin{tabular}{ll}
Inhibitor & $K_i$ ($\mu$M) \\
5NINDN(TP) & 47 30 \\
5NINDN(DP) & 46 50 \\
5NINDN(MP) & 45 no inhibition \\
5NINDN & 44 no inhibition
\end{tabular}

Figure 3-2: Synthesis of 5NINDN(MP) (46) and 5NINDN(DP) (45).
3.2.4 Detection of Intracellular Phosphorylation of 5NINDN (44)

Although biochemical experiments revealed the ability of 5NINDN(TP) 47 and 5NINDN(DP) 45 to inhibit PV 3D\textsuperscript{pol}, demonstration of intracellular phosphorylation is required to correlate the cellular antiviral effect with \textit{in vitro} data. Toward this end, HeLa cells were treated with the nucleoside 5NINDN (44) for 3 hours and lysed with subsequent nucleotide extraction. Briefly, HeLa cells extracts from treatments with and without 5NINDN 44 and sulconazole 54 were analyzed by reversed-phase HPLC. These experiments revealed phosphorylation of 5NINDN to the nucleoside monophosphate 46 only. Exhaustive evaluation of conditions for extraction and detection demonstrated no reproducible result of higher phosphorylation for the artificial nucleoside 5NINDN (44).
3.2.5 Syntheses of 5NINDN Mechanism of Action Probes

To further probe the mechanism of action of 5NINDN (44) a non-phosphorylatable analog, 5´-O-methyl-5NINDN 48 was synthesized and studied.

Figure 3-3: Detection of 5NINDN(MP) (44) in HeLa cell extracts. A) Analysis of untreated HeLa cell extracts. B) Analysis of 5NINDN (44)-only treated cell extracts. C) Analysis of 5NINDN (44) and sulconazole (54) treated HeLa cell extracts. D) Analysis of a mixture of 5NINDN (TP) (47), 5NINDN (DP) (45), 5NINDN (MP) (46) and 5NINDN (44) standards. The absorbance for all wavelengths is 325 nm.
Based on an analogous route for 5’ substituted indole nucleosides,\textsuperscript{14,15} 5NINDN 44 was protected as the 2’, 3’ isopropylidene ketal by standard conditions, namely, 2,2-dimethoxy-propane and \( p \)-toluenesulfonic acid hydrate as a catalyst in acetone, which provided nucleoside 55 in 81\% yield.\textsuperscript{16} The NMR shift difference between the methyl groups was 0.26 ppm, thus confirming the isolation of the \( \beta \)-anomer.\textsuperscript{17} Deprotonation of the 5’ hydroxyl was achieved with sodium hydride, followed by methylation with methyl iodide afforded protected 5’O-methyl intermediate 56 in 52\% yield, which was deprotected with aqueous trifluoroacetic acid to afford 5’OMe-5NINDN 57 in 81\% yield.

![Figure 3-4: Synthesis of 5’OMe5NINDN (48).](image)

We hypothesized that the nucleobase may play a role in antiviral activity, thus we decided to evaluate the nucleobase 5-nitroindole 49 as an antiviral. However, to prevent complicating issues resulting in the indole being scavenged by the purine salvage pathway to the indole nucleoside as seen in other
studies,\textsuperscript{18} we synthesized $N$-Me 5-nitroindole. Unfortunately, $N$-methylation afforded an insoluble derivative. To circumvent these solubility issues, an alkylated and soluble $N$-ethanol-5-nitroindole derivative was synthesized. In brief, 5-nitroindole $49$ was deprotonated with sodium hydride and treated with 2-bromoethyl acetate to access protected intermediate $57$ which was deprotected under basic conditions to afford probe $50$.\textsuperscript{19}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{synthesis.png}
\caption{Synthesis of $N$-5NI-EthOH ($50$).}
\end{figure}

3.2.6 Toxicity and Antiviral Evaluation of Mechanistic Probes

The 5NINDN mechanism of action probes $48$-$50$ were evaluated for cellular toxicity against HeLa S3 cells grown in cell culture as described in section 2.5. As seen in Figure 3-6, probes $48$-$50$ mimic the toxicity of 5NINDN $44$ and the toxicity is potentiated by addition of sulconazole ($54$).
Antiviral evaluation of 5NINDN mechanism probes revealed a surprising result, namely, a non-phosphorylatable 5’OMe5NINDN analog (48).

Figure 3-6: 5NINDN (44) and 5NINDN mechanistic probes 48-50.

Figure 3-7: Cytotoxicity of 5NINDN mechanistic probes.
demonstrated a more potent antiviral effect than parent nucleoside 5NINDN (44). Additionally, the 5-nitroindole pseudobase (49) and the alkylated 5-nitroindole 50 demonstrated antiviral activity. Therefore, the mechanism probes revealed that phosphorylation may not be required for activity, inferring a non-lethal mutagenesis mode of action. Also, since the simple indole pseudobase had antiviral activity, it may be that the target protein has a binding pocket for the indole moiety.

![Graph](image)

**Figure 3-8:** Antiviral activity of 5NINDN mechanistic probes (48-50) in the presence of 10 µM sulconazole (54).

### 3.2.7 Translation Inhibition
Antiviral evaluation of the mechanism of action probes demonstrated the authentic target of 5NINDN metabolites may not be virus-specific. Thus, 5NINDN mechanism probes were evaluated for inhibition cellular targets. Toward this end, a luciferase-based reporter assay for PV replication was performed. HeLa S3 cells were transfected with the subgenomic replicon pRLucRA, which contains the wild-type PV sequence with the capsid-coding region replaced by the luciferase reporter gene. Additionally, guanidine hydrochloride, a reversible inhibitor of PV replication, provided another control. As shown in Figure 3-9, cells treated with 2.0 mM 5NINDN (44) and 5NINDN analogs (48-50) demonstrated luciferase activity lower than in the presence of guanidine which represents the translation of input RNA. Therefore, inhibition of a cellular target is probable. Furthermore, a cell-free system was used to evaluate the efficiency of PV subgenomic replicon to be translated after 12 h (Figure 3-10). Marked decrease in translation was observed for both 5NINDN (44) and 5NI (48), which is also the concentration at which maximal antiviral effect was observed. Therefore the mechanism of action of 5NINDN (44) is likely inhibition of a cellular target involved in translation.
Figure 3-9: 5NINDN inhibition of cellular translation. Replication of PV quantified by a luciferase reporter assay. HeLa S3 cells were transfected with pRLucRA. Nucleoside analogs at 2 mM were employed. Data contributed by Jason D. Graci.
3.3 Design of 5-Halo Indole Ribonucleosides as Antiviral Agents

Although 5NINDN (44) is an interesting lead compound in our investigations of indole ribonucleosides as antiviral agents, it is a problematic model nucleoside to study mechanism of action due to its unstable nitroaromatic ring system. However, indole nucleosides continue to be attractive as novel potential antiviral agents due because they are isosteric with the purine scaffold. Attempts to resolve compound instability though inhibition of cytochrome P450 was also complicated. Therefore, we designed a series of more stable 5-substituted indole ribonucleosides as antiviral agents.

Figure 3-10: 5NINDN(44) and 5-nitroindole (49) inhibit in vitro translation of RNA. Cell free-reactions were programmed with PV replicon RNA containing luciferase. Translation was measured via a luciferase assay. 2 mM nucleoside analog. Data contributed by Jason D. Graci.
The first generation indole analogs we designed included 5-methyl indole ribonucleoside analog 5MINDN (58). To our delight, 5MINDN (58) retained activity similar to sulconazole-treated 5NINDN giving credence to importance of steric bulk at the 5-position of indole as a determinant of antiviral activity. Consistent with this idea, unsubstituted indole ribonucleoside (69) is not an antiviral agent. Additionally, 5MINDN(58) did not demonstrate inhibition of translation in the PV replicon assay (data not shown). 5MINDN(5P) (59) was synthesized by standard phosphorylation methods\textsuperscript{21} and shown not to be an efficient substrate for PV 3D\textsubscript{pol}. No inhibition studies have been conducted to date.
with 5MINDN(TP) (59), however, a 5MINDN-resistant mutant has been isolated and is being sequenced to determine the location of the mutation (Figure 3-11).

**Figure 3-12: Synthesis of 5MINDN(TP) (52).**

Encouraged by an increase in antiviral activity and apparent *difference* in mechanism, we chose to design a series of 5-halo indole ribonucleosides as antiviral agents. We hypothesized that 5-chloro and 5-bromo indole ribonucleosides 5ClINDN (51) and 5-bromo (52) might retain antiviral activity and exhibit mechanisms similar to 5MINDN (58) and possibly be more soluble than the hydrophobic and sparingly soluble 5MINDN (58). Additionally, we designed the 5-haloindoles to investigate the effect of electron withdrawing substituents on the indole ring.
3.3.1 Syntheses of 5-ClINDN (51) and 5-BrINDN (52)

The synthesis of 5-halo derivatives 51 and 52 was achieved by the indoline-indole method of Preobrazhenskaya\textsuperscript{22} and Walton,\textsuperscript{23} which involves the coupling of more nucleophilic reduced indoline to a ribosylation agent. Once the coupled and desired product is isolated, oxidation with MnO\textsubscript{2} followed by deprotection affords the desired indole nucleoside. To access 5-chloroindole nucleoside (5ClINDN, 51), 5-chloroindoline (62) was required. Reduction of 5-chloroindole (63) to 5-chloroindoline (62) can be achieved with sodium cyanoborohydride. Additionally, an economical synthesis of 5-chloroindoline can be achieved by chlorination of acetyl indoline 60 with sulfuryl chloride in carbon tetrachloride followed by basic deprotection to access the requisite amount of 5-chloroindoline (62) in good overall yield.

Figure 3-13: Structures of novel 5-halo indole ribonucleosides 51 and 52.
Subsequent coupling of 5-chlorindoline (62) to common ribosylation agent 24 in refluxing ethanol containing acetic acid gave coupled indoline-protected ribonucleoside 65 in 17% yield. Oxidation of indoline nucleoside to indole nucleoside 67 with MnO₂ followed by deprotection afforded 5ClINDN (51) in 91% yield. 5-Bromoindole nucleoside (5BrINDN, 52) was prepared by a similar route. Namely, commercially available 5-bromoindoline was coupled to sugar 24 in 31% yield, dehydrogenated to 68 and deprotected to afford 5BrINDN (52).
3.3.2 Cellular Toxicity of 5ClINDN (51) and 5BrINDN (52)

The cellular toxicity of analogs 51 and 52 toward cultured HeLa S3 cells was examined at varying concentrations from 100 µM to 2 mM after a 7 hour treatment. Following 24 hour recovery, a trypan blue exclusion assay was utilized to evaluate toxicity by manual counting of viable cells providing the data in Figure 3-16. 5ClINDN (51) and 5BrINDN (52) demonstrated similar toxicities with 50% cell viability at 1 mM which is similar to the toxicity observed for other base-modified nucleosides analyzed in this work.
Figure 3-16: Cellular toxicity of 5CIINDN (51) and 5BrlNDN (52) against HeLa cells.
3.3.4 Antiviral Evaluation of Indole Ribonucleoside Analogs and Structure Activity Relationships (SAR)

The antiviral activity of the Indole ribonucleoside analogs 51 and 52 was measured in poliovirus infected HeLa S3 cells grown in various concentrations of analog was compared to selected indole analogs (69, 70, 58, 44, 71 and 72) and RBV (1) to make structure-activity comparisons. The most potent compounds of the series are 5ClINDN (51), 5BrINDN (52) and TFMINDN (72) followed by 5MINDN (58) which were all more active than RBV (1). In fact 51, 52 and 72 demonstrate a greater than three-log reduction in viral titer at 1 mM. Surprisingly, INDN (69) and 5FINDN (70) which do not have large substituents on the indole scaffold are inactive as antiviral agents indicating the need for steric

Figure 3-17: Chemical Structures of Indole Ribonucleosides Analyzed as Antiviral Agents.
bulk at the 5-position of the indole ring for activity. However, the 5-amino analog is also inactive, indicating that it may be metabolized in cells. 5NINDN (44) was also previously shown to be activated by inhibition of cytochrome P450. Therefore, we demonstrate a clear SAR demonstrating that substitution at the 5 position is necessary, but not sufficient for antiviral activity.

![Figure 3-18: Antiviral Evaluation of Indole Ribonucleoside Analogs compared to Ribavirin. Data compiled in collaboration with Jason Graci and Runzhi Wu.](image)

3.4 Conclusions and Future Directions

The evaluation of indole ribonucleosides as antiviral agents has provided significant insight into the design of antiviral agents. Mechanistic studies of 5NINDN (44) revealed a few subtle lessons. First, the ability of nucleoside
analogs to be phosphorylated by cellular kinases is a key prerequisite of potential lethal mutagens. Second, the metabolism pathways of base-modified nucleoside analogs is an intricate and crucial factor in the design of antivirals. Third, subtle molecular substitutions may reveal drastic changes in biological activity and also mechanism of action. Therefore the design of novel nucleoside analogs as antiviral agents requires an appreciation for the intricacies of the cellular machinery that may tightly discriminate between slightly modified nucleoside analogs.

