Glyoxal Caging of Nucleoside Antivirals Toward Self-Activating, Extended-Release Prodrugs

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Supporting Information Placeholder

ABSTRACT: Nucleoside antivirals are a leading class of compounds prescribed as a first-line treatment for viral infections. However, inherent limitations such as low solubility and circulation lifetime can necessitate multi-intraday dosing. Here, we deploy the 1,2-dialdehyde glyoxal to generate antiviral nucleoside prodrugs with enhanced pharmacokinetic properties and extended-release activity to combat poor patient adherence. The near-quantitative reaction of glyoxal with acyclovir (ACV) drastically improves ACV solubility and enables subsequent drug release with a half-life of 2.3 h under physiological conditions. Further, glyoxal caging thermoreversibly disrupts ACV activity against HIV-1 reverse transcription *in vitro* and HSV-1 pathology *in cellulo*. Finally, the amenability of a panel of nucleoside reverse transcriptase inhibitors to glyoxal caging showcases the potential of this highly versatile method for achieving timed-release activation of a clinically important class of antiviral therapeutics.

INTRODUCTION

Global health threats from viral infections such as SARS-CoV-2 and HIV-1, combined with the rising prevalence of antiviral drug resistance, pose a significant biomedical challenge. Among the most effective treatments for viral infections are nucleoside analogues, which mimic naturally occurring nucleosides and cause selective termination of the nascent DNA chain during viral replication or reverse transcription (Figure 1).[1-2] Despite their therapeutic efficacy, many nucleoside analogues have a short mean plasma half-life and poor solubility, leading to intravenous (IV) dosing regimens up to five times per day.[3-5] This results in inadequate patient adherence and contributes to drug resistance.[6-8] Meanwhile, the development of novel therapeutics requires extensive time and resources, and discovery and optimization steps are specific to each chemical entity.

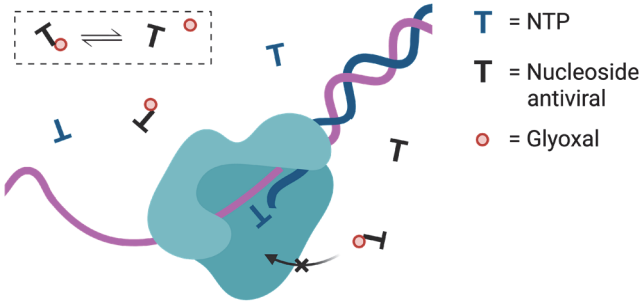


Figure 1. Nucleoside antivirals induce chain termination in viral polymerases by competing with endogenous nucleoside triphosphates (NTPs) for incorporation into the growing strand. Glyoxal caging thermoreversibly inhibits nucleoside antiviral activity. Created with BioRender.com.

Herein, we report a novel prodrug strategy that uses the simplest dialdehyde, glyoxal, to generate nucleoside antivirals that undergo spontaneous activation over several hours (Figure 1). Extended-release formulations offer a promising pharmacokinetic solution to combat poor drug adherence, as they can obviate the need for multi-intraday dosing.[9] Glyoxal is highly electrophilic and thus susceptible to nucleophilic addition under mild aqueous conditions. The reaction is favorable with the

nucleobases cytosine, adenine, and especially guanine, as an addition from guanidine or amidine functional groups yields a cyclic bis-hemiaminal structure.[10-13] Our lab recently deployed glyoxal caging to thermoreversibly tune the activity of diverse oligonucleotides, including aptamers, CRISPR/Cas gRNAs, and antisense oligonucleotides.[14] We now extend this principle to nucleoside-based small-molecule therapeutics, characterizing the reactivity of glyoxal with acyclovir (ACV) and using glyoxal to modulate ACV bioactivity in the context of viral DNA polymerase and reverse transcriptase (RT) inhibition. Finally, we show that a wider panel of nucleoside antivirals is compatible with glyoxal caging, underscoring the generalizability of this approach toward improving the pharmacokinetic properties of antiviral drugs that are otherwise limited by solubility and permeability constraints.

