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KP-1212/1461, a nucleoside designed for the treatment of HIV by viral mutagenesis

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Abstract

We report the activities of a novel nucleoside analog against HIV. This nucleoside (KP-1212) is not a chain terminator but exerts its antiviral effects via mutagenesis of the viral genome. Serial passaging of HIV in the presence of KP-1212 causes an increase in the mutation rate of the virus leading to viral ablation. HIV strains resistant to KP-1212 have not yet been isolated. Quite to the contrary, virus treated with KP-1212 exhibited an increased sensitivity not only to KP-1212 but also to another nucleoside reverse transcriptase inhibitor (NRTI), zidovudine. HIV strains resistant to other NRTIs (e.g. zidovudine, lamivudine, stavudine, abacavir, etc.) exhibited no cross-resistance towards KP-1212. Multiple assays confirmed that KP-1212 has a favorable (low) genotoxicity profile when compared to some approved antiviral nucleosides. In addition, KP-1212 is not toxic to mitochondria nor does it exhibit any inhibitory effects on mitochondrial DNA synthesis.

Keywords: HIV; Mutagenesis; Novel nucleoside analog

1. Introduction

The error-prone nature of retroviral reverse transcriptases (RT) like that of HIV provide a selective advantage for the virus to evade host immune responses and chemotherapy. Because of the error proneness of the viral RT (Preston et al., 1988; Roberts et al., 1988) and the high rate of HIV replication (Coffin, 1995), the viral genome in patients is not homogenous but rather exists as variants of nucleic acid sequences otherwise known as a quasispecies (Eigen, 1993). The high error rate of RT also has a detrimental effect on the virus. Indeed most of the HIV virus particles found in infected blood are nonviable, most likely due to an accumulation of debilitating mutations (Coffin, 1995). It has been proposed that the mutation rate could be artificially increased by the introduction of a mutagenic nucleoside (Loeb et al., 1999). This increase in mutation rate would lead the viral population to cross the threshold for error catastrophe and ablate the viral population as a whole. Experiments with 5-hydroxy-2′-deoxycytidine (5-OH-dC) first demonstrated it was possible to extinguish HIV by repeated passaging in the presence of a mutagenic nucleoside analog, although this was only achieved at high drug concentrations (0.5–1 mM).

In this article, we describe the activity of another mutagenic nucleoside analog, 5-aza-5,6,-dihydro-2′-deoxycytidine (KP-1212), which is several orders of magnitude more potent than 5-OH-dC and has a very attractive safety profile. KP-1212 is the 2′-deoxy derivative of 5-aza-5,6-dihydrocytidine (DHAC) which has been studied in oncology clinical trials (Creagan et al., 1993; Curt et al., 1985; Samuels et al., 1998). KP-1212 has been shown to be a metabolite of DHAC (Kees and Avramis, 1995). KP-1212 can ablate HIV after 8–13 passages at concentrations in the nanomolar to low micromolar range. It typically increases the viral mutation rate by 50–100% without evidence of genotoxic effects on mammalian cells under a variety of assay conditions. Most importantly, there is no evidence of cross resistance with HIV strains resistant to other nucleoside analogs or evidence of development of de novo resistance by HIV to

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KP-1212. In addition, HIV treated with KP-1212 shows increased sensitivity toward not only toward KP-1212 but also to another nucleoside analog (zidovudine, AZT). It appears to be non-toxic to mitochondria, as judged by lactate production and mitochondrial DNA synthesis.

2. Materials and methods

2.1. Cytotoxicity assays

MT-2 cells were treated with half log increasing concentrations of KP-1212 for 5 days at 37 °C. Cell viability was determined by an MTT assay. A cytotoxic concentration exhibiting 50% toxicity (CC_{50}) was determined.

2.2. Cells and viruses

MT-2 cells were maintained in RPMI 1640 medium-10% fetal bovine serum (GIBCO). HIV type 1 (HIV-1) was generated by transfecting the HIV-containing plasmid pNL4-3 into MT-2 cells. Stocks of NL4-3 virus were prepared and titered on polybrene-treated (10 μg/ml) MT-2 cells. Cell-free viral supernatants were serially diluted and added to fresh MT-2 cells in a 96-well plate format to determine the viral titer or tissue culture infectious dose 50% ($TCID_{50}$). Wells positive for HIV infection were scored by visualization of HIV-induced syncytia and confirmed by p24 ELISA (Beckman–Coulter). Drug-resistant HIV strains were obtained from NIH AIDS Research and Reference Reagent Program. In particular, the AZT-resistant strain RTMC, and the multi-drug resistant strain, 1617 (Kantor et al., 2001; Larder et al., 1990, 1991) were used.