3.5 Experimental Section

3.5.1 Cytoxicity Assays

HeLa S3 cells were maintained in DMEM/F-12 media supplemented with 2% dialyzed fetal bovine serum and penicillin/streptomycin (1X, Invitrogen). Nucleosides were freshly suspended in 100% DMSO (200 mM) immediately prior to use. Ribavirin was suspended in deionized water. For cytotoxicity studies, HeLa S3 cells (1 X 10^5) were plated the day before in 24-well plates. Cells were incubated with ribonucleosides at various concentrations for 7 hours at 37 °C. All wells were adjusted to a final concentration of 1% DMSO. Media was removed and cells were washed with PBS (0.5 mL). Cells were allowed to grow for an additional 24 h in the absence of either compound. Cell monolayers were
washed in PBS (0.5 mL), dissociated by treatment with trypsin (1X, Invitrogen), and viable cells were counted by trypan blue exclusion using a hemacytometer.

3.5.2 Antiviral Evaluation

Infection with PV employed HeLa S3 host cells (1 X 10^5) plated 1 day prior to treatment in 24-well plates. Cells were pretreated by addition of nucleoside at the specified concentration in fresh media adjusted to a final concentration of 1% DMSO. After a 1-hour incubation at 37 °C, media was removed and cells were infected with PV (1 X 10^6 PFU) in PBS (0.1 mL). Plates were incubated for 15 min. at 23 °C, PBS was removed by aspiration, and fresh, prewarmed (37 °C) media containing the specified amount of nucleoside was added. The infection was allowed to proceed at 37 °C for 6 hours. Cells were washed with PBS and collected after treatment with trypsin. Cells were pelleted by centrifugation, resuspended in PBS (0.5 mL), and subjected to 3 freeze-thaw cycles. Cell debris was removed by centrifugation and the supernatant containing the cell-associated virus was saved. Titer was determined by applying serial dilutions of supernatant to HeLa S3 monolayers (plated in 6-well plates 1 day before at 5 X 10^5 cell/well) and overlaying with growth media containing low melting point agarose (1% for V, 0.5% for CVB3). Plates were incubated for 2 days at 37 °C, at which time the agar was removed and plaques were visualized by staining with crystal violet (1%) in aqueous ethanol (20%).
3.5.3 Determination of Inhibition Constants

Figure 3-19: Model of competitive inhibition used for the kinetic simulation. ER: 3D\textsuperscript{pol}-s/s template complex; N: NTP; X: NTP analogues such as 47 and RTP.

Figure 3-20: Raw fluorescence data obtained by stopped-flow kinetic studies of inhibition of incorporation of ATP catalyzed by PV RdRP. [3Dpol] = 0.5 \mu M, [s/s-U-2AP] = 0.25 \mu M (duplex).[ATP], [RTP], [45] and [47] = 100 \mu M. A) Combined fluorescence data for ATP, RTP, and 47. B) Combined fluorescence data for ATP, RTP, and 45.
Stopped-flow kinetic assays used to determine Ki. These assays follow the decrease in fluorescence generated as a result of nucleotide incorporation onto a s/s primer-template that contains the fluorescent probe 2-aminopurine (2AP) on the next position downstream of the templating base. The following RNA sequence, termed s/s-U-2AP, was employed: 5’-GC(2AP)UGGCC-3’. All fluorescence experiments were performed using an SF-2001 model stopped-flow apparatus (KinTek Corp, Austin, TX) equipped with a recirculating water bath. The excitation wavelength was 313 nm, and the emission was captured above 370 nm using a cut-on filter (Chroma Technologies, Rockingham, VT). All reactions were run in HEPES buffer (50 mM, pH 7.5) containing 2-mercaptoethanol (10 mM), MgCl₂ (5 mM) and ZnCl₂ (60 µM). Reactions were performed at 30 °C. 3Dpol was diluted immediately prior to use in HEPES (50 mM, pH 7.5), 2-mercaptoethanol (10 mM), ZnCl₂ (60 µM), and glycerol (20 %). 3Dpol-s/s complexes were assembled by mixing annealed s/s-U-2AP (1.0 µM) with 3Dpol (1.0 µM) in the reaction buffer for 3 min at 23 °C. This mixture was placed in one of the stopped-flow syringes and allowed to equilibrate for 2 min at 30 °C. The other stopped-flow syringe contained ATP alone (200 µM), or with ribavirin triphosphate (200 µM), 45 (200 µM), or 47 (200 µM) in the reaction buffer. The reaction was initiated by mixing the 3Dpol-s/s complex with the nucleotide solution and recording the fluorescence decay for at least 5 times the
reaction half-life. After mixing, the reactant concentration was reduced by 50%. The experimental data shown in Figure 3-16 was normalized to the initial concentration of 3D\textsuperscript{pol}-s/s complex present in the mixture. In contrast to nucleotide 47, diphosphate 45, or RTP, neither ribavirin (1), nucleoside 44, nor UTP were inhibitors of 3D\textsuperscript{pol} under these conditions (data not shown). Data in Table 3-1 was obtained by fitting data shown in Figure 3-16 against a nonlinear regression model to a single exponential using KaleidaGraph 3.5 software (Synergy Software, Reading, PA).

**Kinetic Simulation.** Kinetic simulations were performed by using KinTekSim Version 2.03 (KinTek Corp., Austin, TX). The experimental data shown in Figure 3-16 was fitted to the competitive inhibition mechanism shown in Figure 3-15. The values of \( K_{d,\text{app}} \) and \( k_{\text{pol}} \) for the uninhibited reaction to be used in the simulation were determined experimentally and are 66 \( \mu \text{M} \) for \( K_{d,\text{app}} \) and 70 s\(^{-1} \) for \( k_{\text{pol}} \). In the simulation, the association rate constant for inhibitor binding was set at 10 \( \mu \text{M}\text{-}1 \) s\(^{-1} \) and only the dissociation rate constant was varied. The agreement between the experimental data and kinetic simulations was determined by visual inspection.
3.5.4 Nucleotide Extraction and HPLC Detection

Nucleotide extraction was modified from a previously published procedure. A total of 7.5 x 10^6 HeLa S3 cells were plated in a 100-mm dish 15 to 18 h before treatment. Cells were treated with 2.5 µg/mL actinomycin D (Sigma) for 15 min at 37°C, and then nucleosides was added to the medium to a final concentration of 2 mM. Cells were incubated for 3 h at 37°C. After incubation, the medium was aspirated, plates were washed with 5 mL PBS, and 1 mL prewarmed trypsin-EDTA solution (Invitrogen) was added to each plate. Cells were incubated for 5 min at 37°C to facilitate detachment, after which cells were collected, pelleted, and resuspended in 0.05 mL ice-cold 0.6 M trichloroacetic acid (Sigma). The cell suspension was incubated on ice for 10 min and then centrifuged at 14,000 x g for 2 min at 4°C. The supernatant was collected and extracted with an equal volume of ice-cold 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane (Sigma). Samples were then vortexed for 30 s and centrifuged for 30 s at 14,000 x g and 4°C. The upper (aqueous) layer was removed and analyzed on a Waters 2850 Separations Module instrument equipped with an Atlantis C_{18} analytical column (2.3 by 150 mm, 5 µm; Waters Corp.) running the following mobile phase Isocratic run of 15% CH₃CN in 2 mM NH₄OAc over 15 min, 50% CH₃CN in 2 mM NH₄OAc for 15-20 min.(Flow rate = 0.8 mL/min).
3.5.5 Luciferase Based Reporter for PV-Replication

RNA transcripts carrying a luciferase reporter gene were generated as described for viral genomes from ApaI-linearized plasmids encoding pRLucRA. HeLa cell cultures were propagated in DMEM/F-12 (Invitrogen) supplemented with 2% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen), and maintained between 20% and 80% confluence. Subconfluent HeLa monolayers were detached from culture flasks by trypsin treatment, washed with 1X phosphate-buffered saline (PBS), adjusted to 2.4 x 10^6 cells/mL suspended in DMEM/F-12 containing varying concentrations of nucleoside analogs. All samples were adjusted to a final concentration of 1% DMSO. After 1 h incubation in a 37 °C water bath with agitation, HeLa cells were again pelleted, then resuspended in PBS, mixed with pRLucRA RNA (9 µg) in a microcentrifuge tube, transferred to an electroporation cuvette (0.2-cm gap width; Bio-Rad), and subjected to an electric pulse (500 microfarads, 0.13 V) with a Gene Pulser system (Bio-Rad). Electroporated cells were immediately transferred to prewarmed (37 °C) DMEM/F-12 containing ribavirin (1), or no nucleotide, with 1% DMSO and plus or minus guanidine hydrochloride (3 mM). The volume of electroporated cells added was calculated by multiplying 33 µL by n + 1 (where n equals the number of time points to be measured). The volume of DMEM/F-12 added to electroporated cells was calculated by multiplying 500 µL by n + 1. After mixing the appropriate volume of electroporated cells with the appropriate volume of medium, 500 µL aliquots were prepared in microcentrifuge tubes for
each time point to be measured. These aliquots were incubated in a 37 °C water bath with agitation. At fixed time points, cells were pelleted by centrifugation (14,000 rpm, 2 min), lysed by addition of 1X cell culture lysis reagent (Promega, 100 µL), and vortexed. Cell lysates were maintained on ice for 2 min, then pelleted by centrifugation (14,000 rpm, 2 min) to remove cellular debris and nuclei. Cell lysates were maintained on ice at 4 °C until all time points were collected. Assays of cell lysates for luciferase activity were conducted by mixing of lysate (10 µL) with the luciferase assay substrate (10 µL, Promega) followed by quantifying in a Lumat LB 9501 luminometer (Berthold). Protein assays were performed to quantify the relative lights units (RLU) per milligram of protein. In this regard, cell lysate (5 µL) was added to Bio-Rad protein assay dye reagent (1 mL, Bio-Rad) and vortexed. Protein concentrations were obtained by measuring the UV absorbance at 595 nm.

3.5.6 General Synthesis Information.

Reagents and solvents were obtained from Aldrich, Acros, TCI, and Fisher and were used without further purification unless otherwise noted. Reactions were performed under an atmosphere of dry nitrogen. Methanol was dried using a solvent purification system and used immediately. Thin-layer chromatography was performed on glass-backed silica gel-coated plates with silica gel 60 F254 (EM Science, 0.25 mm thick). ICN SiliTech Silica Gel (32-63 µm) was employed
in column chromatography. TLC plates were visualized by UV irradiation (254 nm) or stained with a solution of methanol, sulfuric acid, and p-anisaldehyde (18:2:1). Infrared spectra were obtained with a Mattson GL-4020 Series FTIR. Bruker DRX 400, DPX 300, CDPX 300, and AMX 360 spectrometers were employed in nuclear magnetic resonance (NMR) spectroscopy. NMR peaks are reported as parts per million (ppm) and are referenced to internal CHCl$_3$ or DMSO. Mass spectral data was obtained from The Pennsylvania State University Mass Spectrometry Facility (ESI). Melting points were determined on a Thomas Hoover apparatus and are reported uncorrected. Internal solvent peaks were referenced in each case. Purification by preparative reversed-phase HPLC employed an Agilent 1100 analytical pump / gradient extension instrument equipped with a Hamilton PRP-1 (polystyrene-divinylbenzene) reverse phase column (7 $\mu$M particle size, 21.5 mm x 25 cm). The HPLC gradient for purity analysis for compounds 48-50, 51, 52 was 1 to 99% CH$_3$CN (containing 0.1% trifluoroacetic acid [TFA]) in double distilled water (ddH$_2$O, 0.1% TFA, 0 to 30 min) then held at 99% from 30-40 min using a Hamilton PRP-1 (polystyrene-divinylbenzene) reversed-phase column (7 $\mu$M particle size, 250 mm x 4.1 cm). Flow rate= 0.8 mL/min. The HPLC gradient for purity analysis for compounds 45, 46 and 58 was 99 to 20% 100mM KH$_2$PO$_4$ in acetonitrile over 30 min. Aquasil column (4.6 by 250 mm, 5 $\mu$m; Thermoelectron corporation).
1-β-D-ribofuranosyl-5-nitroindole diphosphate (45).