RESULTS AND DISCUSSION

ACV is a guanosine analogue that undergoes intracellular phosphorylation before inducing chain termination in herpes simplex virus type 1 (HSV-1) DNA polymerase and other viral polymerases.[15] However, the poor solubility, permeability, and bioavailability of ACV limit its therapeutic potential.[16-17] Namely, ACV has a maximum solubility of 2.5 mg/mL at pH 7, suffers from a mean plasma half-life of only 2.5 h, and induces nephropathy in 5–10% of patients receiving IV administration due to precipitation of needle-shaped crystals.[3, 18-20]

Envisioning that glyoxal caging could improve these properties, we first explored the reactivity of 100 mM ACV with 1 M glyoxal in pH 6 buffer, analyzing reaction progress over 24 h by HPLC (Figure 2a). Caging led to complete ACV consumption and conversion into a new major product in <1 h (Figure 2b). A similar experiment conducted with 0.8–1 equivalents of glyoxal revealed near-quantitative formation of a product with two new singlet peaks, corresponding to hemiaminal protons of the caged ACV species with a 90° dihedral angle, by ^{1}H NMR in D_2O (Figure S1).[21] These data suggest that while two diastereomers of the caged species are possible, the product either exists as a single diastereomer or is able to undergo stereoisomerization that is fast on the NMR timescale. The ^{13}C-NMR and high-resolution ESI-MS spectra were also consistent with the expected product (Figure S2, Figure S3).

We then proceeded to assess the decaging kinetics of the glyoxal-modified species. During the incubation of 10 or 100 µM caged ACV in 1X PBS (pH 7.4) at 37 °C over 0.5–36 h, equilibrium increasingly favored disassociation of the glyoxal cage and regeneration of the parent ACV compound, with a half-life slightly under 2 h (Figure 2c, Figure 2d). These data provide encouraging evidence that in a therapeutic context, low intracellular concentrations of caged ACV would deliver the active antiviral compound over a time span of several hours, consistent with other extended-release systems.[3]

We anticipated glyoxal caging would provide several improvements to the pharmacokinetic properties of ACV. Strikingly, 24 h incubation of 23 mg of ACV with an equimolar amount of glyoxal in 1 mL of 1X PBS enabled complete solubilization of the precipitate, representing a near 10-fold improvement in solubility (Figure 2e).[18] This improvement could drastically reduce patient nephropathy by preventing circulatory precipitation of ACV.[19] Likewise, the calculated logP value of caged ACV is equivalent to that of valacyclovir, the L-valyl ester of ACV developed for its significantly improved oral bioavailability (Table S1).[22-23] Finally, 4 h treatment of HEK293T cells with 0.5 mM ACV, caged ACV, or glyoxal had no measurable effect on cell viability, nor was there an impact with up to 20 mM caged ACV (Figure S4). This was not entirely surprising, as glyoxal has been reported to have low cytotoxicity (IC50 = 310 ± 83 mM),[24] and in mammalian cells, the glyoxalase enzyme system degrades excess glyoxal and other metabolic dicarbonyls to mitigate the accumulation of advanced glycation end products that drive disease processes such as neuropathy and aging.[25] Additionally, glyoxal is naturally produced by metabolism and is commonly found in fermented food products and baked goods at concentrations on the order of several mg/kg. Indeed, routine glyoxal consumption is on the order of mg per day, and the typical blood concentration of glyoxal for healthy individuals is 2.5–6.5 mg/L.[26-27] Given this literature precedent and our data showing good cell viability at mM concentrations of glyoxal, we are encouraged that toxicity of glyoxal will not pose a significant challenge for practical application of our prodrug strategy.

