Published in final edited form as:

Bioorg Med Chem. 2013 November 15; 21(22): 7222–7228. doi:10.1016/j.bmc.2013.08.023.

5,6-Dihydro-5-aza-2'-deoxycytidine potentiates the anti-HIV-1 activity of ribonucleotide reductase inhibitors

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Abstract

The nucleoside analog 5,6-dihydro-5-aza-2'-deoxycytidine (KP-1212) has been investigated as a first-in-class lethal mutagen of human immunodeficiency virus type-1 (HIV-1). Since a prodrug monotherapy did not reduce viral loads in Phase II clinical trials, we tested if ribonucleotide reductase inhibitors (RNRI) combined with KP-1212 would improve antiviral activity. KP-1212 potentiated the activity of gemcitabine and resveratrol and simultaneously increased the viral mutant frequency. G-to-C mutations predominated with the KP-1212-resveratrol combination. These observations represent the first demonstration of a mild anti-HIV-1 mutagen potentiating the antiretroviral activity of RNRI and encourage the clinical translation of enhanced viral mutagenesis in treating HIV-1 infection.

Keywords

Lethal mutagenesis; Error catastrophe; Resveratrol; KP-1212; 5,6-Dihydro-5-aza-2'-deoxycytidine

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1. Introduction

—Human immunodeficiency virus type-1 (HIV-1) infects about 34 million individuals globally and resulted in the deaths of 1.7 million in 2011 alone.¹ Despite great strides in antiretroviral therapy, current drugs are still limited by toxicity, cross-resistance, and the transmission of drug-resistant viral variants.² HIV-1 has a high mutation rate (3 to 9×10^{-5} mutations/base pair/cycle),³⁻⁵ and it has been postulated to replicate near the error threshold—a theoretical upper limit to the mutation rate beyond which genetic information cannot be maintained.⁶⁻⁸ Thus, it is conceivable to exceed the error threshold with small molecule viral mutagens. This antiviral strategy, called lethal mutagenesis, was first investigated using mutagenic ribonucleoside analogs and RNA-damaging agents in poliovirus and vesicular stomatitis virus by Holland and colleagues in the 1990s.^{9, 10} It was later demonstrated in HIV-1 using 5-hydroxy-2'-deoxycytidine,¹¹ and subsequently explored in several other RNA viruses.¹²⁻¹⁵

HIV-1 lethal mutagenesis has not been clinically exploited, but a prodrug of 5,6-dihydro-5-aza-2'-deoxycytidine (KP-1212) has advanced to clinical trials. KP-1212 potently inhibited HIV-1 replication in culture (EC_{50} of ~ 10 nM) with low cellular, mitochondrial, and genome toxicities, as well as activity against both wildtype (wt) and drug resistant strains.¹⁶ KP-1212 was shown to induce primarily G-to-A and A-to-G (and to a lesser extent T-to-C and C-to-T) transition mutations within HIV-1. The prodrug of KP-1212 (KP-1461) was well tolerated in clinical trials, but was not effective in reducing viral loads despite adequate drug bioavailability.^{17, 18} Nonetheless, there was a detectable increase in the number of private mutations (i.e., those most likely to have occurred in the latest round of replication) and evidence of an altered mutation spectrum in clinical samples.¹⁹

We have previously demonstrated that combinations of nucleoside analogs that act as viral mutagens and ribonucleotide reductase inhibitors (RNRIs) can result in synergistic decreases in viral infectivity.^{20, 21} Similarly, combining nucleoside reverse transcriptase inhibitors (NRTIs) with RNRIs results in potentiation of anti-HIV-1 activity.²²⁻²⁴ RNRIs likely have a threefold antiretroviral mechanism in such combinations: 1) inhibition of retroviral DNA synthesis *via* depletion of deoxynucleoside triphosphate (dNTP) pools;^{22, 25-27} 2) many RNRIs do not inhibit synthesis of all four natural dNTPs equally, thus generating dNTP pool imbalances that increase the viral mutation rate;^{23, 26-28} and 3) decreases in cellular dNTP concentrations result in the upregulation of nucleotide kinases, leading to more efficient activation of nucleoside analogues such as KP-1212.²⁰⁻²⁴ Given this, the combination of an RNRI with KP-1212 or a KP-1212 prodrug should result in potent inhibition of HIV-1 infectivity.