To a solution of 1-(β-D-ribofuranosyl)-5-nitroindole-5′-monophosphate (46, 11 mg, 0.03 mmol) in DMF (1 mL) was added anhydrous Bu3N (8 µL, 0.03 mmol). The solution was stirred for 10 min then concentrated in vacuo. The resulting oil was redissolved in DMF (1 mL) and carbonyl diimidazole (27 mg, 0.17 mmol) was added. The reaction mixture was stirred for 3 h at 23 °C, then quenched by addition of MeOH (10 µL). After stirring for an additional 30 min, a solution of anhydrous H3PO4 and Bu3N in DMF (0.5 M, 1.5 mL) was added. The solution was stirred for 12 h at 23 °C, then concentrated in vacuo. The crude reaction products were purified by preparative HPLC. The method utilized an Aquasil C18 preparative column (21.2 x 250 mm, 5 µm; Thermo Electron Corporation), mobile phase method: (flow rate = 20 mL/min) linear gradient of 10% to 20% CH3CN in TEAA buffer (0 to 20 min, 20 mM TEAA, pH 6) followed by 20% to 90% CH3CN in TEAA buffer (20 to 30 min, 20 mM TEAA, pH 6); yielding diphosphate 45 (tR = 9-10 min). The purified material was then concentrated in vacuo, redissolved in deionized H2O and lyophilized to dryness to afford yield diphosphate 45 (12 mg, 0.14 mmol, 50 % yield). 1H NMR (360.1 MHz, D2O): δ 8.45 (d, J = 2.2 Hz, 1H), 7.99 (dd, J = 2.2Hz, J =9.2 Hz, 1H), 7.66 (d, J = 3.5 Hz, 1H), 7.53 (d, J = 9.2 Hz, 1H), 6.72 (d, J = 3.4 Hz, 1H), 6.00 (d, J =
7.0 Hz, 1H), 4.40 (m, 1H), 4.17 (m, 1H), 3.95 (m, 2H), 3.00 (q, \( J = 7.3 \) Hz, 26H, TEAA salt), 1.80 (s, 2H, TEAA salt), 1.12 (t, \( J = 7.3 \) Hz, 37H, TEAA salt). \(^{13}\)C NMR (90.6 MHz, D2O): \( \delta \) 141.6, 140.0, 128.5, 128.2, 118.3, 117.8, 110.1, 106.0, 88.0, 73.6, 70.4, 45.5 (TEAA salt), 8.2. (TEAA salt). \(^{31}\)P NMR (145.8 MHz, D2O): \( \delta \) -7.38 (m), -10.19 (m). HRMS (ESI-) calcd for \( \text{C}_{13}\text{H}_{15}\text{NOP}^- \) [\( M-\text{TEAA}+2\text{H} \)] 453.0100. Found: 453.0078.

Analysis of 45 revealed > 91% purity:

Analytical reversed-phase HPLC profile of 5NINDN(DP) (45) after purification (retention time = 11.8 min).

1-\((\beta-D\text{-ribofuranosyl})\)-5-nitroindole-5'-monophosphate (46).
To a partially dissolved solution of 1-(β-D-ribofuranosyl)-5-nitroindole (44, 66 mg, 0.22 mmoles) in trimethylphosphate (2.2 mL) was added Proton-Sponge (1,8-bis(dimethylamino)naphthalene, 83.0 mg, 0.39 mmol). The solution was cooled to 2 °C and POCl₃ (50 mL, 0.55 mmoles) was added dropwise. The yellow solution was stirred for 2 h at 2 °C, then quenched with triethylammonium bicarbonate (TEAB, 1.0 M solution, 5 mL). This solution was lyophilized to dryness and purified by preparative HPLC. The HPLC method used utilized an Atlantis C18 preparative column (19 x 150 mm, 5 µm; Waters Corporation), mobile phase method: (flow rate = 25 mL/min) linear gradient of 10% to 30% CH₃CN in TEAA buffer (0 to 20 min, 20 mM TEAA, pH 6) followed by 20% to 90% CH₃CN in TEAA buffer (20 to 30 min, 20 mM TEAA, pH 6) to yield monophosphate 46 (tᵣ = 14 min). The purified material was concentrated in vacuo, redissolved in deionized H₂O and lyophilized to dryness to afford monophosphate 46 as a yellow, oily solid (29 mg, 24% yield). ¹H NMR (300.1 MHz, D₂O): δ 8.48 (d, J = 2.2 Hz, 1H), 8.00 (dd, J = 2.2 Hz, J = 9.2 Hz, 1H), 7.65 (d, J = 3.5 Hz, 1H), 7.58 (d, J = 9.2 Hz, 1H), 6.75 (d, J = 3.4 Hz, 1H), 6.02 (d, J = 7.0 Hz, 1H), 4.54 (m, 1H), 4.31 (m, 1H), 4.20 (m, 1H), 3.95 (m, 2H), 3.04 (q, J = 7.3 Hz, 20H, TEAA salt), 1.80 (s, 8H, TEAA salt), 1.12 (t, J = 7.3 Hz, 30H, TEAA salt). ¹³C NMR (75.5 MHz, D₂O): δ 180.6 (TEAA salt), 142.0, 140.0, 128.8, 128.6, 118.7, 118.1, 110.5, 106.3, 88.5, 84.2, 84.1, 74.0, 71.1, 65.1, 47.0 (TEAA salt), 22.9 (TEAA salt), 8.6(TEAA salt). ³¹P NMR (145.8 MHz, D₂O): δ 1.12 (s). HRMS (ESI-) calcd for C₁₃H₁₄N₂O₉P⁻ [M-TEAA+H]⁻ 373.0437, found 373.0436.
Analysis of 46 revealed > 94% purity:

![Graph showing HPLC profile](image)

Analytical reversed-phase HPLC profile of 5NINDN(DP) (46) after purification (retention time = 14.8 min).

1-(5-O-methyl β-D-ribofuranosyl l)-5-nitro-1H-indole (48)

Protected nucleoside 1-[2,3-O-(1-methylethylidene) β-D-ribofuranosyl]-5-nitro-1H-indole (55, 70 mg, 0.21 mmoles) was dissolved in dry THF (2.0 mL) and cooled to 4 °C. To the cooled solution, NaH (10.0 mg, 0.50 mmoles, 2 eq) was added and let stir at 4 °C. The solution turned dark red. After 7 min, iodomethane
(40 µl, 0.57 mmoles, 3.1 eq) was added and let stir at rt for 16 h as the solution returned to a bright yellow color. The reaction was quenched with the addition of MeOH (0.10 mL), concentrated in vacuo, redissolved in CH₂Cl₂, washed with deionized H₂O, and dried over MgSO₄. The solution was filtered, concentrated in vacuo and purified by column chromatography (20% to 50% EtOAc in Hexanes) to afford 48 (38 mg, 52 % yield). This material was partially dissolved in ddH₂O (3.0 mL). To this solution, trifluoroacetic acid (8.0 mL, 60%) was added dropwise into the stirring solution and allowed to stir for 30 min. Reaction mixture was concentrated in vacuo and purified by column chromatography (5% MeOH in CH₂Cl₂) to afford desired 5′OMe nucleoside (27 mg, 80%) Further purification by HPLC (30 to 99% CH₃CN (containing 0.1% trifluoroacetic acid [TFA]) in double distilled water (ddH₂O, 0.1% TFA, 0 to 30 min, flow rate= 20 mL/min) tᵣ= 18.4 min afforded 48 (20 mg, 31% over 2 steps). ¹H NMR (400.1 MHz, DMSO): δ 8.63 (d, J = 2.0, 1H), 8.05 (d, J = 7.4 Hz, 1H), 7.78 (m, 2H), 6.84 (m, 1H), 5.87 (m, 1H), 5.46 (d, J = 6.5 Hz, 1H), 5.30 (d, J = 4.1 Hz, 1H), 4.28 (m, 1H), 4.0 (m, 2H), 3.56 (m, 2H), 3.32 (s, 3H). ¹³C NMR (100.6 MHz, DMSO): δ 140.1, 139.6, 130.1, 128.9, 118.4, 117.7, 113.8, 110.5, 108.0, 105.7, 90.2, 84.2, 75.0, 73.3, 71.5, 59.5. IR(film): 3406, 1655 cm⁻¹. HRMS(ESI-) calcd for C₁₄H₁₅N₂O₆ [M-H]⁻ 307.0930. Found 307.0930.

Analysis of 48 revealed > 96% purity:
Analytical reversed-phase HPLC profile of 5OMe5NINDN (48) after purification (retention time = 18.2 min).

2-(5-Nitro-indol-1-yl)-ethanol (50)$^{19}$

5-nitroindole (49, 1.0 g, 6.2 mmoles) was dissolved in DMF (10 mL) followed by NaH addition (164 mg, 6.8 mmoles, 1.1 eq). After 30 min, 2-bromoethylacetate (0.75 mL, 6.8 mmoles, 1.1 eq dissolved in 2 mL DMF) was added dropwise and allowed to stir at room temperature for 3 h. Reaction was stopped with addition of sat. aqueous NH$_4$Cl. The solution was extracted with EtOAc and washed with deionized water, sat. aqueous NaCl, dried over MgSO$_4$, filtered and concentrated in vacuo. The crude product was partially purified in 20% EtOAc hexanes to afford 400 mg which was dissolved in MeOH (24 mL) followed by addition of K$_2$CO$_3$ (s, 0.80 g). The reaction mixture was slowly stirred
as deionized H$_2$O (8 mL) was added and let stir for 16 h. The reaction mixture was concentrated in vacuo, diluted with EtOAc, washed with deionized H$_2$O, dried over MgSO$_4$, and concentrated in vacuo. The crude product was purified in 3:2 EtOAc:Hexanes to afford 142 mg (12% over 2 steps). $^1$H NMR (400.1 MHz, DMSO): $\delta$ 8.56 (s, 1H), 8.00 (d, $J = 8.7$ Hz, 1H), 7.68 (d, $J = 9.1$ Hz, 1H), 7.62 (d, $J = 2.6$ Hz, 1H), 6.73 (d, $J = 2.3$ Hz, 1H), 4.95 (t, $J = 4.8$ Hz, 1H), 4.30 (t, $J = 3.7$ Hz, 1H), 3.74 (d, $J = 5.0$ Hz, 1H). $^{13}$C NMR (100.6 MHz, DMSO): $\delta$ 140.5, 139.1, 133.0, 127.3, 117.4, 116.1, 110.7, 103.3, 60.3, 48.8. LRMS(ESI+) calcd for C$_{10}$H$_{10}$N$_2$NaO$_3$ [M+H]$^+$ 229.1. Found 228.8.

![Absorbance wavelength = 215 nm](image)

Analytical reversed-phase HPLC profile of 5NIEthOH (50) after purification (retention time = 19.6 min).

![Structure of compound](image)
1-β-D-ribofuranosyl-5-chloroindole (51).

A solution of 1-(2’,3’,5´-tri-O-benzoyl-β-D-ribofuranosyl)-5-chloroindole (65, 82 mg, 0.14 mmoles) and manganese (IV) oxide (360 mg, 4.1 mmoles, 30 eq) in benzene (5 mL) was refluxed through a Dean Stark trap for 2 h. The reaction was then filtered though a pad of Celite and washed thoroughly with CH₂Cl₂ and concentrated in vacuo. The crude material was purified with 2% EtOAc in benzene to afford pure protected chloro-indole nucleoside 67. (58 mg, 71%) which was taken on to the next step. Protected nucleoside 1-(2’,3’,5´-tri-O-benzoyl-β-D-ribofuranosyl)-5-chloroindole (67, 34 mg, 0.05 mmoles) was partially dissolved in dry MeOH. To this stirring solution, NaOCH₃ (25 % wt in MeOH, 0.1 mL) was added and the solution went slightly yellow and clear. The reaction was quenched with the addition of NaHCO₃ (s) to pH 8 and concentrated in vacuo. The crude product was purified by column chromatography to afford pure nucleoside 51 (13 mg, 91% yield). ¹H NMR (400.1 MHz, DMSO): δ 7.68 (d, J = 3.3 Hz, 1H), 7.62 (m, 2H), 7.13 (dd, J = 8.8, J = 1.9, 1H), 6.50 (d, J = 3.2, 1H), 5.85 (d, J = 6.1 Hz, 1H), 5.31 (d, J = 6.6, 1H), 5.12 (d, J = 4.9, 1H), 4.99 (t, J = 5.3, 1H), 4.23 (m, 1H), 4.06 (m, 1H), 3.91 (m, 1H), 3.60 (m, 2H). ¹³C NMR (75 MHz, CDCl₃): δ 134.3, 129.7, 127.4, 124.2, 121.3, 119.6, 111.9, 101.8, 88.8, 84.9, 74.0, 70.2, 61.4. IR(film): 3273 cm⁻¹ C₁₃H₁₅ClN₂O₄ [M+H]⁺ 284.0684. Found 284.0690.

Analysis of 51 revealed > 97% purity:
Analytical reversed-phase HPLC profile of 5CIINDN(51) after purification (retention time = 15.8 min).

1-β-D-ribofuranosyl-5-bromoindole(52).

1-(2΄,3΄,5΄-tri-O-benzoyl- β-D-ribofuranosyl)-5-bromoindole (68, 250 mg, 0.39 mmoles) was added to a pressure tube and partially dissolved in NH₃ in MeOH (20 mL, c.a. 7N), sealed and stirred at room temperature for 16 h. The tube was then vented, and the contents concentrated in vacuo. The crude material was purified by column chromatography (5% MeOH in CH₂Cl₂) to afford 5 bromoindole nucleoside 52 (72 mg, 56.2 % yield). ¹H NMR (300.1 MHz, DMSO): 7.75 (d, \( J = 2.3 \) Hz, 1H); 7.66 (d, \( J = 4.3 \) Hz, 1H), 7.57 (d, \( J = 8.8 \), 1H), 7.24 (dd, \( J = 8.6, J = 1.7 \), 1H), 6.51 (d, \( J = 3.0 \), 1H), 5.85 (d, \( J = 6.0 \) Hz, 1H), 5.30 (m, 1H), 5.12 (m, 1H), 4.99 (m, 1H), 4.24 (m, 1H), 4.07 (m, 1H), 3.93 (m,
1H), 3.60 (m, 2H). $^{13}$C NMR (75 MHz, DMSO): $\delta$ 134.6, 130.4, 127.2, 123.8, 122.6, 112.4, 112.1, 101.7, 88.8, 84.9, 74.1, 70.2, 61.4. IR (KBr): 3357, 605 cm$^{-1}$.