2.3. Single passage EC_{50}

10^7 MT-2 cells were suspended in 1 ml of RPMI and infected with 2×10^3 $TCID_{50}$ HIV resulting in a multiplicity of infection (MOI) of 1:5000. The virus-cell suspension was incubated for 1 h at 37 °C. During the 1 h incubation, 1 ml of KP-1212 at a 2× concentration was added to each well of a 24-well plate. After virus-cell incubation, 24 ml of RPMI was added to this mixture to give a final volume of 25 ml. One millilitre of the virus-cell suspension was added to each well of the 24-well plate containing KP-1212 to yield the following final concentrations: 0, 0.01, 0.1, 1, 10, and 100 μM (in quadruplicate). The plate was then spinoculated at 1200 × *g* RPM for 2 h (O'Doherty et al., 2000). Thereafter, the plate was placed in a 37 °C incubator containing 5% CO_2 for 4 days. The cell-free supernatants were collected for p24 measurement and for $TCID_{50}$ determination. Based on these results, an effective concentration showing 50% inhibition could be determined (EC_{50}). EC_{50} values and CC_{50} values were calculated using BioDataFit (Chang Bioscences, Castro Valley, CA) and the model [a $(1–10^{-bx})$], which gives an r^2 value of 0.9 or better with our data sets.

2.4. Serial passage experiments

The serial passage experiments were initiated in much the same manner as the single passage experiment except that the starting MOI was 1:1000 or 1:100. After the cell-free supernatant was harvested from each well (∼2 ml), the p24 levels were measured. Then three 0.4 ml aliquots were stored at −80 °C for future passages and a 0.3 ml aliquot was used to determine the viral titer. The cells were stored at −80 °C and used for sequencing proviral DNA. Preliminary experiments comparing p24 levels to $TCID_{50}$ values indicated that 3 ng of p24 was approximately equal to 1000 $TCID_{50}$ (data not shown). The volume of cell-free supernatant to transfer for the next passage was dependent on the average p24 levels present in the negative drug controls (NDC). The supernatants from the quadruplicate NDC were pooled and used to infect MT-2 cells at an MOI of 1:1000. The same was done for each of the five KP-1212-treated supernatants. For example, if 100 μl of pooled cell-free supernatant was equivalent to 2000 $TCID_{50}$ then this volume would be added to 2×10^6 fresh MT-2 cells to end up with a final MOI of 0.001. This same volume, 100 μl, was applied for the cell-free supernatants with the other concentrations (i.e. 0.01, 0.1, 1, 10 and 100 μM). This process was continued until a loss of infectivity was observed in a KP-1212-treated passage. An additional passage was performed to confirm viral ablation.

2.5. Cross resistance

The cross-resistance studies were conducted in a similar manner as the single passage EC_{50} studies described above. The drug-resistant strains used in this study were RTMC and 1617. RTMC is an AZT-resistant strain containing the following resistant mutations: 67N, 70R, 215F and 219Q (Larder et al., 1990, 1991). 1617 is a multi-drug resistant (ABC, DDI, 3TC, D4T, TDF, DDC and AZT) strain containing the following resistant mutations: 69K, 70G, 75I, 77L, 116Y, 151M and 184V (Kantor et al., 2001). These viral isolates were obtained from the NIH AIDS Research and Reference Reagent Program.

2.6. Sequencing

Two regions of the HIV genome were targeted for sequencing. The reverse transcriptase (RT) gene was sequenced because it is relatively conserved. The V3 loop of the envelope (ENV) gene was sequenced because of its high tolerance for mutations, as evidenced by its sequence heterogeneity. The primer sets used to amplify each sequence (Invitrogen Life Technologies) were as follows:

RT Sense 5′-ATTTTCCCATTAGTCCTATTGAGACTGTACC-3′; RT Antisense 5′-CTGTTAGTGGTACTACTTCTGTTAGTGCTTTGG-3′; ENV Sense 5′-AATTCCCATACATTATTGTGCCCCGGC-3′; ENV Antisense 5′-GTGTCACTTCCTTCAGTGTTATTTGACCC-3′.