We report here that while KP-1212 alone had minimal antiviral activity in our single round replication assay, it potentiated the activity of the RNRIs gemcitabine and resveratrol. The combination of KP-1212 with resveratrol, in particular, reduced the EC_{50} by 1.9-fold relative to resveratrol alone (i.e., from $99.6 \mu\text{M}$ to $52.8 \mu\text{M}$). The KP-1212-RNRI combinations led to corresponding increases in the viral mutant frequency in the absence of cell cytotoxicity relative to that of RNRIs alone. Taken together, these observations represent the first demonstration of a mild anti-HIV-1 mutagen having the ability to potentiate the antiretroviral activity of RNRIs. These findings may improve the potential for clinical translation of KP-1212 in the treatment of HIV-1 infection.

2. Results & discussion

2.1. Development of an mCherry/GFP dual reporter HIV-1 vector

In order to efficiently assess both HIV-1 infectivity and mutant frequency, we constructed a HIV-1 vector expressing two fluorescent proteins—mCherry and the green fluorescence protein (GFP) (Figure 1A). The resulting vector expresses all viral proteins except Env and Nef. Infectious vector virus was produced by *trans*-complementing with the vesicular stomatitis virus G protein (VSV-G) *via* co-transfection with the vector virus plasmid. This strategy limits virus replication to a single cycle of replication. The utility of the construct for detecting drug-induced increases in virus mutant frequency was confirmed using 5-azacytidine (5-AZC), a ribonucleoside analog previously shown to have antiretroviral activity against HIV-1.²⁹ We found that treatment of target cells with 5-AZC reduced HIV-1 infectivity in a concentration-dependent manner, with an EC₅₀ of 53.0 μM, comparable to the previously reported value of 57.0 μM (Figure 1B).²⁹ We also observed a concentration-dependent elevation of the viral mutant frequency to a maximum of 19.8-fold relative to the no drug control, confirming previous findings with 5-AZC.²⁹

2.2. Individual effects of RNRI and KP-1212 on HIV-1 infectivity & mutant frequency

We initially combined KP-1212 at 100 μM with a panel of several anti-metabolites and found that KP-1212 potentiated the antiretroviral activity of gemcitabine (2',2'-difluoro-2'-deoxycytidine, dFdC), resveratrol, and deferoxamine (data not shown). These three compounds have all been previously reported to have RNRI activity as well as antiretroviral activity, though inhibition of ribonucleotide reductase has not been clearly HIV-1.^{20, 21, 24, 27, 30-33} Resveratrol, in particular, has been reported to interact with a wide variety of cellular targets in exerting its anti-inflammatory, anti-tumorigenic, and antiviral effects.³⁴ We next characterized the individual effects of each compound on HIV-1 infectivity and mutant frequency. We found that gemcitabine, resveratrol, and deferoxamine all reduced HIV-1 infectivity (Figure 2A & Table 1) and elevated the viral mutant frequency in a concentration-dependent fashion (Figure 2B).

We observed that KP-1212 exerted only a mild antiviral effect alone, without concentration dependence in the concentration range of 5 to 200 μM (Figure 2A). However, we observed a slight dose-dependent increase in mutant frequency (up to ~1.6-fold) (Figure 2B). Our observations using this assay are somewhat different than previous findings of KP-1212 being a potent inhibitor of HIV-1 replication.¹⁶ We tested multiple batches of KP-1212 to rule out the possibility of degradation of our liquid drug stocks (data not shown). Additionally, ¹H NMR and combustion analysis confirmed the purity and stability of KP-1212 in our hands (data not shown). The differences in observed activity of KP-1212 may be due to the previous study using a very low multiplicity of infection (MOI), ranging from 1:1000 to 1:5000.¹⁶ These authors found that higher MOIs led to higher EC₅₀ values, and were not able to obtain a reproducible EC₅₀ at MOIs of 1:300 or greater. In line with this, we used a much higher MOI (~1:3 to 1:5) and were unable to obtain an EC₅₀. Additionally, the previous study used a different cell line (MT-2) and centrifuged the drug onto cells, potentially altering the uptake and/or phosphorylation of KP-1212. Finally, the virus was likely not limited to a single round of replication, which potentially enhanced the observed effects of KP-1212. Despite the apparent discrepancies in antiretroviral potency, our findings are in relatively good agreement with the Phase II clinical trials on KP-1212, which found no clinically significant decline in viral loads but some evidence of mutagenicity (increases in private mutations and altered mutation spectra).^{18, 19}