HRMS(ESI+) calcd for C$_{13}$H$_{15}$BrNO$_4$ [M+H]$^+$ 328.0179. Found 328.0184.

Analysis of 52 revealed > 94% purity:

Analytical reversed-phase HPLC profile of 5BrINDN(52) after purification (retention time = 13.2 min).

1-[2,3-O-(1-methylethylidene) $\beta$-D-ribofuranosyl]-5-nitro-1H-indole (55)
5-nitroindole nucleoside 44 (100 mg, 0.34 mmoles) was prepared as previously published from our group in 3 steps\(^1\) and dissolved in dry acetone (14.0 mL) and dimethoxy propane (0.34 mL, 2.7 mmoles) and pTsOH (50 mg, 0.28 mmoles) was added and let stir 2 h. Reaction was quenched with addition of NaHCO\(_3\) (s) and poured into deionized H\(_2\)O, extracted with CH\(_2\)Cl\(_2\). dried over MgSO\(_4\), filtered and concentrated in vacuo to afford protected nucleoside 55 (92 mg, 81% yield). \(^1\)H NMR (400.1 MHz, CDCl\(_3\)): \(\delta\) 8.59 (d, \(J = 2.2\) Hz, 1H), 8.05 (dd, \(J = 2.3\) Hz, \(J = 9.1\) Hz, 2H), 7.88 (d, \(J = 3.4\) Hz), 7.83 (d, \(J = 9.1\) Hz, 1H), 6.85 (d, \(J = 3.3\) Hz, 1H), 6.23 (d, \(J = 3.4\) Hz, 1H), 5.08 (m, 2H), 4.93 (dd, \(J = 6.3\) Hz, \(J = 2.8\) Hz, 1 H), 4.19 (m, 1H), 3.52 (m, 2H), 1.59 (s, 3H), 1.33 (s, 3H), \(^1\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 141.4, 138.4, 129.5, 128.1, 117.5, 116.9, 113.6, 111.2, 105.0, 90.6, 85.1, 83.5, 80.8, 61.2, 27.0, 25.2. IR (film): 3341.5 cm\(^{-1}\). HRMS(ESI+) calcd for C\(_{16}\)H\(_{19}\)N\(_2\)NaO\(_6\) [M+Na]\(^+\) 357.1057. Found 357.1063.

\[ \text{1-\(\beta\)-D-ribofuranosyl-5-methylindole triphosphate (59)} \]

Ribonucleotide 59 was prepared by the widely-utilized “one-pot, three-step” methodology for nucleotide synthesis.\(^21\) A solution of 1-\(\beta\)-D-ribofuranosyl-5-methylindole (58, 75 mg, 0.29 mmol), Proton-Sponge (1,8-bis(dimethylamino)napthalene, 93 mg, 0.42 mmol), and trimethylphosphate (2.5
mL) was cooled to 2 °C. POCl₃ (45 μL, 0.48 mmol) was added dropwise, and the solution was stirred for 2 h. Anhydrous Bu₃N (250 μL, 1.05 mmol) was injected followed by a solution of tributylammonium pyrophosphate (280 mg) in DMF (1.1 mL). The reaction was stirred for 2 min then hydrolyzed by addition of triethylammonium bicarbonate (TEAB, 1.0 M, 5 mL). The crude reaction products were purified by preparative HPLC. The HPLC method used utilized an Aquasil C18 preparative column (19 x 150 mm, 5 μm; Thermoelectron Corp.), mobile phase method: (flow rate = 20 mL/min) linear gradient of 10% to 40% CH₃CN in TEAA buffer (0 to 20 min, 20 mM TEAA, pH 6) followed by 20% to 90% CH₃CN in TEAA buffer (20 to 30 min, 20 mM TEAA, pH 6). The purified material was concentrated in vacuo, redissolved in deionized H₂O and lyophilized to dryness to afford triphosphate 59 as the triethylammonium salt (tᵣ = 6-7 min.). This material was concentrated in vacuo, redissolved in TEAB (100 mM, 10 mL) and lyophilized to dryness Triphosphate 59 was isolated as a white oily solid (40 mg, 13% yield). ¹H NMR (360.1 MHz, D₂O): δ 7.5-7.4 (m, 3H), 7.09 (d, J = 7.9 Hz, 1H), 6.55 (m, 1H), 6.01 (d, J = 7.0 Hz, 1H), 4.44 (m, 1H), 4.22 (m, 1H), 4.14 (m, 1H), 4.08 (m, 1H), 3.08 (q, 3H, TEAA salt), 2.33 (s, 3H), 1.17 (t, 5H, TEAA salt). ¹³C NMR (90.6 MHz, D₂O): δ 134.8, 130.3, 129.2, 129.1, 125.6, 123.8, 120.5, 109.9, 103.3, 87.7, 83.1, 72.9, 70.5, 65.6, 45.5 (TEAA), 8.2 (TEAA). ³¹P NMR (145.8 MHz, D₂O): δ -9.01 (m), -10.51 (m), -22.04. HRMS (ESI-) calcd for C₁₄H₂₀NO₁₃P₃[M-H]: 502.0075. Found: 502.0069.

Analysis of 59 revealed > 90% purity:
Analytical reversed-phase HPLC profile of 5MINDN(TP) (59) after purification (retention time = 14.8 min).

1-Acetyl-5-chloroindoline (61)

Acetylindoline (2.0 g, 12.3 mmoles) was synthesized by known methods and was partially dissolved in carbon tetrachloride (50 mL) to produce a pink solution and cooled to 4 °C. Sulfuryl chloride (1.8 mL, 22.2 mmoles, 1.8 eq) was added dropwise. Solution turned to cloudy white and was diluted with CH$_2$Cl$_2$ after 1 h to dissolve product, washed with deionized H$_2$O and aqueous NaOH (20%), dried over MgSO$_4$, filtered, and concentrated in vacuo and purified by
column chromatography (100% CH₂Cl₂) to afford product (2.10 g, 87% yield). \(^1\)H NMR (400.1 MHz, CDCl₃): \(\delta 8.12\) (d, \(J = 8.0\) Hz, 1H), \(7.13\) (m, 2H) 4.04 (t, \(J = 8.5\) Hz, 2H), 3.15 (t, \(J = 8.5\) Hz, 2H) 2.20 (s, 3H). \(^1\)C NMR (100.6 MHz, CDCl₃): \(\delta 169.1, 142.0, 133.5, 128.7, 127.8, 125.0, 118.1, 49.3, 28.1, 24.5.\) LRMS(ESI+) calcd. for C₁₀H₁₁ClNO [M+H]\(^+\) 196.1. Found 195.8.

5-Chlorindoline (62)

**Method A.** Acetyl-5 chloroindoline (61 1.1 g, 5.6 mmoles) was partially dissolved in MeOH (10 mL) and aqueous NaOH (40% by wt., 10 mL) was added and allowed to reflux for 16 h.\(^{26}\) The reaction mixture turned yellow, cloudy, then clear orange. Reaction was quenched by diluting with CH₂Cl₂, washed with deionized H₂O, aqueous sat. NaCl, dried over MgSO₄, filtered, concentrated *in vacuo* to afford 5-chloroindoline (62) orange oil. Purification by column chromatography (100% toluene to 50% EtOAc in toluene) afforded desired product 62 (720 mg, 83 % yield) as a yellow-orange oil which darkens upon standing.

**Method B.** 5-Chloroindole (63 3.8 g, 25.1 mmoles) was partially dissolved in acetic acid (75 mL) and cooled to 4 °C. Sodium cyanoborohydride (4.5 g, 72.6 mmoles, 2.9 eq) was added portionwise and the solution was allowed to warm to rt.\(^{19}\) After 4 h, the reaction mixture was concentrated *in vacuo*, redissolved in CH₂Cl₂, eashed with deionized water, sat. aqueous NaCl, dried over MgSO₄,
filtered, dried over MgSO₄ and concentrated in vacuo to afford orange oil 62 which was purified by column chromatography (100% CH₂Cl₂) to afford pure 5-chloroindoline 62 (2.1 g, 51%). ¹H NMR (400.1 MHz, CDCl₃): δ 7.07 (s, 1H), 6.97 (dd, J = 8.2 Hz, J = 1.4 Hz, 1H), 6.53 (d, J = 8.2 Hz, 1H), 3.57 (t, J = 8.4 Hz, 2H), 3.02 (t, J = 8.4 Hz, 2H). ¹³C NMR (100.6 MHz, CDCl₃): δ 150.7, 131.7, 127.3, 125.2, 123.4, 110.3, 48.0, 30.2. LRMS(ESI+) calcd. for C₈H₈ClN [M+H]+ 153.6. Found 153.8.

1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)-5-chloroindoline. (65)

5-chloroindoline (62, 500 mg, 3.26 mmoles, 2.7 eq) was added to abs ethanol (10 mL) and commercially available β-D-ribofuranose-1-acetate-2,3-5 tribenzoate (24, 600 mg, 1.19 mmoles) was added and partially dissolved. To this solution, acetic acid (0.45 mL, 7.8 mmoles, 6.6 eq) was added and heated to reflux for 16 h. The reaction mixture was concentrated in vacuo and redissolved in CH₂Cl₂, washed with sat. aqueous NaHCO₃, deionized water, sat. aqueous NaCl, dried over MgSO₄, filtered, concentrated in vacuo to afford syrup which was purified in 2% EtOAC in benzene to afford protected indoline ribonucleoside 65 (120 mg, 17% yield) ¹H NMR (300.1 MHz, CDCl₃): δ 8.12 (m, 2H), 7.99 (m, 4H), 7.60-7.35 (m, 9H), 7.02 (m, 2H), 6.69 (d, J = 8.9 Hz, 1H), 5.89 (m, 2H), 5.80 (m, 1H), 4.73 (m, 1H), 4.55 (m, 2H), 3.63 (m, 2H), 2.93 (d, 2H). ¹³C NMR (75
136 MHz, CDCl₃): δ 166.4, 165.7, 165.5, 148.5, 133.8, 133.7, 133.5, 132.4, 130.06, 129.98, 129.91, 129.8, 129.3, 129.2, 128.8, 128.7, 128.6, 127.2, 125.3, 124.4, 109.0, 88.5, 78.6, 72.2, 70.9, 64.6, 46.0, 28.2. IR(film): 1727 cm⁻¹. HRMS(ESI+): calcd. for C₃₈H₂₈ClNO₇ [M+H]⁺ 598.1627. Found 598.1633.

1-(2΄,3΄,5΄-tri-O-benzoyl-β-D-ribofuranosyl)-5-bromoindoline (66).

5-bromolindoline (65, 3.0 g, 15.1 mmoles) was dissolved in abs ethanol (100 mL) followed by commercially available β-D-ribofuranose-1-acetate-2,3-5 tribenzoate 24 (7.5 g, 14.18 mmoles). To this stirring solution, acetic acid (30 mL) was added and refluxed for 16 h. The crude reaction mixture was concentrated in vacuo and redissolved in CHCl₃, washed with deionized water and sat. aqueous NaCl, dried over MgSO₄, filtered, concentrated in vacuo to afford crude product 66 as a foam. Purification by column chromatography (2% EtOAc in benzene afforded protected indoline nucleoside 66 (2.9 g, 31% yield) as a foam.¹H NMR (300.1 MHz, CDCl₃): 8.12 (m, 2H), 7.99 (m, 4H), 7.60-7.35 (m, 9H), 7.17 (m, 2H), 6.66, (d, J = 8.9 Hz, 1H), 5.92-5.77 (m, 3H), 4.70 (m, 1H), 4.6 (m, 2H), 3.64 (m, 2H), 2.94 (m, 2H). ¹³C NMR (75 MHz, CDCl₃): 166.2, 165.6, 165.4, 148.9, 133.7, 133.6, 133.4, 133.2, 132.8, 130.02, 129.96, 129.9, 129.8, 129.7, 129.2, 129.1, 128.8, 128.7, 128.6, 128.5, 128.0, 111.4, 109.5, 88.3, 81.0, 72.1, 70.8, 64.5,
45.9, 28.0. IR (film): 1728, 709, cm$^{-1}$. HRMS(ESI$^+$) calcd for C$_{34}$H$_{29}$BrNO$_7$ [M+H]$^+$
642.1122. Found 642.1127.