The respective PCR products were amplified from proviral DNA, cloned into a TA vector (Invitrogen) and sequenced using a CEQ 8000 capillary electrophoresis unit (Beckman–Coulter). The raw data were analyzed using Seqman (DNASTAR, Madison, WI).

2.7. Genotoxicity

Genotoxicity was determined by quantifying the mutations occurring in the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene in Chinese hamster ovary (CHO) cells and in male lymphoblasts. The CHO/HGPRT and the human male lymphoblast/HGPRT assays have been described (O'Neill et al., 1977; O'Neill and Hsie, 1977; Sussman et al., 1999).

2.8. Mitochondrial toxicity studies

CEM cells were passaged in the presence of ddC or KP-1212 for 9 days. Every 3 days the cells were passaged into fresh RPMI (GIBCO) media containing either ddC (0.1, 0.3, 1, 3, 10 and 30 μM) or KP-1212 (10, 30, 100, 300 μM and 1 mM). KP-1212 was synthesized in-house by Koronis Pharmaceuticals, Inc and was >99.8% pure. A positive control, ddC, was purchased from Sigma, St Louis, MO (D5782, Lot no. 020K3459) and prepared as a 0.1 M solution in DMSO. A suspension of CEM cells in RPMI at a concentration of 3×10^5 cells/ml was prepared. A 10× solution of each drug concentration for the two drugs to be tested was prepared from the 0.1 M stock solution of ddC or KP-1212 in RPMI. One hundred microlitres of the 3×10^5 cells/ml suspension were seeded into each sample well of a 96-well flat-bottomed tissue culture plate. Eighty microlitres of RPMI was added to each well. Twenty microlitres of 10× drug solution were added to the corresponding sample well. All samples were tested in triplicate. After 72 h of incubation at 37 °C/90% humidity, the CEM cell culture solution in each well was mixed thoroughly and the CEM cells in each well were counted on a hemocytometer. The cell count for each well was recorded and used to determine the volume of cell suspension from that well needed to be transferred to obtain 3×10^4 cells in each well of a new 96-well flat bottom tissue culture plate. A volume of RPMI was added to each well to bring the final volume in the well to 180 μl. 10× drug solutions were prepared as on day 1, and 20 μl of each were added to the appropriate sample wells. This process was repeated for a third time for a total of 9 days. The lactic acid concentration present in the tissue culture supernatant was determined by spectrophotometric determination of NADH using the lactate dehydrogenase/NAD^+ assay (Sigma–Aldrich Company Ltd., procedure no. 826-UV).

Passaging of CEM cells for the mtDNA study was done as described below except that T-25 flasks were used instead of 96-well plates in order to extract enough cellular DNA for Southern analysis. In addition, only one concentration of ddC (3.2 μM) and KP-1212 (320 μM) were used. Each condition, NDC, 3.2 μM ddC and 320 μM KP-1212, was tested in triplicate. Total cellular DNA was purified using DNeasy™ tissue kit (Qiagen Cat. no. 69506). One microgram of total DNA was utilized in dot-blot Southerns to quantify the amount of nuclear DNA and mitochondrial DNA (mtDNA) in CEM cells treated with nucleoside analogs versus untreated controls. The probe specific for nuclear DNA was the β-globin gene, and the probe specific for mtDNA was the D-loop 2 segment.

3. Results

3.1. Cytotoxicity

Concentrations of KP-1212 inhibiting 50% of cellular proliferation (CC_{50}) were determined utilizing various cell types. The CC_{50} was 0.83 mM for CHO cells and >1 mM for all lymphoid cells, namely, MT-2, TK6 and CEM cells (data not shown).

3.2. Single passage inhibition of HIV growth by KP-1212

KP-1212 (Fig. 1a) was studied for its ability to inhibit HIV growth in vitro. At a multiplicity of infection (MOI) of 1:5000, the viral titer of drug treated samples produced a no-