2.3. KP-1212 potentiates the effects of RNRI on HIV-1 infectivity & mutant frequency

To further investigate the interaction between RNRI and KP-1212, we combined a fixed low concentration of each RNRI with varying concentrations of KP-1212 (i.e., ranging from 5 to 200 μ M). For the gemcitabine and KP-1212 drug combination, we observed an unexpected trend: low concentrations of KP-1212 (5 to 50 μ M) enhanced the antiviral activity of gemcitabine, whereas high concentrations partially (100 μ M) or fully (200 μ M) antagonized the antiviral activity (Figure 3A). Similarly, the corresponding mutant frequencies increased to a maximum of 4.8-fold at 25 μ M KP-1212 and then decreased to levels lower than for gemcitabine alone (Figure 3B). KP-1212 likely antagonized gemcitabine at high concentrations because they are both deoxycytidine analogs that require transport and metabolic conversion to their active forms. Specifically, they may utilize the same transporter proteins to breach the plasma membrane (either through facilitated diffusion or active transport), and they are both phosphorylated by deoxycytidine kinase to become active within cells.³⁵⁻³⁸ Furthermore, the drugs were simultaneously added to cells, and KP-1212 was used at a much higher concentration than gemcitabine (10,000-fold higher for 200 μ M KP-1212). Our observation of potential drug interference could likely be circumvented by 1) using a more potent mutagenic deoxycytidine analog, 2) using a mutagenic nucleoside analog that is not phosphorylated by deoxycytidine kinase, 3) applying the drugs sequentially rather than simultaneously, or 4) using an RNRI that is not a nucleoside analog.

We therefore examined the interactions of KP-1212 with two non-nucleoside RNRI—resveratrol and deferoxamine. The addition of KP-1212 (5 to 200 μ M) to resveratrol (50 μ M) resulted in a concentration-dependent decline in viral infectivity, with the greatest potentiation observed at 200 μ M KP-1212 (Figure 3A). We observed a similar, though less concentration-dependent, trend for the combination of KP-1212 with deferoxamine. For both of these combinations, we also observed increases in the viral mutant frequencies (to a maximum of 6.2-fold for resveratrol and 4.7-fold for deferoxamine relative to that of no drug) (Figure 3B). These results indicate, in contrast to gemcitabine, high concentrations of KP-1212 were superior compared to that of low concentrations for potentiating the antiviral activity of resveratrol and deferoxamine. The elevation of the viral mutant frequency by the drug combinations is consistent with an increase in virus mutational load as a major mechanism of antiretroviral activity.

2.4. KP-1212 potentiates the anti-HIV-1 activity of RNRI without enhancing cytotoxicity

To further characterize these drug combinations, we investigated the extent to which KP-1212 can augment the potency of RNRI. We found that the addition of KP-1212 at a fixed amount (5 μ M for gemcitabine or 200 μ M for resveratrol) enhanced the potency of both gemcitabine and resveratrol, as evidenced by the significant decrease in EC_{50} values upon addition of KP-1212 (Table 1). The greatest enhancement was observed for resveratrol, with the EC_{50} approximately halved. The effect was much less for gemcitabine, as only low concentrations of KP-1212 could be used due to antagonism. The effect of KP-1212 in combination with deferoxamine was not statistically significant compared to deferoxamine alone. We then examined the effect of the drugs (alone and in combination) on cell viability in order to determine whether drug cytotoxicity could explain the antiviral effects observed. For gemcitabine, some degree of cytotoxicity was observed at all concentrations tested, but the CC_{50} was higher than the EC_{50} (62.0 μ M vs. 27.5 nM), indicating that the antiviral activity of gemcitabine cannot be explained by cytotoxicity (Figure 4 & Table 1). We also performed combustion analysis to confirm high purity of drug stocks, and conducted a separate cytotoxicity assay (trypan blue staining) to confirm cytotoxicity results (data not shown). Taken together, we conclude that gemcitabine has low cytotoxicity in this cell line, possibly due to inactivation by rapid deamination of