1-(2΄,3΄,5΄-tri-O-benzoyl- β-D-ribofuranosyl)-5-bromoindole (68)

A solution of 1-(2΄,3΄,5΄-tri-O-benzoyl- β-D-ribofuranosyl)-5-bromoindoline (66, 1.0 g, 1.56 mmoles) and manganese (IV) oxide (6.0 g, 69 mmoles, 44 eq) in benzene (15 mL) was refluxed through a Dean Stark trap for 16 h. The reaction was then filtered though a pad of Celite and washed thoroughly with CH$_2$Cl$_2$ and concentrated in vacuo. The crude material was purified with 2% EtOAc in benzene to afford pure protected bromo-indole nucleoside 68. (500 mg, 50%). $^1$H NMR (300.1 MHz, CDCl$_3$): 8.18 (m, 2H), 8.01 (m, 4H), 7.75-7.24 (m, 14H), 6.52 (d, $J$ = 3.2, 1H), 6.46 (d, $J$ = 5.0, 1H), 6.04 (m, 2H), 4.94-4.73 (m, 3H). $^{13}$C NMR (75 MHz, CDCl$_3$): 166.3, 165.5, 165.2, 134.8, 133.8, 133.6, 131.2, 129.9, 129.5, 128.9, 128.8, 128.6, 125.5, 125.3, 123.9, 114.0, 111.6, 103.9, 87.7, 80.0, 74.1, 71.5, 63.9. IR (film): 1726, 685 cm$^{-1}$. HRMS(ESI$^+$) calcd for C$_{34}$H$_{27}$BrNO$_7$ [M+H]$^+$
640.0965. Found 640.0971.
3.6 References


20. Harki, D. A.; Graci, J. D.; Galarraga, J. E.; Chain, W. J.; Cameron, C. E.; Peterson, B. R., Synthesis and antiviral activity of 5-substituted cytidine


Chapter 4
Ribosylation Strategy to Access Indole Nucleosides

4.1 Overview

Nucleoside analogs have occupied a central role in medicine and biomedical research. Not only do nucleosides comprise a substantial class of antiviral and anticancer agents, they also have found utility in numerous applications such as gene therapy, biochemical probes, and modern DNA sequencing. Therefore, the development of better methods to synthesize nucleosides is of universal value. Known methods of nucleoside synthesis consist mainly of key glycosylation strategies, however, such methods are plagued by substrate specificity, limiting access to purine and indole nucleosides. Few commonly used glycosylation agents exist for nucleoside coupling reactions. Therefore, by altering the electronic substituents on ribose protecting groups, we designed a series of ribosylation agents designed to improve access to biologically active indole nucleosides. Additionally, there was a decrease in an undesired orthoamide byproduct and an increase in the yield of the nucleoside,
indicating that the methodology may be broadly applicable to nucleoside synthesis.

4.2 Nucleoside Coupling Reactions

Currently, there are three major classes of nucleoside-forming reactions, namely, 1) the Fusion reaction, 2) the Metal Salt Procedure and the Vorbrüggen Reaction. (Figure 4-1)\textsuperscript{106} The Fusion reaction involves melting the peracylated sugar of interest and a heterocyclic nucleobase in the presence of a lewis acid catalyst at high temperatures\textsuperscript{107} The metal salt procedure entails reaction of sodium, silver or mercury salt of the heterocyclic base with a chloroglycoside.\textsuperscript{108} The most common coupling reaction is the Vorbrüggen procedure where a persilylated heterocyclic base is reacted with a peracylated sugar with a Lewis acid catalyst.\textsuperscript{65} Whereas this procedure has found widespread application, synthesis of purines and indole analogs by this route remains challenging. Additionally, the indoline-indole method pioneered by Preobrazhenskaya\textsuperscript{103} and Walton\textsuperscript{104} has been developed for synthesis of indole nucleosides which suffer from low glycosylation yields. Although this route typically involves an additional one or two steps compared to a direct glycosylation, often the increased nucleophilicity of the indoline nucleobase enables a successful coupling reaction compared to the indole. To circumvent this issue, a direct glycosylation onto a
deoxy-chlorosugar with subsequent conversion of the deoxyribonucleoside to the ribonucleoside has been explored, however, this approach is lengthy and demonstrates poor yield for many indoles of interest.

Figure 4-1: Nucleoside coupling strategies.
Currently, the most common glycosylation agents for use in nucleoside synthesis are listed in Figure 4-2. Peracetylated sugar $73^{110}$ has found significant application in melt fusion reactions because of its low melting temperature. Glycosyl halides $76^{111}, 25^{112}, 22^{113}$ and $38^{114}$ are optimal coupling partners for metal salt glycosylation. Additionally, $\beta$-1-O-acetyl-2,3,5-O-benzoyl ribofuranose (24) is commercially available and is by far the most common ribosylation agent in use today due to the stability of the benzoyl protecting group.

Additionally, the stereoselectivity of the nucleoside coupling reaction is highly dependent on the sugar moiety utilized. For example, using $\alpha$-chloroglycoside 22 or 38 affords the desired $\beta$-configuration due to Walden inversion on the C1 of ribose ring. However, the use of $73, 76^{111}, 25^{112}$, and $24^{114}$ will afford the desired $\beta$-nucleoside due to the anchimeric assistance of the C2′ benzoyl which stabilizes the oxocarbenium intermediate.

![Chemical structures](image)

Figure 4-2: Common ribosylation agents.
4.3 Undesired Side Product from Nucleoside Coupling Reactions

In our efforts to design novel hydrophobic universal ribonucleosides as antiviral agents, a major drawback has been developing synthetic routes that allow production of sufficient material for biological testing and mechanism of action studies. In a previous paper from the Peterson and Cameron laboratories,\(^8^5\) 3-nitropyrrrole ribonucleoside triphosphate (3NPNTP, \(\text{11}\)) was synthesized and analyzed as a lethal mutagen. However, the coupling strategy suffered from poor yields due to the formation of significant amounts of an unwanted orthoamide byproduct that was formed presumably by attack of the 3-nitropyrrrole sodium salt onto the stabilized carbocation intermediate as depicted in Figure 4-3.\(^{11^5}\) The chemical structure was confirmed by x-ray crystal structure. Additionally, other coupling strategies described throughout this work also uniformly suffered from decreased yield due to this side reaction.\(^{11^6, 11^7}\)
Because antiviral mechanistic studies warrant large quantities of nucleoside of interest, we wondered if we could limit the side reaction that produced the orthoamide by-product. Since the carbocation intermediate 77 is partially stabilized by resonance through the benzoyl protecting group, we hypothesized that the use of even greater electron releasing substituents on the benzoyl protecting group might allow greater stabilization and increased yield.

Figure 4-3: Orthoamide by-products in nucleoside couplings.

4.4 Tuning the Electronic-Releasing Properties of Protected Ribosylation Agents to Increase Access to Indole Ribonucleosides
Although optimization of glycosylation yields via altered protecting group strategies has been well developed and investigated in hexopyranoside literature,\textsuperscript{118} application of this concept to ribofuranosides has been limited. Therefore, we investigated the use of \(p\)-methoxy benzoyl and \(p\)-toluyl protecting groups and compared these to commercially available benzoyl sugar 24. As a negative control donor, we also designed a \(p\)-nitro ribosylation agent bearing an electron-withdrawing group.

4.2 Synthesis of Ribosylation Agents

To test this hypothesis, electron donating benzoylated ribosylating agents 84\textsuperscript{119} and 85\textsuperscript{120} as well as electron withdrawing benzoylated derivative 86\textsuperscript{121, 122} were synthesized by previously reported procedures. In brief, methyl ribose was protected by substituted benzoyl chloride to afford protected 1-\(O\)-methylated sugars (81-83) which were converted to their respective 1-\(O\)-acetyl-ribosylating agents (84-86).
4.5. Optimized Yields of Indole Glycosylations with Electron Donating Protecting Groups

Indoline was coupled to these ribosylating agents to examine the role of electron releasing and withdrawing groups on the benzoyl protecting groups of the ribose. Standard indoline-indole conditions were utilized, namely, refluxing in ethanol containing acetic acid for 6.5 h\textsuperscript{103} with the sugar of interest. As shown in Figure 4-5, the coupling yield increased significantly from 33 to 75\% yield when p-methoxybenzoyl glycosyl donor \textit{84} was utilized. Additionally, even the slight electron releasing ability of the methyl subsituent in the case of the p-toluyl agent \textit{85} afforded an increase in yields to 60\%. Control glycosylation agent \textit{86} only provided 20\% of the coupled indoline \textit{89} and also followed the overall trend correlating electron donating ability with increased yield, presumably due to
decreased orthoamide by-product. A potentially more electron releasing donor, namely, dimethyl amino benzoyl protected sugar was also coupled. However, the yield was poor due to apparent solubility in the reaction solvent used. Also, a \( p \)-chloro benzoyl ribosylation agent was synthesized and evaluated as a glycosyl donor and afforded a poor yield as expected. However, the resulting indoline adduct was extremely difficult to isolate and characterize. In addition to indoline, 5-chloroindoline was coupled utilizing this methodology and produced 60% yield of 67 demonstrating this methodology is useful for substituted indolines.

![Figure 4-5: Comparison of glycosylation yields with altered benzoyl protecting groups.](image-url)
4.6 Preliminary Characterization of the Ratio of Desired Nucleoside to Undesired By-Product by Reversed Phase HPLC

Encouraged by the increased yield of indoline coupling, we were intrigued to confirm whether the increased yield was attributable to decreased orthoamide byproduct. A preliminary investigation was conducted with the caveat that indoline-ribose adducts are unstable and difficult to purify and characterize, especially the indoline orthoamide. Therefore, crude reaction mixtures were quantified by reversed-phase HPLC. Figure 4-6 demonstrates a clear relationship between electron donation of the benzoyl substituent and favorable ratio for formation of desired indoline ribonucleoside. The best ratio is exhibited by the most electron releasing donor 84 which provides a 77:23 ratio of desired to undesired orthoamide. For the control glycosylation agent, a reversal of selectivity (40:60) is seen as the undesired orthoamide is produced at a higher amount than desired nucleoside.
4.7 Ribosylation Agents Provide Increased Overall Yields to Indole Nucleosides

To demonstrate the utility of this approach to access biologically active indole nucleosides, this methodology was compared to existing methodology to access INDN (69) and antiviral agent 5CIINDN (51). Namely, the indoline was coupled, oxidized to indole ribonucleoside and deprotected to demonstrate the ease of oxidation and deprotection of the $p$-methoxy benzoyl protecting group. As shown in Figure 4-6 the overall yields from indoline increased from 11.6% to 26.6% for INDN (69) and from 11.0% to 33.4% for 5CIINDN (51), an increase of 2.3 and 3.0-fold in overall yield over 3 steps, respectively.

Figure 4-6: Quantitation of ratios of Indoline-ribosylation adducts by reversed-phase HPLC.

<table>
<thead>
<tr>
<th>R</th>
<th>Ratio of desired to undesired products by HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMe</td>
<td>77 : 23</td>
</tr>
<tr>
<td>Me</td>
<td>70 : 30</td>
</tr>
<tr>
<td>H</td>
<td>61 : 38</td>
</tr>
<tr>
<td>NO₂</td>
<td>40 : 60</td>
</tr>
</tbody>
</table>
Optimization of nucleoside coupling methodologies is paramount to the development of nucleosides for a variety of applications. Indole nucleosides have

\[ R = \text{H}, \quad R_1 = \text{H} \quad 75 \]
\[ R = \text{H}, \quad R_1 = \text{Cl} \quad 65 \]
\[ R = \text{OCH}_3, \quad R_1 = \text{H} \quad 87 \]
\[ R = \text{OCH}_3, \quad R_1 = \text{Cl} \quad 90 \]

**Figure 4-7:** Glycosylation strategy increases overall yield to INDN (69) and 5CIINDN (51).

4.8 Conclusions and Future Directions

Optimization of nucleoside coupling methodologies is paramount to the development of nucleosides for a variety of applications. Indole nucleosides have
attracted interest in recent years and are of particular interest in our ongoing project toward the design of antiviral nucleosides. Our key observation of a significant orthoamide byproduct present in many of our coupling reactions has led to the development of ribosylation agents with altered protecting groups to tune the electronics of the ribose and increase overall yield. We have shown that two ribosylation agents 84 and 85 are efficient ribosyl donors with increased yield with indolines compared to commercially available 24. Future work should investigate these tuned ribosylation agents in the Vorbrüggen procedure and other standard nucleoside coupling reactions.

4.9 Experimental Section

4.9.1 Determination of Ratios of Desired Nucleoside to Orthoamide by Reversed-Phase HPLC

The HPLC gradient for analysis for orthoamide formation was 1 to 99% CH₃CN (containing 0.1% trifluoroacetic acid [TFA]) in double distilled water (ddH₂O, 0.1% TFA, 0 to 30 min) using a Hamilton PRP-1 (polystyrene-divinylbenzene) reversed-phase column (7 µM particle size, 250 mm x 4.1 cm). Flow rate= 0.8 mL/min. Injection volumes were 10 µL and the injection port was fitted with a 20 µl injection loop. The desired protected nucleosides were less polar and eluted between tₛ = 28-33 min. The orthoamides eluted earlier tₛ = 17-
19 min. The reactions were monitored for the disappearance of the starting material ribosylation agents which was of intermediate polarity (t_R = 20-23 min. Peak areas were obtained by standard integration of the appropriate peaks by the ChemStation for LC 3D software (Rev. A.09.03, Agilent Technologies).