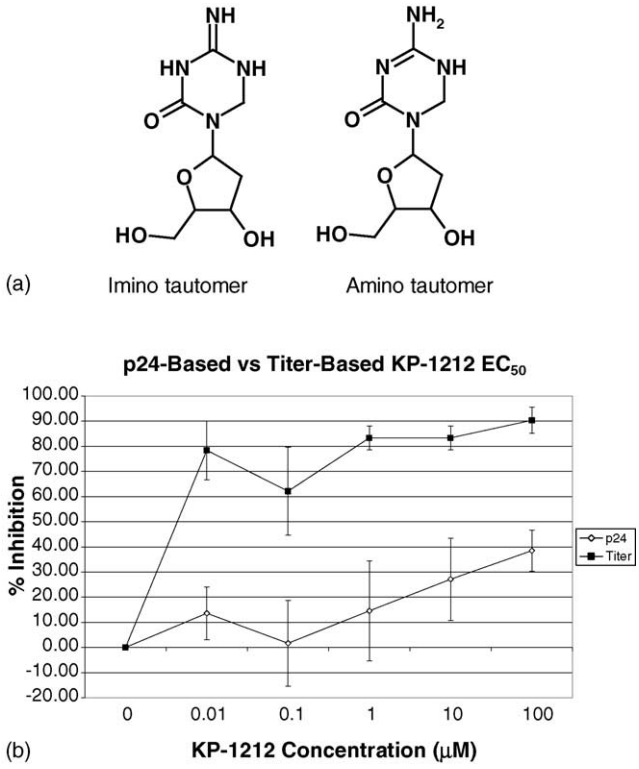

Fig. 1. (a) Imino and amino tautomers of KP-1212. (b) Single passage inhibition of HIV growth by KP-1212. Comparison of p24-based vs. titer-based inhibition curves.

tably different inhibition curve from that achieved with p24 (Fig. 1b). Inhibition based on the levels of p24 present in the cultures treated with KP-1212 versus the negative drug control (NDC) was almost 50% at 100 μM. However, when using viral titer as a basis for inhibition, 70–90% inhibition was achieved at KP-1212 concentrations from 0.01 to 100 μM with a maximum inhibition of 90% at 100 μM.

3.3. Serial passage of HIV in the presence of KP-1212 leads to viral ablation and an increase in HIV DNA mutations

In an effort to see if KP-1212 can eradicate HIV growth in vitro, the virus was serially passaged in the presence of varying concentrations of KP-1212. The potency of KP-1212 against HIV increased from the first passage to the 12th passage (see Fig. 2 and below). The first passage at an MOI of 1:100, demonstrated a difference in the inhibition curves when the p24 results were compared to HIV titers (Fig. 2a). The p24/titer difference became less apparent as the passage study progressed (Fig. 2b). Tracking the viral titer and p24 concentration of treated versus non-treated virus heralded the eventual viral ablation in the case of HIV treated with 10 μM KP-1212 by passage 13 (Fig. 2c and d). Viral cultures treated with the other KP-1212 concentrations (0.1, 1, and 100 μM, data not shown) exhibited viral ablation by the 14th passage, except for 0.01 μM KP-1212-treated virus which was minimally inhibited. In an effort to rescue viable virus from KP-1212-treated cultures, cell-free supernatants from passage 13 of 10 μM KP-1212 were used to inoculate fresh cells. These cells were then incubated for two weeks with refeeding in the absence of drug. Viable virus was not detected after several attempts, indicating that viral ablation was achieved. A similar experiment was carried out with an initial MOI of 1:1000 (data not shown). Viral ablation was first noted at passage 8 at a drug concentration of 10 μM.

3.4. Sequencing of viral genome following treatment with KP-1212

Sequencing of proviral DNA from the 11th passage of virus treated with 10 μM KP-1212 and virus from the negative drug control (NDC) exhibited an increase in the mutation frequency of the viral DNA treated with 10 μM KP-1212 by 45% for the RT gene (N.S., χ^2-test) and 91% for the ENV gene ($P < 0.025$, χ^2-test) (see Table 1). It is interesting to note that the majority of the mutations were transitions (purine to purine or pyrimidine to pyrimidine, i.e. A to G, G to A, C