gemcitabine to its major metabolite, 2',2'-difluoro-2'-deoxyuridine (dFdU).^{39, 40} Unlike gemcitabine, KP-1212 was non-toxic up to 200 μ M (data not shown), and the addition of KP-1212 (5 μ M) did not alter the cytotoxicity of gemcitabine

(Figure 4 & Table 1). These results indicate that enhanced cytotoxicity cannot explain the potentiation of the antiviral activity of gemcitabine by KP-1212. Similarly, the antiviral activity of resveratrol also could not be explained by cytotoxicity, as the CC₅₀ was markedly higher than the EC₅₀ (>400 μ M; Figure 4 & Table 1). Furthermore, the addition of KP-1212 (200 μ M) did not significantly alter the cytotoxicity of resveratrol. In summary, KP-1212 potentiates the antiviral activity of gemcitabine and resveratrol without augmenting cytotoxicity, which improves their selectivity indices against HIV-1 (Table 1).

2.6. Characterization of viral mutation spectra in the presence of KP-1212 and RNRI

To determine the types of mutations resulting from these drugs (i.e., individually and in combination), we sorted out mCherry+/GFP- cells infected in the presence or absence of drug. We then amplified and performed Sanger sequencing of the *gfp* gene. We chose not to include deferoxamine in this analysis due to its poor potency against HIV-1 (Table 1). We obtained a total of 376 unique mutations across all treatments, but several G-to-A hypermutants (likely a result of APOBEC3 activity) were excluded from further analysis (see Section 3.7 for details), lowering the total number of mutations to 303. In the absence of drug, G-to-A mutations were the most common, followed by A-to-G, T-to-C, and C-to-T transitions, with the remainder representing transversions (Figure 5). In the presence of only gemcitabine or resveratrol, no statistically significant ($p < 0.05$; 2-way Fisher's exact test) change in the relative appearance of each mutation type within the HIV-1 mutation spectrum was observed compared to the no drug control, suggesting that these drugs did not cause an increase in any particular mutation type. KP-1212, despite having only a modest ability to elevate the mutant frequency of HIV-1, caused a significant increase (p -value = 0.004) in the frequency of G-to-C mutations (from 1% to 22% of all mutations identified, relative to that of no drug), a transversion mutation rarely observed during HIV-1 replication. Strikingly, the combination of KP-1212 with resveratrol led to an even greater increase in the frequency of G-to-C mutations (up to 41% of all substitutions identified), which is significantly different from either resveratrol alone (p -value < 0.0001) or KP-present 1212 alone (p -value = 0.048). The combination of KP-1212 and gemcitabine did not result in a significant increase in the frequency of G-to-C mutations (p -value = 0.130), likely due to a lower concentration of KP-1212 used in this combination (i.e., 5 μ M versus 200 μ M) due to drug antagonism observed at higher concentrations of KP-1212.

KP-1212 has not been previously found to induce G-to-C transversions, but 5-aza-2'-deoxycytidine (decitabine) and its ribonucleoside counterpart (5-AZC), have been reported to cause G-to-C transversions in HIV-1 and cellular genomic DNA.^{20, 29, 41} These molecules decompose in water at physiological temperature and pH by hydrolytic opening and deformylation of the triazine ring.⁴²⁻⁴⁷ The resulting ring-opened guanylurea derivatives are thought to pair with cytosine leading to G-to-C transversions.⁴¹ It is unclear how KP-1212 might induce G-to-C transversions, as KP-1212 should be resistant to hydrolysis.