4.9.2 General Synthesis Information.

Reagents and solvents were obtained from Aldrich, Acros, TCI, and Fisher and were used without further purification unless otherwise noted. Reactions were performed under an atmosphere of dry nitrogen. Methanol was dried using a solvent purification system and used immediately. Thin-layer chromatography was performed on glass-backed silica gel-coated plates with silica gel 60 F_{254} (EM Science, 0.25 mm thick). ICN SiliTech Silica Gel (32-63 μm) was employed in column chromatography. TLC plates were visualized by UV irradiation (254 nm) or stained with a solution of methanol, sulfuric acid, and p-anisaldehyde (18:2:1). Infrared spectra were obtained with a Mattson GL-4020 Series FTIR. Bruker DRX 400, DPX 300, CDPX 300, and AMX 360 spectrometers were employed in nuclear magnetic resonance (NMR) spectroscopy. NMR peaks are reported as parts per million (ppm) and are referenced to internal CHCl_3 or DMSO. Mass spectral data was obtained from The Pennsylvania State
University Mass Spectrometry Facility (ESI). Melting points were determined on a Thomas Hoover apparatus and are reported uncorrected. Internal solvent peaks were referenced in each case.

1-[2,3,5-tris-O-(4-methoxybenzoyl) β-D-ribofuranosyl]indoline (87)

A solution of 1-O-acetyl-2,3,5-tris-O-(4-methoxybenzoyl) β-D-ribofuranoside (84, 83 mg, 0.14 mmol) and indoline (50 μL, 0.45 mmoles, 3.1 eq) in abs. EtOH (1.0 mL) containing glacial AcOH (50 μL) was refluxed for 6.5 hr. The reaction was poured into sat. aqueous NaHCO₃ then diluted in CH₂Cl₂ (5 mL). The organic layer was washed with deionized H₂O, sat. aqueous NaCl, dried over MgSO₄, filtered, concentrated in vacuo to afford syrup which was purified in 2 % EtOAC in hexanes to afford protected indoline ribonucleoside 87 (68 mg, 75% yield) as a white foam. $^1$H NMR (400.1 MHz, CDCl₃): δ 8.09 (d, $J = 8.9$ Hz, 2H), 7.95 (m, 2H), 7.07 (d, $J = 7.5$ Hz, 2H), 6.97 (d, $J = 8.9$ Hz, 2H), 6.90 (d, $J = 7.9$ Hz, 2H), 6.83 (d, $J = 8.0$ Hz, 2H), 6.80 (d, $J = 7.8$ Hz, 1H), 6.74 (t, $J =
7.1 Hz, 1H), 5.91-5.89 (m, 2 H), 5.78-5.76 (m, 1H), 4.68 (d, $J = 11.3$ Hz, 1H), 4.55-2.52 (m, 2H), 3.90-3.83 (m, 9H), 3.63 (t, $J = 8.5$ Hz, 2H), 2.98 (t, $J = 9.1$ Hz, 2H). $^{13}$C NMR (100.6 MHz, CDCl$_3$): $\delta$ 166.4, 165.7, 165.5, 164.23, 164.18, 164.1, 132.4, 132.34, 132.27, 131.1, 130.8, 127.7, 125.4, 122.5, 122.1, 121.9, 120.0, 114.5, 114.3, 114.2, 114.1, 108.3, 88.5, 82.4, 78.9, 72.4, 70.8, 64.8, 55.9, 45.9, 28.6. IR(film): 1643 cm$^{-1}$ C$_{37}$H$_{36}$NO$_{10}$ [M+H]$^+$ 654.2234. Found 654.2239.

5-chloro-1-[2,3,5-tris-O-(4-methoxybenzoyl) $\beta$-D-ribofuranosyl] $\beta$-D-indoline (90)

A solution of 1-O-acetyl-2,3,5-tris-O-(4-methoxybenzoyl) $\beta$-D-ribofuranoside (84, 144 mg, 0.24 mmol) and 5-chloroindoline 62 (115, 0.75 mmole, 3.1 eq) in abs. EtOH (2.0 mL) containing glacial AcOH (100 $\mu$L) was refluxed for 6.5 hr. The reaction was poured into sat. aqueous NaHCO$_3$ then diluted in CH$_2$Cl$_2$ (5 mL). The organic layer was washed with deionized H$_2$O, sat. aqueous NaCl, dried over MgSO$_4$, filtered, concentrated in vacuo to afford syrup which was purified in 10 % EtOAC in hexanes to afford protected indoline ribonucleoside 90 (144 mg, 67% yield) as a white foam. $^1$H NMR (400.1 MHz,
CDCl₃): δ 8.10 (d, J = 8.8 Hz, 2H), 7.97 (dd, J = 8.8 Hz, J = 11.2 Hz, 4H), 7.38 (s, 1H), 7.07-6.85 (m, 6H), 6.70 (d, J = 9.0 Hz, 1H), 6.54 (d, J = 8.2 Hz, 1H), 5.88-5.75 (m, 2H), 4.68-4.53 (m, 3H), 3.94-3.81 (m, 9H), 3.68-3.54 (m, 2H), 3.05-2.93 (m, 2H). ¹³C NMR (75.5 MHz, CDCl₃): δ 166.3, 165.6, 165.4, 164.21, 164.18, 164.0, 150.7, 148.9, 132.7, 132.4, 132.2, 131.7, 128.8, 127.4, 127.3, 125.5, 125.2, 124.4, 123.4, 122.3, 121.9, 121.7, 114.3, 114.2, 114.1, 110.3, 109.2, 79.0, 72.3, 70.8, 64.6, 55.9, 48.0, 30.2. IR(film) 1643 cm⁻¹. C₃₇H₃₄ClNO₁₀ [M+H]+ 688.1944. Found 688.1949.

1-[2,3,5-tris-O-(4-methylbenzoyl) β-D-ribofuranosyl]indoline (88)

A solution of 1-O-acetyl-2,3,5-tris-O-(4-methylbenzoyl) β-D-ribofuranoside (85, 300 mg, 0.55 mmol) and indoline (0.15 mL, 1.33 mmoles, 2.4 eq) in abs. EtOH (5.0 mL) containing glacial AcOH (0.19 mL) was refluxed for 6.5 hr as the ribosylating agent went into solution at higher temperatures. The reaction was concentrated in vacuo and then redissolved in CHCl₃ (5 mL). The organic layer was washed with saturated NaHCO₃, sat. aqueous NaCl, dried over MgSO₄, filtered, concentrated in vacuo to afford syrup which was purified in 3%
EtOAC in benzene to afford protected indoline ribonucleoside 88 (199 mg, 60% yield) as an off-yellow foam, $^1$H NMR (300.1 MHz, CDCl$_3$): $\delta$ 8.04 (d, $J = 8.1$ Hz, 2H), 7.90 (dd, $J = 9.9$ Hz, $J = 8.3$ Hz, 4H), 7.31-7.17 (m, 7H), 7.08 (d, $J = 7.4$ Hz, 2H), 6.81 (d, $J = 7.7$ Hz, 1H). 6.75 (t, $J = 7.2$ Hz, 1H), 5.94-5.93 (m, 2H), 5.80 (m, 1H), 4.73-4.69 (m, 1H), 4.58-4.56 (m, 2H), 3.66-3.62 (m, 2H), 3.00-2.96 (m, 2H), 2.44-2.41 (m, 9H). $^{13}$C NMR (100.6 MHz, CDCl$_3$): $\delta$ 166.8, 166.0, 165.9, 150.1, 144.7, 144.6, 144.4, 130.8, 130.4, 130.3, 130.2, 129.8, 129.7, 129.6, 127.7, 127.4, 127.0, 126.9, 125.4, 120.0, 88.6, 78.9, 72.5, 71.0, 64.9, 45.9, 28.6, 22.2 IR(film): 1643 cm$^{-1}$. C$_{37}$H$_{36}$NO$_7$ [M+H]$^+$ 606.2486. Found 606.2492.

1-[2,3,5-tris-O-(4-nitrobenzoyl) β-D-ribofuranosyl]indoline (89)

A solution of 1-O-acetyl-2,3,5-tris-O-(4-nitrobenzoyl) β-D-ribofuranoside (86, 679 mg, 1.1 mmol) and indoline (0.30 mL, 2.67 mmoles, 2.4 eq) in abs. EtOH (10 mL) containing glacial AcOH (0.40 mL) was refluxed for 6.5 h as the solution turned red. The reaction was concentrated in vacuo, then redissolved in CH$_2$Cl$_2$ (5 mL). The organic layer was washed with saturated NaHCO$_3$, deionized H$_2$O, sat. aqueous NaCl, dried over MgSO$_4$, filtered, concentrated in vacuo to
afford syrup which was purified in 10 to 20% EtOAC in hexanes to afford protected indoline ribonucleoside 89 (199 mg, 24% yield) as a white foam. $^1$H NMR (300.1 MHz, CDCl$_3$): $\delta$ 8.73-8.15 (m, 10H), 7.11 (d, (d, $J = 6.1$ Hz, 2H), 6.84-6.78 (m, 2H), 6.00-5.85 (m, 3H), 4.80-4.62 (m, 3H), 3.66-3.59 (m, 2H), 3.03 (m, 2H). $^{13}$C NMR (100.6 MHz, CDCl$_3$): $\delta$ 164.7, 164.14, 164.09, 151.54, 151.49, 151.3, 149.5, 135.2, 134.5, 134.4, 131.4, 131.32, 131.26, 131.7, 127.8, 125.6, 124.33, 124.27, 124.21, 120.7, 88.4, 78.3, 73.0, 71.6, 65.5, 45.9, 28.6. IR(film): 1642 cm$^{-1}$. C$_{35}$H$_{26}$N$_4$O$_{13}$ [M+H]$^+$ 699.1569. Found 699.1575.

![Chemical Structure](image)

1-O-acetyl-2,3,5-tris-O-(4-methoxybenzoyl) β-D-ribofuranoside (84)

Methyl-D-ribose (3.3 g, 20.0 mmoles) was dissolved in distilled pyridine (10.0 mL) and cooled to 4 °C. To this stirring solution, $p$-Anisoyl chloride (9.0 mL, 63.3 mmoles, 3.2 eq)) was added dropwise with the solution turning cloudy while warming to room temperature. After 16 h, reaction was quenched with addition of deionized water (1.0 mL), concentrated in vacuo, washed with sat. aqueous NH$_4$Cl, deionized H$_2$O, sat aqueous NaCl, dried over MgSO$_4$, filtered and concentrated in vacuo to afford white solid (7.0 g). This crude product (3.0 g) was taken onto the next step by dissolving in glacial acetic acid (24 mL) and
acetic anhydride (10 mL). The reaction mixture was cooled to 4 °C, and sulfuric acid (3.8 mL) was added dropwise. Reaction was allowed to warm to room temperature. After 2 h, reaction was quenched with addition of ice cold sat aqueous NaHCO₃ and let stir 15 min. Reaction mixture was extracted with EtOAc, washed again with sat aqueous NaHCO₃, deionized H₂O, dried over MgSO₄, filtered and concentrated in vacuo to afford foam. Purification by column chromatography (10% EtOAc in Hexanes) yielded 84 (500 mg, 10% yield). ¹H NMR (400.1 MHz, CDCl₃): δ 8.04-7.96 (m, 6H), 6.92 (m, 6H), 6.42 (s, 1H), 5.87-5.75 (m, 2H), 4.75-4.72 (m, 3H), 3.88-3.83 (m, 9H), 2.10-2.0 (m, 3H). ¹³C NMR (100.6 MHz, CDCl₃): δ 169.6, 169.1, 165.7, 165.0, 165.0, 164.7, 164.6, 163.8, 163.8, 163.6, 163.5, 131.9, 131.82, 131.77, 131.7, 122.0, 121.7, 121.7, 121.1, 121.1, 121.0, 113.8, 113.8, 113.71, 113.69, 113.62, 113.58, 98.5, 94.4, 82.3, 80.0, 74.7, 71.1, 70.9, 70.5, 63.7, 63.5, 55.43, 55.41, 55.39, 21.0, 20.9. LRMS(ESI)C₃₁H₃₀NaO₁₀ [M+Na]^+ 617.2. Found 616.8.