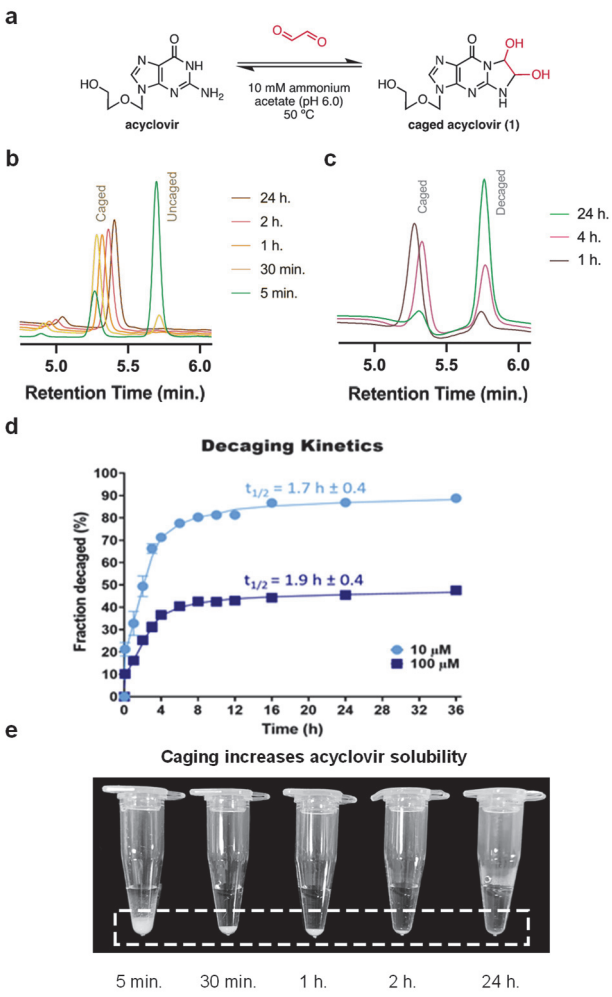
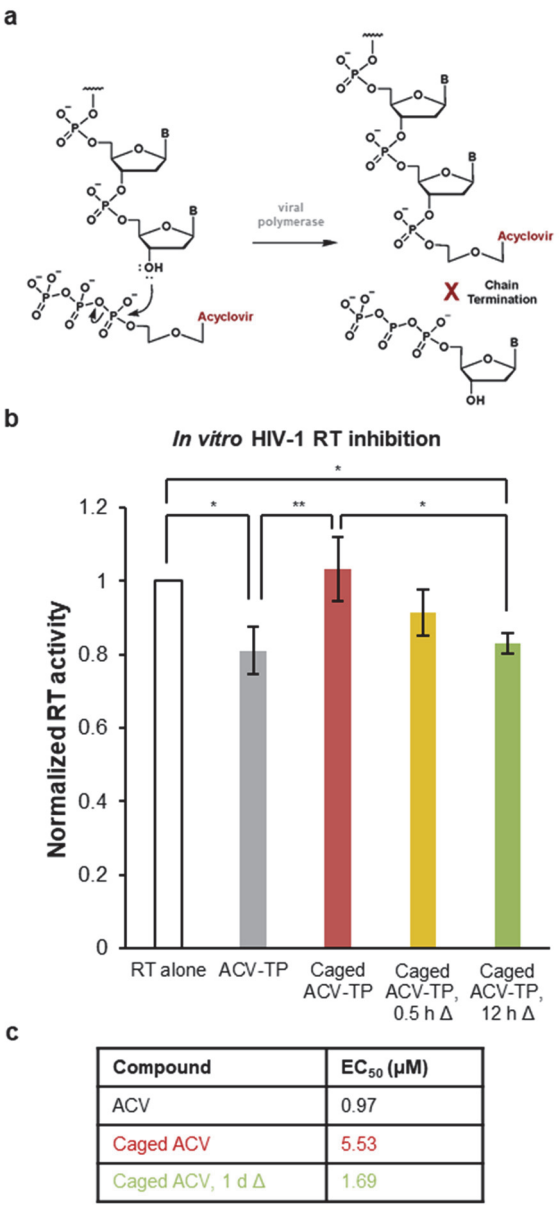


Figure 2. ACV caging and decaging with glyoxal. (a) Reaction scheme. (b) Caging of ACV (100 mM) over 5 min–24 h with 10 equiv. of glyoxal. (c) Reconversion of purified, caged ACV (10 µM) into ACV after 1–24 h incubation in 1X PBS (pH 7.4) at 37 °C. (d) Concentration of caged ACV impacts fraction reconverted over 36 h (n = 3). Error bars indicate mean and standard deviation. (e) Near 10-fold increase in solubility of ACV (100 mM) upon incubation with 1 equiv. of glyoxal.