2.7. Conclusions

While KP-1212 exerted minimal antiviral and mutagenic activity against HIV-1 in our assay, it was able to enhance the antiviral activities of the RNRI gemcitabine and resveratrol, leading to robust reductions in HIV-1 infectivity and improvement of their selectivity indices. We found that the combinations simultaneously elevated the mutant frequency of HIV-1, suggesting enhancement of virus mutational loads as a major mechanism of the drug combinations. While the RNRI gemcitabine and resveratrol do not

appear to cause a specific type of mutation, the addition of KP-1212 to resveratrol led to a pronounced increase in G-to-C transversion mutations. Further studies of the antiretroviral mechanisms of these combinations are warranted, as are efforts to identify prodrug forms and derivatives exhibiting reduced toxicity, improved potency, and prolonged stability. Our findings suggest that the enhancement of viral mutant frequency is a major contributor to these antiviral mechanisms, but do not preclude other activities – such as inhibition of reverse transcription. Resveratrol, while well-tolerated, has low bioavailability due to rapid and extensive metabolism in a number of clinical trials⁴⁸ but may serve as a lead for further development. It would also be of great interest to investigate the interplay of RNRIs ± KP-1212 with conventional anti-HIV-1 inhibitors. Previous studies have shown that these inhibitors can accelerate mutagen-driven viral extinction. This has been demonstrated for HIV-1 using AZT and 5-hydroxy-2'-deoxycytidine in combination and in foot-and-mouth disease virus, in which case sequential treatments were superior to combination therapy.^{49, 50} Overall, our findings are the first to demonstrate that a mild anti-HIV-1 mutagen can significantly potentiate the antiretroviral activity of RNRIs. These observations may enhance the potential for clinical translation of KP-1212 for the treatment of HIV-1 infection.

3. Materials and methods

3.1. Plasmids, cell lines, and reagents

The molecular clone of HIV-1 we used to produce virus, pNL4-3 MIG, was created from pNL4-3 HIG, which has been previously described.²⁰ The virus encoded by this plasmid contains *mCherry*, an internal ribosome entry site (IRES), and enhanced green fluorescent protein (GFP). We pseudotyped virions with VSV-G expressed from pHCMV-G, a kind gift from J. Burns (University of California, San Diego). The human embryonic kidney (HEK 293T) cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) from Cellgro (Manassas, VA) with 10% HyClone FetalClone III (FC3) from Thermo Scientific (Waltham, MA) and 1% penicillin/streptomycin from Invitrogen (Carlsbad, CA). U373-MAGI-CXCR4_{CEM} cells were obtained from Michael Emerman through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.⁵¹ U373-MAGI cells were maintained similarly to 293T cells but with addition of 1.0 µg/mL puromycin, 0.1 mg/mL hygromycin B, and 0.2 mg/mL neomycin to the medium. The chemicals investigated were obtained from Sigma-Aldrich (deferrioxamine and resveratrol; St. Louis, MO), Carbosynth (5-AZC and gemcitabine; Compton, UK), and Berry & Associates (KP-1212; Dexter, MI), dissolved in the appropriate solvent, and stored at –20 °C. For cloning, PCR was performed using the Platinum PCR Supermix from Invitrogen, while restriction enzymes, Klenow fragment, and T4 DNA ligase were from New England Biolabs (Ipswich, MA). For transfections, poly-L-lysine was from Newcomer Supply (Middleton, WI), and polyethylenimine (PEI) was from Polysciences, Inc. (Warrington, PA).

3.2. Construction of an mCherry/GFP HIV-1 vector for rapid mutation detection

The *mCherry* gene was swapped with the mouse *heat stable antigen (hsa)* in pNL4-3 HIG to generate pNL4-3 MIG as follows. From NL4-3 HIG, an IRES-GFP fragment was amplified *via* PCR and cloned into a pEGFP-N1-based mCherry vector using NotI and XbaI. The NotI site was then destroyed by fill-in with large Klenow fragment. The entire MIG cassette was then amplified using primers that added a NotI site to the 5' end and a XhoI site to the 3' end of the cassette. The PCR product was directly digested with NotI and XhoI and ligated into NotI and XhoI-digested pNL4-3 HIG using T4 DNA ligase. The resulting plasmid, pNL4-3 MIG, expresses mCherry and GFP as well as all viral proteins except Env and Nef.