![Structure](image)

1-O-acetyl-2,3,5-tris-O-(4-methylbenzoyl) β-D-ribofuranoside (85)

Methyl-D-ribose (500 mg, 3.05 mmoles) was dissolved in distilled pyridine (8.0 mL) and cooled to 4 °C. To this stirring solution, p-Toluoyl chloride (2.0 mL,
15.0 mmoles, 5 eq)) was added dropwise with the solution turning light pink while warming to room temperature. After 16 h, reaction was quenched with addition of deionized water (1.0 mL), concentrated in vacuo, washed with sat. aqueous NH₄Cl, deionized H₂O, sat aqueous NaCl, dried over MgSO₄, filtered and concentrated in vacuo to afford clear syrup. Purification by column chromatography (10% EtOAc in Hexanes) yielded 82 (1.10 g, 70% yield). Tituration with EtOAc afforded white foam. This product was taken onto the next step by dissolving in glacial acetic acid (23 mL) and acetic anhydride (10 mL). The reaction mixture was cooled to 4 °C, and sulfuric acid (1.5 mL) was added dropwise. Reaction was allowed to warm to room temperature. After 26 h, reaction was quenched with addition of ice cold sat aqueous NaHCO₃ and let stir 15 min. Reaction mixture was extracted with EtOAc, washed again with sat aqueous NaHCO₃, deionized H₂O, dried over MgSO₄, filtered and concentrated in vacuo to afford foam. Purification by column chromatography (20% EtOAc in Hexanes) yielded 85 (1.10 g, 96% yield). ¹H NMR (400.1 MHz, CDCl₃): δ 8.02 (d, J = 7.9 Hz, 2H), 7.95 (d, J = 7.9 Hz, 2H), 7.84 (d, J = 7.4 Hz, 2H), 7.32-7.18 (m, 6H), 6.48 (s, 1H), 5.95 (t, = 6.3 Hz, 1H), 5.82 (d, J = 4.6 Hz, 1H), 4.83-4.80 (m, 2H), 4.56 (m, 1H), 2.27-2.42 (m, 9H), 2.07 (s, 3H). ¹³C NMR (100.6 MHz, CDCl₃): δ 169.1, 165.0, 166.x, 166.x, 165.0, 144.4, 144.3, 143.9, 129.8, 129.7, 129.2, 129.0, 126.8, 126.1, 126.0, 98.4, 80.0, 74.8, 71.1, 63.6, 21.7, 21.6, 20.9. LRMS(ESI)C₃₁H₃₀NaO₉ [M+Na]⁺ 569.2. Found 569.8.
1-O-acetyl-2,3,5-tris-O-(4-nitrobenzoyl) β-D-ribofuranoside (86)

Methyl-D-ribose (2.3, 14.0 mmoles) was dissolved in distilled pyridine (15.0 mL) to afford a yellow solution. To this stirring solution, p-nitrobenzoyl chloride (8.1 g 43.6 mmoles, 7.1 eq)) was added with the solution turning brown and cloudy. After 16 h, reaction was quenched by concentration in vacuo, and dissolved in HCl (aq, 5%) and EtOAc. The solution was extracted with EtOAc, washed with deionized H2O, sat aqueous NaCl, dried over MgSO4, filtered and concentrated in vacuo to afford crude white foam. Purification by column chromatography (100% CH2Cl2) yielded 81 (7.29 g, 85% yield). This product was taken onto the next step by dissolving in glacial acetic acid (150 mL) and acetic anhydride (30 mL). The reaction mixture was cooled to 4 °C, and sulfuric acid (15 mL) was added dropwise. Reaction was allowed to warm to room temperature and turned crystalline. After 16 h, reaction was quenched by carefully pouring into sat aqueous NaHCO3 and let stir 30 min. Reaction mixture was extracted with EtOAc, washed with deionized H2O, dried over MgSO4, filtered and concentrated in vacuo to afford foam. Purification by column chromatography (100% CH2Cl2)
yielded 86 (5.0 g, 66% yield). $^1$H NMR (400.1 MHz, CDCl$_3$): $\delta$ 8.32-8.04 (m, 12H), (6.72(d, J = 4.5 Hz), 6.49 (s), 1H), 5.93-5.86 (m, 1H), 5.83-5.67 (m, 1H), 4.89-4.77 (m, 2H), 4.69-4.59 (m, 1H), 2.19-2.09 (m, 3H). $^1$H NMR (100.6 MHz, CDCl$_3$): $\delta$ 169.7, 169.2, 164.6, 164.5, 164.2, 164.0, 163.7, 163.7, 151.5, 151.44, 151.38, 151.2, 151.2, 135.2, 135.0, 134.6, 134.1, 134.0, 131.3, 131.3, 131.3, 131.2, 131.1, 124.3, 124.2, 124.16, 124.06, 98.5, 94.4, 82.3, 80.0, 76.0, 72.4, 71.9, 71.7, 65.1, 64.9, 21.5, 21.4. LRMS(ESI)C$_{28}$H$_{21}$N$_3$NaO$_{15}$ [M+Na]$^+$ 662.1. Found 661.7.
4.10 References


Chapter 5

Detection of Intracellular Phosphorylation of Nucleoside Analogs

5.1 Overview

Nucleoside analogs developed as antiviral and anticancer agents require intracellular phosphorylation for biological activity. Conversion to nucleoside 5’-triphosphates necessitates recognition of modified nucleosides by cellular kinases. Mechanism of action studies of nucleoside analogs call for evaluation of phosphorylation efficiency by modern analytical methods. This chapter describes the application and optimization of reversed phase HPLC methods to detect phosphorylation of nucleosides. The lack of antiviral activity for nucleoside P (91) is attributed to undetectable conversion to the 5’ triphosphate. Additionally, purine analog JA28 (92), is a lethal mutagen which is efficiently phosphorylated to JA28 (TP) (100). Structural comparisons between phosphorylated and non-phosphorylated nucleosides reveal insights into the future design of lethal mutagens.
5.2 Intracellular Phosphorylation of Nucleoside Analogs

Most clinically utilized nucleoside analogs are prodrugs that require intracellular metabolism to active 5‘-phosphorylated species.\(^1\) Stepwise phosphorylation is mediated by cellular nucleoside kinases and is sensitive to cell type,\(^2\) ratio of modified nucleoside triphosphate to natural triphosphate,\(^3\) cell cycle,\(^4\) and activation status.\(^5\) Natural nucleoside phosphorylation pathways are shown in Figure 5-1 and are highly regulated systems.
Non-natural nucleoside analogs follow cellular phosphorylation pathways. For ribonucleoside analogs, the first phosphorylation step is mediated by ribonucleoside kinases (rNKS) such as adenosine kinase (ADK) and uridine-cytidine kinase (UCK 1 and 2) which are irreversible reactions. ADK and UCK2 have a cytosolic subcellular localization. ADK is known to have broad substrate specificity tolerant of both sugar and base modifications. However, the
monophosphates can be dephosphorylated by their parent nucleosides by 5′-nucleotidases (5′NTs). The second phosphorylation step is reversible and mediated by nucleoside monophosphate kinases (NMPKs). This family includes UMP-CMP kinase (UMP-CMPK), 5-isozymes of adenylate kinase (AK) and several guanylate kinases (GUK). The last phosphorylation step is mediated by the nucleoside diphosphate kinases (NDPK) for which there are 8 isozymes in humans (Figure 5-1).

The rate limiting step for nucleoside analog phosphorylation is usually monophosphorylation; however, studies of antiretrovirals have demonstrated marked differences in flux though kinase pathways. For example, for AZT, diphosphorylation is rate limiting compared to d4T, which exhibits monophosphorylation as the rate determining step, although both are substrates for the same enzymes.

5.2 Development of an HPLC Method to Analyze Intracellular Phosphorylation

Numerous methods have been developed to analyze nucleoside phosphorylation including isocratic-HPLC-radioimmunoassay (RIA), cartridge-RIA, LCMS-MS and conventional HPLC methods. Although the use of radiolabeled nucleoside has become routine in pharmaceutical companies, synthesis of the desired radiolabel can be cost-prohibitive and impractical on large scale. LCMS techniques were explored; however, the experiments with volatile buffers suffered from poor peak shape and separation. Therefore, since
non-volatile buffers were LCMS incompatible, we investigated conventional HPLC methods. Ion-exchange chromatography was explored; however, reversed-phase C18 columns demonstrated improved retention for base-modified ribonucleosides.

Evaluation of nucleoside phosphorylation involves extraction of nucleotides and analysis by reversed-phase HPLC. The nucleoside of interest was incubated with HeLa cells for 2-3 hours to allow accumulation of the nucleoside 5’-phosphate, and cells were subsequently trypsinized, pelleted and resuspended in aqueous trichlororacetic acid (TCA). At 4 °C, the supernatant was collected and tri-n-octylamine in trichlorofluorethane was added. The solution was vortexed and centrifuged. The resulting aqueous layer was

![Figure 5-2: Schematic representation of method to detect nucleoside phosphorylation by HPLC.](image)
analyzed by reversed-phase HPLC. In addition, extractions with ethanol, methanol, water and dimethylsulfoxide were also attempted and examined as extraction reagents.

Each nucleoside analyzed required a unique HPLC method. Challenges to HPLC method development for nucleoside 5´- triphosphate are numerous. Specifically, the intracellular ATP concentration is significant, and it is challenging to distinguish micromolar concentrations of base-modified nucleoside from endogenous nucleotides due to poor retention of polar, highly charged nucleotides. Fortunately, polar-end capped HPLC columns have been developed for such difficult separations. Additionally, base-modified ribonucleoside 5´-triphosphates are temperature sensitive and decompose readily to the monophosphate complicating analysis. However, we developed an efficient method to detect ribonucleoside 5´-triphosphates in HeLa cell extracts, and we applied this methodology to the evaluate hydrogen-bonding lethal mutagens rP (91) and JA28 (92).

5.3 Evaluation of Nucleoside P as a Lethal Mutagen

Nucleoside P (rP, 91) is a ribonucleoside analog with ambiguous base pairing arising from imino-amino tautomerization. Although not demonstrating equal ratios of imino to amino prevalence, the known 11:1 ratio\textsuperscript{11} may induce lethal mutagenesis.
In a series of elegant experiments conducted by our collaborators, a systematic evaluation of the steps of lethal mutagenesis was conducted. To summarize the key experiments conducted in the study, T7 RNA polymerase was utilized to produce synthetic RNA containing rPMP in the viral genome. This resulted in a dose-dependent decrease in specific infectivity as shown in Figure 5-3. Additionally, the 5’ triphosphate of rP (91) was an efficient substrate for PV 3D<sup>pol</sup> and could be incorporated across both cytidine and uridine during poliovirus RNA replication. Chain termination was not observed.

Figure 5-3: Structures of nucleoside P (91) and rPTP (101). P basepairs with adenine and guanine. P exists as two tautomers: imino-P and amino-P. The former hydrogen bonds with adenine; the latter hydrogen bonds with guanine. The imino-P to amino-P ratio is approximately 11:1.12
Figure 5-4: rPMP incorporation into PV genomic RNA results in a dose-dependent decrease in specific infectivity. (A) HeLa S3 cells were transfected with varying concentrations of in vitro transcribed RNA and serially diluted on HeLa S3 monolayers. Resulting plaques increased linearly up to ~5 μg. (B) PV genomic RNA was transcribed in vitro in the presence of varying concentrations of rPTP and infectivity was determined by infectious center assay. The number of rPMP incorporations per genome is plotted on the x-axis, as determined by extrapolating the data in Figure 3B for transcription in the presence of varying amounts of rPTP. Specific infectivity was normalized such that for each experiment the number of plaques resulting from RNA transcribed in the absence of rPTP was set to 100. The mean and standard deviation of at least 3 independent samples are shown for each data point. Data compiled by Jason Graci.
5.4 Analysis of Phosphorylation of rP(91) by HPLC

Therefore, since rP (91) demonstrated promise as a lethal mutagen in vitro, rP (91) was evaluated as an antiviral in cell culture. Surprisingly, 91 did not cause a reduction in viral titer up to 2 mM nucleoside. We hypothesized that the lack of antiviral activity maybe due to inefficient phosphorylation by cellular nucleoside kinases. Thus, nucleoside P (91) was evaluated in vitro as a substrate for HSV-1 thymidine kinase (TK) and was found to be an efficient substrate. Hence, evaluation of phosphorylation in a HeLa cell line expressing HSV-1 TK was conducted.

Intracellular phosphorylation was analyzed in our research group by reversed phase HPLC. Figure 5-5 demonstrates that no phosphorylation of rP was detected. Panel A displays separation of untreated HeLa TK cells. Panel B shows separation of extracts from HeLa- TK cells that were treated with 2 mM rP for 3 hours. Unmodified rP (tR= 11.8 min) is clearly observed in treated extracts. However, no additional peaks with the retention time for rP 5’ triphosphate (tR= 2.2 min) or characteristic UV trace were observed. The absorbance wavelength is 295 nm. Therefore the nucleoside was definitively transported across the cellular membrane. However, it was not phosphorylated. Not surprisingly, nucleoside P again failed to demonstrate antiviral activity in the poliovirus infected HeLa -TK cells. Although further experiments revealed that nucleoside P is indeed an effective lethal mutagen in vitro, inefficient phosphorylation in cell
culture precludes development of 91 as an antiviral agent without the development of an effective rP prodrug.
Figure 5-5: Phosphorylation is not observed in HeLa-TK cells treated with rP. (A) Separation of extracts from untreated HeLa-TK cells (100 μL injection). (B) Separation of extracts from HeLa-TK cells that were treated with 2 mM rP for 3 hours (100 μL injection). rP (t_R =11.8 min) is clearly observed in treated extracts. No new peaks with rP 5’- triphosphate retention (t_R =2.2 min) or characteristic UV trace are observed. The absorbance wavelength for all traces is 295 nm.
5.5 Evaluation of N\textsuperscript{6}-Purine Analogs as Lethal Mutagens

In light of the lack of phosphorylation of pyrimidine analog rP (91), N\textsuperscript{6}-modified purine analogs that act as ambiguous bases were analyzed as lethal mutagens by our collaborators.\textsuperscript{14} Some of the purine analogs (JA24, JA25, JA28 and JA30) are antiviral agents demonstrating a 90% reduction in viral titer. Methylated analogs (JA23, JA27, JA26, JA31) exhibited a reduction in antiviral activity.