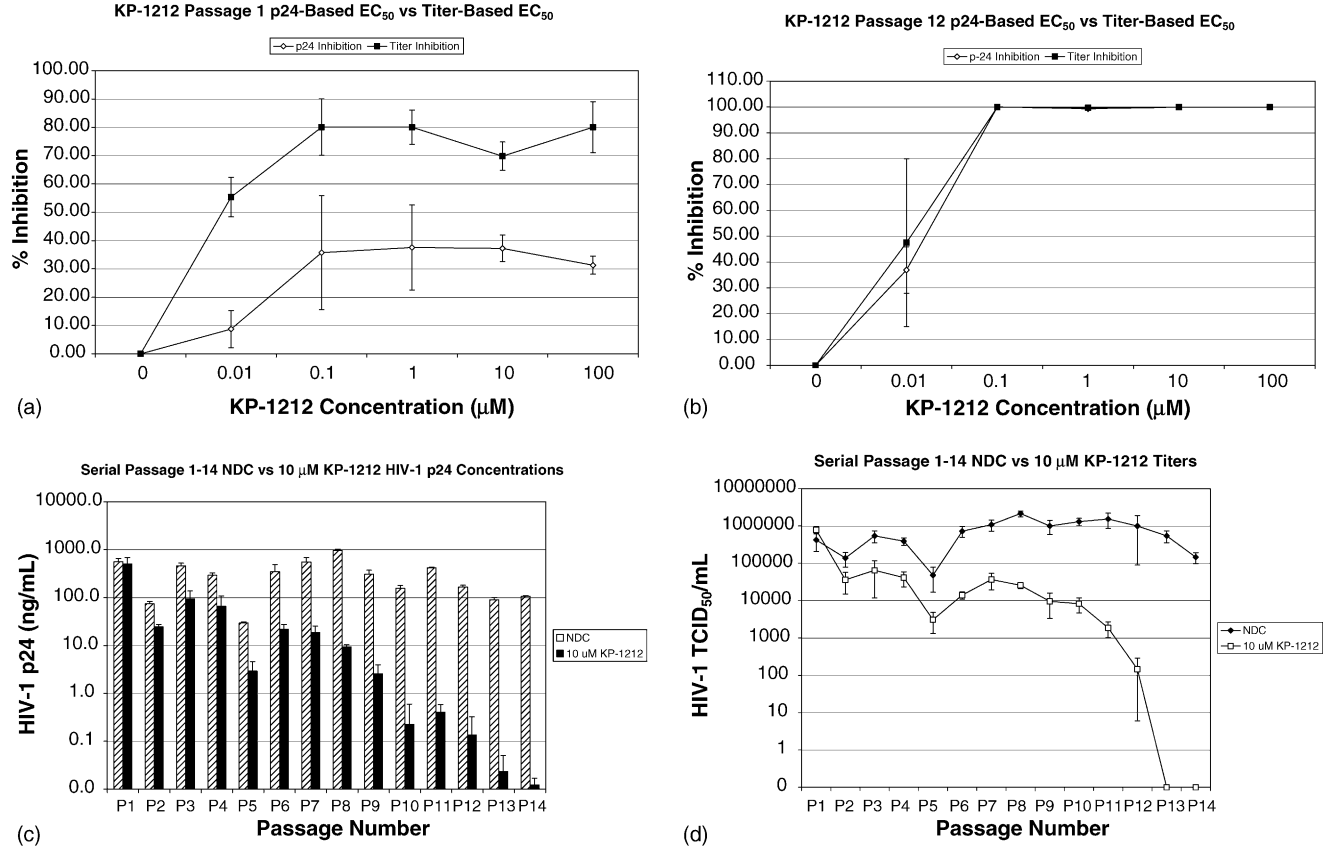

Fig. 2. (a) Serial Passaging HIV-1 in the presence of KP-1212. Comparison of the p24-based inhibition curves vs. titer-based curves from passage 1. (b) Serial Passaging HIV-1 in the presence of KP-1212. Comparison of the p24-based inhibition curves vs. titer-based curves from passage 12. (c) Comparison of the HIV-1 p24 concentrations from the NDC and 10 μM KP-1212 tissue culture supernatants. (d) Comparison of the HIV-1 titers ($TCID_{50}$/mL) from the NDC and 10 μM KP-1212 tissue culture supernatants.