3.3. Virus production and titering

Virus was produced by co-transfecting pNL4-3 MIG and pHCMV-G into 293T cells *via* the PEI method.⁵² PEI stocks were prepared at 1 mg/mL by dissolving PEI in water, adjusting the pH to 7.0, and filtering through a 0.2 μ M filter. Stocks were divided into 1 mL aliquots and frozen at -80°C . On the day before transfection, 4 million 293T cells were plated on each 10 cm plate (pre-coated with poly-L-lysine) to be transfected. For each plate, 10 μ g pNL4-3 MIG + 1 μ g pHCMV-G + 33 μ L 1 mg/mL PEI were mixed in a 1.6 mL microcentrifuge tube to a final volume of 1 mL using serum-free DMEM. After 20 minutes of incubation, the medium on the 293T cells was replaced and the DNA-PEI mixture was added. The medium was replaced the morning after transfection, and virus was collected ~48 hours post-transfection by filtering the supernatant through a 0.2 μ m filter. Viral stocks were divided into 1 mL aliquots and frozen at -80°C .

Prior to any drug treatments, viral stocks were first titrated in U373-MAGI cells. The day before infection, 62,500 cells/well were plated in 12-well plates. The next day the media was replaced and varying amounts of virus ranging from 1.25 to 40 μ L were added. The media was replaced again 24 hours post-infection and cells were collected at 72 hours post-infection for analysis by flow cytometry. The infectious titer was calculated from the flow data by adding all positive quadrants (mCherry+ only, GFP+ only, and mCherry+/GFP+) to determine infectivity and then plotting the volume of virus against infectivity.

3.4. Drug treatments and infections

Drug treatments were performed using U373-MAGI cells in 12-well plates similar to the way in which viral stocks were titrated (Section 3.3). Two hours prior to infection, drugs (1-2 μ L) were added to a final volume of 0.5 mL media in each well. Uninfected cells and no drug (DMSO only-treated cells) were included as controls. After two hours, virus and additional media were added, resulting in a final volume of 1 mL in each well. Drug concentrations were based on the final volume of 1 mL. The amount of virus added was targeted to achieve 15-30% infection (for the no drug control) based on the previously determined viral titer. Drugs were removed by replacing the media 24 hours post-infection. The cells were collected 72 hours post-infection for flow cytometry by treating with trypsin, pelleting at 500xg for 5 minutes, resuspending in 200 μ L Dulbecco's Phosphate-Buffered Saline (DPBS) + 2% FC3, and transferring to 96-well plates.

3.5. Infectivity and mutant frequency determination by flow cytometry

Cells were analyzed for mCherry and GFP expression on a BD LSR II flow cytometer (BD Biosciences; San Jose, CA). Cells were first gated based on forward scatter and side scatter, and a minimum of 10,000 gated cells were analyzed per sample. GFP was excited with a blue 488 nm laser and emission detected using 505LP and 525/50 filters. mCherry was excited by a custom green 561 nm laser and emission detected using 595LP and 610/20 filters. It should be noted that early on in our work we determined that very little fluorescent compensation was necessary with this particular combination of fluorophores, lasers, and filters (data not shown). These control experiments were performed by transfecting mCherry or GFP into 293T cells and then analyzing the transfected cells (either kept separately or mixed together) by flow cytometry. Flow cytometry data were examined in FlowJo v. 7.6.1 (Ashland, OR). Infectivity was determined by adding all three positive populations: mCherry+ only, GFP+ only, and mCherry+/GFP+. Mutant frequency was calculated by dividing the single positive populations (mCherry+ only and GFP+ only) by all infected cells. Double negative (mCherry-/GFP-) cells were excluded due to the difficulty in detecting these rare events; mutant frequency calculations are therefore underestimates. Infectivity and mutant frequency data were normalized to the no drug controls.