![Chemical structures of N\textsuperscript{6}-modified purine nucleoside analogs.](image)

Figure 5-6: Chemical structures of N\textsuperscript{6}-modified purine nucleoside analogs.
Further analysis of mechanism of action revealed an increase in the guanidine resistance frequency when treated with purine analogs, pointing toward a lethal mutagenesis mechanism. Triphosphates of the purine analogs were examined as substrates for PV 3D<sup>pol</sup> and demonstrated rapid incorporation. JA28(TP) demonstrated equal kinetics of incorporation across C and U showing great promise as a lethal mutagen.

Table 5-1: Biological and biochemical properties of nucleoside analogs. Adapted from Graci et al.\textsuperscript{14}

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>% Reduction in titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Cell viability (HeLa S3)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fold Gua&lt;sup&gt;c&lt;/sup&gt; increase&lt;sup&gt;c&lt;/sup&gt;</th>
<th>% Triphosphate incorporation by PV 3D&lt;sup&gt;pol&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt; vs U vs C</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBV</td>
<td>98</td>
<td>75</td>
<td>74</td>
<td>0.13</td>
</tr>
<tr>
<td>JA24</td>
<td>97</td>
<td>57</td>
<td>1.9</td>
<td>0.9</td>
</tr>
<tr>
<td>JA23</td>
<td>88</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>0.54</td>
</tr>
<tr>
<td>JA25</td>
<td>99.7</td>
<td>56</td>
<td>1.8</td>
<td>ND</td>
</tr>
<tr>
<td>JA27</td>
<td>35</td>
<td>ND</td>
<td>ND</td>
<td>0.54</td>
</tr>
<tr>
<td>JA28</td>
<td>98</td>
<td>61</td>
<td>35</td>
<td>0.92</td>
</tr>
<tr>
<td>JA28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81</td>
<td>ND</td>
<td>ND</td>
<td>0.79</td>
</tr>
<tr>
<td>JA30</td>
<td>99.97</td>
<td>37</td>
<td>65</td>
<td>0.89</td>
</tr>
<tr>
<td>JA31</td>
<td>45</td>
<td>ND</td>
<td>ND</td>
<td>0.92</td>
</tr>
</tbody>
</table>

<sup>a</sup> For antiviral and cytotoxicity studies, the JA25 and JA28 data shown are for 0.5 mM treatment. All other analogues are from the 2 mM treatment.

<sup>b</sup> Results are reported as percent reduction in titer or percent viability compared to untreated control. Cellular toxicity was measured via Alamar blue assay after 24 h of continuous exposure.

<sup>c</sup> Increase in guanidine-resistant virions compared to untreated infection.

<sup>d</sup> Percent incorporation relative to the correct, natural nucleotide after 60 s.

<sup>e</sup> ND, not determined.

<sup>f</sup> —, below the limit of detection.
5.6 Detection of JA28(TP) by Reversed-Phase HPLC Analysis

Since the N\textsuperscript{6}-purine analogs may be phosphorylated by different kinases in the cellular pathways, we hypothesized that the modified purines would be phosphorylated in cell culture. Therefore, in an effort to elucidate the cellular mechanism of action of such analogs, we investigated cellular phosphorylation by HPLC. We synthesized nucleoside JA28 (92) by existing methodology to access sufficient material for the extraction experiment. Subsequently, HeLa cells were treated with 0.5 mM of (92) for 2 h and extracted by utilizing the previously described procedure. Reversed-phase HPLC analysis indicated substantial phosphorylation to the nucleoside 5\textsuperscript{-}-triphosphate (100) (Figure 5-8, panel C). Pure JA28(TP) elutes at 3.6 min (panel A). Separation of untreated HeLa cells lacked a detectable peak at 3.6 min (Panel B). However, a strong peak was definitively observed with JA28 treated HeLa cell extracts with a characteristic UV absorption spectrum (inlay)

![Chemical structure of JA28(TP) (100)](image)

Figure 5-7: Chemical structure of JA28(TP) (100),
Figure 5-8: (A) Analysis of pure JA28-TP including the full UV spectrum of the JA28-TP peak at 3.6 min (inlay). mAU, milli-absorbance units. (B) Analysis of extracts of untreated HeLa S3 cells including the full UV spectrum of the eluent at 3.6 min. (C) Analysis of extracts of HeLa S3 cells treated with JA28 (0.5 mM) including the full UV spectrum of the eluent at 3.6 min (inlay). The long-wavelength absorption characteristic of JA28-TP is illustrated by the arrows to the right. Peaks were detected by $A_{325}$.
5.7 Summary of Nucleoside Analog Phosphorylation

Pyrimidine Analogs

![Chemical structures of pyrimidine analogs]

Purine Analogs

![Chemical structures of purine analogs]

Figure 5-9: Structural comparison of chemical structures of nucleoside analogs analyzed for phosphorylation compared to natural substrates. Boxed structures with lines indicate nucleoside analogs that were phosphorylated to the corresponding 5'-triphosphate. Box with dashed lined indicates phosphorylation to the 5'-monophosphate.

In summary, the nucleoside analogs analyzed in this work have revealed surprising results and give insight into intracellular phosphorylation. Of the pyrimidine analogs, only ICS-R(TP) (41) was detected in HeLa cell extracts. Also
significant is the presence of mono- and di-phosphorylated ICS-R. (Figure 2-7) indicating a slower flux through kinase pathways than JA28 (92). Subtle methyl substitution on the anti face of the nucleoside in MICS-R(9) significantly affected phosphorylation as did the thio-substitution in SICS-R(10). Phosphorylation of rP (91) was not detected, explaining the lack of antiviral activity. rP has steric bulk on the opposite face of the pyrimidine nucleobase revealing kinase recognition is sensitive to several modes of substitution. For the ICS-R analogs 8-10, analysis of phosphorylation revealed the difference in the observed inhibition of PV 3Dpol and the observed antiviral activity. Additionally, of the purine analogs, JA28 (92) was robustly phosphorylated to the 5’- triphosphate, correlating the observed antiviral data with the biochemical experiments confirming mechanism of action to be lethal mutagenesis. However, for universal nucleoside 5NINDN (44), although inhibition of PV 3Dpol by 5NINDN(TP) (47) and 5NINDN(DP) (48) was measured, a viral-specific mode of action could not be definitively assigned due to the lack of detectable phosphorylation. Structurally, although the indole scaffold is isosteric with purines, there is a definitive difference in efficiency through cellular phosphorylation pathways.
5.8 Conclusion and Future Directions

Lethal mutagenesis as an antiviral strategy requires a minimal recognition by cellular phosphorylation pathways for activation of nucleoside analogs. Throughout this work, an increased appreciation for the selectivity of nucleoside kinases has emerged. In order for a lethal mutagen to function in vivo, it is not sufficient to possess ambiguous base pairing or to demonstrate recognition and incorporation by the viral polymerases. Rather, a preliminary analysis for nucleoside analog phosphorylation in necessary to screen for nucleosides which cannot operate by lethal mutagenesis. Therefore, we have developed an efficient assay for nucleoside phosphorylation to more efficiently evaluate lethal mutagens. Therefore, in order to bypass flux though cellular phosphorylation pathways which can be rate limiting, future studies toward the development of lethal mutagens should include novel prodrug strategies.\textsuperscript{15, 16}

5.9 Experimental Section
5.9.1 Nucleotide Extraction and HPLC Analysis Procedure for rP (91)\textsuperscript{17}

Nucleotide extraction was modified from a previously published procedure\textsuperscript{10}. A total of $7.5 \times 10^6$ HeLa S3 or HeLa-TK cells were plated in a 100-mm dish 15 to 18 h before treatment. Cells were treated with 2.5 µg/ml actinomycin D (Sigma) for 15 min at 37 °C, and then rP was added to the medium to a final concentration of 2 mM. Cells were incubated for 3 h at 37 °C. After incubation, the medium was aspirated, plates were washed with 5 ml PBS, and 1 ml prewarmed trypsin-EDTA solution (Invitrogen) was added to each plate. Cells were incubated for 5 min at 37 °C to facilitate detachment, after which cells were collected, pelleted, and resuspended in 0.05 ml ice-cold 0.6 M trichloroacetic acid (Sigma). The cell suspension was incubated on ice for 10 min and then centrifuged at 14,000 x g for 2 min at 4 °C. The supernatant was collected and extracted with an equal volume of ice-cold 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane (Sigma). Samples were then vortexed for 30 s and centrifuged for 30 s at 14,000 x g and 4 °C. The upper (aqueous) layer was removed and analyzed on a Hewlett Packard 1100 series instrument equipped with an Aquasil C\textsubscript{18} analytical column (4.6 by 250 mm, 5 µm; Keystone Scientific Inc., Thermo Electron Corp.) running the following mobile phase (flow rate, 1 ml/min): gradient of 1% to 15% CH\textsubscript{3}CN in 100 mM potassium phosphate buffer (KH\textsubscript{2}PO\textsubscript{4}, pH 6.0) over 20 min, followed by 15 to 80% CH\textsubscript{3}CN in 100 mM KH\textsubscript{2}PO\textsubscript{4} buffer over 10 min. All injections were 50 µL.
5.9.2 Nucleotide Extraction Procedure and HPLC Analyses for JA28 (92)

Cell extracts were prepared and analyzed based on previously published methods. HeLa S3 cells were adapted to suspension and maintained in spinner flasks at a density of 1 x 10^6 to 2 x 10^6 cells/ml in S-MEM (minimum essential medium modified for suspension cultures) containing 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). Cell density was adjusted to 5 x 10^6 cells/ml immediately prior to treatment in S-MEM, to which was added 2% dialyzed fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were treated with 2.5 μg/ml actinomycin D (Sigma) for 15 min at 37 °C, and then nucleotide was added to the medium to a final concentration of 0.5 mM. One percent dimethyl sulfoxide (DMSO) was present in both treated and control samples. Samples were incubated for 2 h at 37 °C in a Lauda circulating water bath with occasional mixing by inversion. Cells were collected by centrifugation at 2,000 X g for 4 min and washed with 2 volumes phosphate buffered saline. Cells were then pelleted and resuspended in 1 volume ice-cold 0.6 M trichloroacetic acid (Sigma). The cell suspension was incubated on ice for 10 min and then centrifuged at 14,000 x g for 2 min at 4 °C. The supernatant was collected and extracted with an equal volume of ice-cold 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane (Sigma). Samples were then vortexed for 30 s and centrifuged for 30 s at 14,000 x g and 4°C. The upper (aqueous) layer of the cell extract was analyzed on a Hewlett Packard 1100 series instrument equipped with an Aquasil C18 analytical column.
(4.6 x 250 mm, 5 μm; Keystone Scientific, Inc. [Thermo Electron Corp.]) running a mobile phase (flow rate of 1 ml/min) gradient of 1% to 15% CH$_3$CN in KH$_2$PO$_4$ buffer (pH 6.0, 0 to 20 min) and 15% to 80% CH$_3$CN in KH$_2$PO$_4$ buffer (20 to 30 min). All injections were 50 μL.

5.9.3 General Synthesis Information

Reagents and solvents were obtained from Aldrich, Acros, TCI, and Fisher and were used without further purification unless otherwise noted. Thin-layer chromatography was performed on glass-backed silica gel-coated plates with silica gel 60 F$_{254}$ (EM Science, 0.25 mm thick). ICN SiliTech Silica Gel (32-63 μm) was employed in column chromatography. TLC plates were visualized by UV irradiation (254 nm) or stained with a solution of methanol, sulfuric acid, and p-anisaldehyde (18:2:1). Infrared spectra were obtained with a Mattson GL-4020 Series FTIR. Bruker DRX 400 spectrometer was employed in nuclear magnetic resonance (NMR) spectroscopy. NMR peaks are reported as parts per million (ppm) and are referenced to internal CHCl$_3$ or DMSO. Mass spectral data was obtained from The Pennsylvania State University Mass Spectrometry Facility (ESI). Melting points were determined on a Thomas Hoover apparatus and are reported uncorrected. Internal solvent peaks were referenced in each case.
To a sealed tube, 6-chloropurine (546 mg, 1.91 mmols) was added and dissolved in ethanol: dH₂O (1:1, 10 mL). To this solution, hydroxylamine (50% in dH₂O, 2 mL) was added, sealed and heated to 50 °C for 16 h. Reaction mixture was concentrated in vacuo and recrystallized from MeOH to afford 92 (394 mg, 73%). ¹³C NMR (100 MHz, CDCl₃): δ 156.8, 148.4, 146.1, 138.9, 124.5, 118.8, 87.7, 85.9, 74.0, 70.7, 61.7. LRMS(ESI+) calcd. for C₁₀H₁₄N₅O₅ [M+H]⁺ 284.1. Found 284.2
5.10 References


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