We next sought to probe the effects of glyoxal caging on drug bioactivity by comparing the inhibitory capacity of caged and uncaged ACV-triphosphate (ACV-TP), the cellularly active antiviral agent (Figure 3a).[15, 28] Interestingly, although ACV is prescribed primarily to treat herpesviruses, clinical and biochemical studies suggest ACV inhibits HIV-1 RT as well.[29-32] We leveraged a commercially available HIV-1 RT assay kit to directly explore the effects of glyoxal caging on ACV-TP inhibition activity. After confirming that ACV-TP exhibits comparable glyoxal caging and decaging behavior to that of ACV (Figure S5), we measured the *in vitro* activity of 1 ng of HIV-1 RT alone or in the presence of 100 µM ACV-TP, caged ACV-TP, or caged ACV-TP that had first been incubated in 1X PBS at 37 °C for 0.5 h or 12 h to simulate spontaneous decaging in a biological system (Figure 3b). While ACV-TP exhibited only modest potency against HIV-1 RT, as is consistent with other reports,[30, 33-34] caging fully disrupted this activity. Excitingly, ACV-TP activity was restored upon decaging in a time-dependent manner (Figure 3b).

To directly probe the relative activity of ACV versus caged and time-release decaged ACV against the canonical viral target, HSV-1, we performed a cytopathic effect (CPE) assay measuring viral-mediated reduction in cell growth and corresponding rescue by antiviral agents.[35] Primary human foreskin fibroblast cells were treated with varying concentrations of ACV, caged ACV, or decaged ACV. The cells were subsequently infected with HSV-1 and incubated for 5 d until 100% CPE was observed in virus-only control wells. The half-maximal effective concentration (EC_{50}) values at which viral activity was inhibited for the various compound treatments could then be extrapolated (Figure 3c, Table S2).[35] As expected, ACV demonstrated strong potency against HSV-1. Meanwhile, this potency was reduced 5.7-fold for the caged compound. We speculate this reduction in antiviral activity was not more pronounced due to partial re-equilibration back to ACV during the 5 d assay incubation period. However, the EC_{50} value for the decaged species lay in between that of ACV and caged ACV, demonstrating intermediate recovery of *in cellulo* activity over the 1 d release time. Notably, neither compound demonstrated cytotoxicity.

As final validation of our ACV glyoxal caging approach, we administered IV bolus dosing of 1 mg/kg ACV or caged ACV in ammonium acetate buffer to mice and measured compound plasma concentration over time by LC-MS/MS. Preliminary results indicated that the initial detectable concentration of caged ACV was much higher than that of ACV. Further, caged ACV circulated in the bloodstream for 4 h, as opposed to 1 h for ACV (Figure S6). These encouraging preliminary data support future work exploring caging with glyoxal or modified 1,2-dicarbonyl species that may further extend circulation lifetime. Together, these studies indicate that glyoxal caging of the Watson-Crick-Franklin base-pairing face can be used to deactivate and spontaneously reactivate the bioactivity of nucleoside antivirals while improving pharmacokinetic properties such as solubility.



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Compound	EC_{50} (μM)
ACV	0.97
Caged ACV	5.53
Caged ACV, 1 d Δ	1.69

Figure 3. Glyoxal caging modulates ACV bioactivity. (a) Mechanism of ACV-TP incorporation into viral DNA. (b) Normalized *in vitro* activity of HIV-1 RT upon 100 μM treatment with ACV-TP, caged ACV-TP, or caged ACV-TP after 0.5 h or 12 h decaging at 1 mM in 1X PBS (pH 6.8) at 37 °C (n = 3). One-way ANOVA (95% confidence limit); post-hoc test (Tukey HSD), *p < 0.05, **p < 0.01. Error bars indicate mean and standard deviation. (c) Half-maximal effective concentration (EC_{50}) of ACV, caged ACV, and caged ACV after 1 d decaging at 100 μM in an HSV-1 cytopathic effect (CPE) assay. Primary human foreskin fibroblast cells were infected with HSV-1 and treated for 5 d with 0.048–150 μM ACV or 0.003–10 μM caged or decaged ACV.

Lastly, we explored whether nucleoside antivirals beyond ACV could be modified with glyoxal for potential therapeutic applications, especially considering the prevalence of multidrug combinations in antiviral therapy.[36] We selected three nucleoside reverse transcriptase inhibitors (NRTIs) to test: entecavir, zalcitabine, and abacavir, which are guanosine, cytidine, and adenosine analogues, respectively.[37] The compounds were analyzed by HPLC-UV at 1 mM concentration before and after glyoxal caging.