3.6. Cellular toxicity analysis

The cytotoxicity of drugs was assessed using the CellTiter-Glo Luminescent Cell Viability Assay from Promega (Madison, WI) following the manufacturer's instructions. 5000 U373-MAGI cells/well were plated in 96-well plates the day before drug treatment. The following day the medium was replaced and drugs (alone or in combination) were added to a final volume of 200 μ L/well. No drug (DMSO only) and no cell wells were included as controls. The luminescence was recorded using an Orion microplate luminometer from Berthold Detection Systems (Huntsville, AL). The data were analyzed by first subtracting the no cell control value and then normalizing to the no drug control.

3.7. Characterization of mutation spectra in HIV-1

Infected mCherry+/GFP- U373-MAGI cells were sorted at the Masonic Cancer Center at the University of Minnesota using a BD FACSARIA II. A minimum of 3000 cells were collected per sample. Genomic DNA was isolated by the High Pure PCR Template kit from Roche (Indianapolis, IN). GFP was amplified in a single PCR reaction using the Platinum PCR Supermix from Invitrogen and the primers 5'-CAAGCGTATTCAACAAGG-3' and 5'-GCAATACAGCAGCTAACAAATG-3'. The PCR product was ligated into pGEM-T (Promega) and the *gfp* gene was sequenced at Functional Biosciences, Inc. (Madison, WI) using the T7 promoter primer. Sequences were aligned to the reference and mutations identified using Lasergene (DNASTAR, Inc., Madison, WI). Duplicate mutations, presumably arising from PCR amplification, were excluded from analyses. Several G-to-A hypermutants (multiple G-to-A mutations within the same sequence) were observed, and these likely resulted from APOBEC3 activity, as they occurred in a GA or GG dinucleotide context and were present in both the no drug as well as drug treatment samples. These rare events were excluded from the analyses due to their potential for creating false differences in the rate of G-to-A mutations between samples.

3.8. Statistical analyses

All graphs were created in Microsoft Excel v 12.3.5 (Redmond, WA) or GraphPad Prism v 5.0 (GraphPad Software, Inc.; La Jolla, CA). EC₅₀ values of drugs (drug concentration at which there is a 50% reduction in viral infectivity) were determined by plotting normalized infectivity against the log-transformed drug concentrations and fitting a non-linear regression curve to the data. Likewise, CC₅₀ values of drugs (drug concentration at which there is 50% cytotoxicity) were determined by plotting normalized cell viability against the log-transformed drug concentrations and fitting a non-linear regression curve to the data. Two-way ANOVA was performed using the statistical package R to compare the impact of drug combinations against single drugs on infectivity and mutant frequency. All analyses were performed on the log-transformed data without normalization. Differences in mutation spectra were assessed using Fisher's exact test in GraphPad Prism.

Acknowledgments

This research was supported by NIH grants R01 GM56615 and R21 AI96937. J. M. R. was supported by the Institute for Molecular Virology Training Program (NIH grant T32AI83196); R. H. H. was supported by the MinnCResT Program (NIH grant T32DE007288); L. B. B. was supported by NIH grant F31DA030249; J. L. M. was supported by a University of Minnesota Basic Sciences PhD Graduate Programs Council Fellowship; M. J. D. was supported by NIH grant T32DA007097. Combustion analysis of KP-1212 and gemcitabine was performed by Atlantic Microlab, Inc. (Norcross, GA). The authors would like to thank Laurent Bonnac for performing ¹H NMR of KP-1212 and Cavan Reilly for suggestions regarding statistical tests and procedures.

Abbreviations

5-AZC	5-azacytidine
dFdC	2',2'-difluoro-2'-deoxycytidine
dFdU	2',2'-difluoro-2'-deoxyuridine
DMSO	dimethylsulfoxide
KP-1212	5-aza-5,6,-dihydro-2'-deoxycytidine
MOI	multiplicity of infection
NRTI	nucleoside reverse transcriptase inhibitor
RNRI	ribonucleotide reductase inhibitor
sd	standard deviation

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Figure 1.

Vector and assay design for rapid determination of HIV-1 infectivity and mutant frequency. (A) A dual-reporter (mCherry/GFP) HIV-1 vector was created and co-transfected with VSVG to produce infectious virus. Target cells were pre-incubated with drugs for 2 h before infection. Drug was removed 24 h post-infection and cells were collected 72 h post-infection for determination of infectivity and mutant frequency *via* flow cytometry, as described in Section 3.5. (B) The utility of the dual reporter HIV-1 vector for detecting alterations in infectivity and mutant frequency was validated using 5-azacytidine, a previously characterized potent mutagen of HIV-1²⁹. The data were normalized to the no drug (DMSO only) control and represent the mean $\pm$ sd of three independent experiments.

Figure 2.

Effect of KP-1212 and RNRIs on HIV-1 infectivity and mutant frequency. Cells were infected with HIV-1 after being treated with KP-1212 or selected ribonucleotide reductase inhibitors (RNRIs) for 2 h. Treatments were concluded at 24 h post-infection by replacing the media, and cells were collected for analysis by flow cytometry at 72 h post-infection. The effects of the drugs on infectivity (A) and mutant frequency (B) were determined and normalized to the no drug control. The data shown are the mean $\pm$ sd of three independent experiments.

Figure 3.

KP-1212 potentiates the antiviral and mutagenic activities of RNRI against HIV-1. Cells were pre-treated with a set amount of each ribonucleotide reductase inhibitor (RNRI) and varying amounts of KP-1212 (from 0 to 200 μM) as described in Section 3.4. Flow cytometry was used to determine HIV-1 infectivity (A) and mutant frequency (B), which were plotted relative to no drug. Statistical significance of drug combinations relative to both single drugs was assessed using a two-way ANOVA. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001. The data shown represent the mean ± sd of three independent experiments.

Figure 4.

The ability of KP-1212 to potentiate RNRIs is not due to enhanced cytotoxicity. U373-MAGI cells were treated with ribonucleotide reductase inhibitors (RNRIs) $\pm$ KP-1212 (5 μ M for gemcitabine, 200 μ M for resveratrol and deferoxamine) for 24 h, then washed and replenished with fresh media. Cell viability was assessed 24 h after molecule removal using a luminescent ATP-based kit, and the data were normalized to the no drug control. The data shown are the mean $\pm$ sd of four independent experiments.

Figure 5.

Characterization of viral mutation spectra in the presence of KP-1212 and RNRI. U373-MAGI cells were pre-treated with KP-1212 and/or ribonucleotide reductase inhibitors (RNRIs) and infected with HIV-1. mCherry+/GFP- cells were sorted to enrich for mutants, and the *gfp* gene was amplified and sequenced. Each number in a wedge of a pie chart represents the specific corresponding mutational type, expressed as the percentage of the total mutations observed for that treatment. The total number of mutations sequenced were: no drug, 45; gemcitabine (Gem), 38; resveratrol (Res), 45; KP-1212, 49; gemcitabine + KP-1212, 57; resveratrol + KP-1212, 69 (303 mutations in total). Statistically significant (p -value < 0.05) changes relative to the no drug control are indicated with an asterisk.

Table 1

KP-1212 improves the antiretroviral potency of RNRIIs without altering cytotoxicity.

Drug	– <i>KP-1212</i>			+ <i>KP-1212</i>		
	EC ₅₀	CC ₅₀	SI	EC ₅₀	CC ₅₀	SI
Gemcitabine	27.5 nM (26.0-29.1)	62.0 μM (59.0-65.2)	2250	21.6 nM (20.9-22.3)	64.2 μM (60.8-67.8)	2970
Resveratrol	99.6 μM (92.5-107.4)	> 400 μM	> 4.0	52.8 μM (39.1-71.2)	> 400 μM	> 7.6
Deferoxamine	384 μM (288-514)	> 400 μM	> 1.0	248 μM (194-316)	> 400 μM	> 1.6

The EC₅₀ and CC₅₀ values for ribonucleotide reductase inhibitors (RNRIIs) in the presence or absence of KP-1212 (5 μM for gemcitabine; 200 μM for resveratrol/deferaxamine) in U373-MAGI cells were calculated as described in Section 3.8. Selectivity indices were determined by dividing the CC₅₀ by the respective EC₅₀. The 95% confidence intervals are indicated in parentheses. The data shown represent the mean ± sd of at least three independent experiments.