Entecavir, like ACV, reacted readily with glyoxal, requiring only 15 equivalents to achieve full conversion of the starting material (Figure 4a). Zalcitabine reacted fully with 1,000 equivalents to form a significantly more polar product (Figure 4b), and abacavir reacted 85% with 1,000 equivalents to form three new species that could be individually isolated by HPLC (Figure 4c, Figure S7). We postulate that the multiple products formed from the reactions with entecavir and abacavir arise from addition of glyoxal at alternate locations on the guanidine and/or amidine moieties (Figure S8). Fortuitously, the formation of multiple species does not preclude the use of these caged NRTIs as therapeutics, as long as the individual species are isolated, structurally characterized, and tested for extended-release antiviral activity in the future. NMR analysis of the NRTI caging products in D_2O revealed the formation of products with two new singlet peaks, corresponding to protons of the caged entecavir, zalcitabine, and abacavir by glyoxal (Figures S9–S10). High-resolution ESI-MS analysis of each reaction mixture confirmed the formation of the expected, glyoxal-caged NRTI products (Figure S11). Thus, a wide array of nucleoside-like small-molecule drugs are amenable to glyoxal caging, representing a convenient strategy to convert existing FDA-approved nucleoside analogs into prodrugs having spontaneous, timed-release activity.

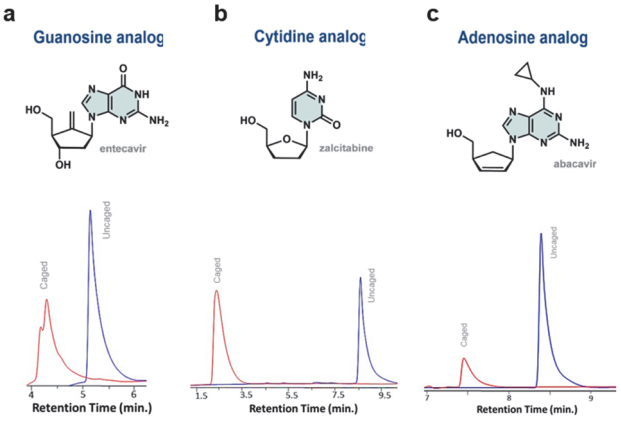

Figure 4. Glyoxal caging of a panel of structurally diverse NRTIs. (a) Entecavir (1 mM) was analyzed before and after 1 d incubation with 15 equiv. of glyoxal in ammonium acetate buffer at 50 °C. (b) Zalcitabine and (c) abacavir were

similarly analyzed after 1 d incubation with 1,000 equiv. of glyoxal and purification by HPLC.

CONCLUSIONS

This work aims to combat low patient adherence to antiviral drug treatment through the glyoxal-based caging of antiviral therapeutics. We envision this approach could provide a mechanism for extended release *in vivo* by temporarily blocking the Watson-Crick-Franklin base-pairing face of the compound. As prior work in our lab utilized glyoxal to modulate the function of varied nucleic acid scaffolds,[14] we recognized the potential to extend this principle to nucleoside-like small molecules. We first characterized the glyoxal caging and decaging kinetics of acyclovir, a competitive inhibitor of HSV-1 DNA polymerase and HIV-1 reverse transcriptase. We found that caging considerably enhanced ACV solubility and thermoreversibly regulated antiviral activity both *in vitro* and *in cellulo*. The method also demonstrated compatibility with a broad substrate scope, extending to a wider panel of guanosine-, adenosine-, and cytosine-like NRTIs with varying nucleobase and sugar structures.

By installing two additional hydroxyl groups, glyoxal caging may more generally enhance pharmacokinetic properties such as solubility and permeability, which are widely viewed as limitations within the nucleoside antiviral drug class.[19] Improvements to these parameters would reduce dosing frequency, thus potentially increasing adherence and enabling superior patient outcomes. Advantageously, the reverse decaging reaction is spontaneous under physiological conditions and does not require exogenous reagents for activation.[38] In sum, this design is innovative in the use of glyoxal as a simple and generalizable approach to generate extended-release antivirals from previously developed pharmaceuticals. This discovery has implications that may advance both the efficacy and levels of adherence to a prominent class of medicines used to treat acute viral disease.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website.

Description of synthetic and experimental methods, Figures S1–S11, Tables S1–S2, and NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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