Table 1
Sequence analysis of the HIV RT and Env genes after 11 serial passages in the presence or absence of KP-1212

NDC

	A	C	G	T
A to	-	1	9	0
C to	0	-	1	2
G to	2	2	-	0
T to	3	8	2	-
Total Mutations			27	
Nucleotides Sequenced			20333	
Mutation Rate			0.1328%	

10 uM KP-1212

	A	C	G	T
A to	-	0	15	0
C to	0	-	1	3
G to	12	2	-	0
T to	1	6	1	-
Total Mutations			41	
Nucleotides Sequenced			21355	
Mutation Rate			0.1920	
Δ in Mutation Rate			+44.58%*	

Envelope Sequencing Data Summary

NDC

	A	C	G	T
A to	-	1	7	2
C to	0	-	0	1
G to	8	0	-	1
T to	0	4	1	-
Total Mutations			25	
Nucleotides Sequenced			20394	
Mutation Rate			0.1226%	

10 uM KP-1212

	A	C	G	T
A to	-	1	23	0
C to	0	-	0	9
G to	2	0	-	0
T to	1	11	0	-
Total Mutations			47	
Nucleotides Sequenced			20095	
Mutation Rate			0.2339	
Δ in Mutation Rate			+90.78%*	

Reverse transcriptase sequencing data summary. $^{*}P < 0.025$, χ^2-test.

to T and T to C) with no increase in transversions (purine to pymidine or pyrimidine to purine).

3.5. Increased sensitivity HIV passaged in the presence to KP-1212 to nucleoside analogs

Virus from the 10 μM KP-1212 11th passage and the cognate passage from the NDC were investigated for their sensitivity toward KP-1212 and a nucleoside reverse trancriptase inhibitor (NRTI), AZT (Table 2). The virus passaged in the presence of KP-1212 demonstrated a seven-fold increase in sensitivity toward KP-1212 and a 14-fold increase in sensitivity toward AZT (Table 2).

3.6. Lack of cross-resistance with other nucleoside-resistant HIV strains

KP-1212 being a nucleoside analog, it seemed necessary to determine whether HIV strains resistant to other nucleoside reverse transcriptase inhibitors (NRTIs) were resistant to KP-1212. RTMC is a strain resistant to zidovudine (Larder et al., 1990, 1991) and 1617 is a multidrug-resistant strain resistant to lamivudine, abacavir, zidovudine, stavudine, zalcitabine, didanosine, and tenofovir (Kantor et al., 2001). These drug-resistant strains were analyzed for their sensitivity against KP-1212 in a single passage format. Resistance to KP-1212 was not observed in either strain (Fig. 3).

Table 2
Increased sensitivity of KP-1212 passaged virus to KP-1212 and AZT

	KP-1212 EC_{50}[a]	AZT EC_{50}[a]
KP-1212 naïve virus (11th passage)	10 nM	8.7 nM
10 μM KP-1212 passaged virus (11th passage)	1.4 nM	0.62 nM

[a] The EC_{50} values were based on the viral titer ($CCID_{50}$).

3.7. Genotoxic studies of KP-1212

Since KP-1212 is a deoxyribonucleoside, theoretically it could be incorporated into the host nuclear DNA. Detection of mutations in the HGPRT gene in CHO cells and in male lymphoblasts was used as an indicator for possible mutagenicity of KP-1212 for host genomes (O'Neill et al., 1977a; O'Neill and Hsie, 1977; Sussman et al., 1999). Treatment of CHO cells with KP-1212 ranging from 100 μM to 1 mM did not exhibit significant genotoxic effects on CHO cells for the duration of the study (Fig. 4a). Similar results were observed when male lymphoblasts were treated with KP-1212 at final concentrations up to 3 mM (data not shown).

3.8. Assessment of mitochondrial toxicity

To determine if exposure to KP-1212 caused mitochondrial toxicity, both a lactate assay and a test of mtDNA synthe-

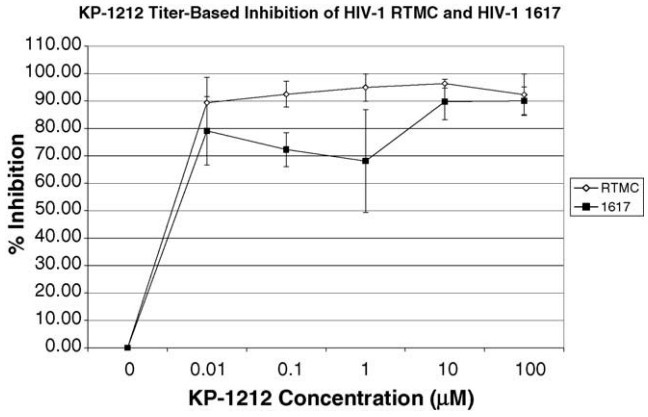

Fig. 3. KP-1212 cross-resistance studies. RTMC is an AZT-resistant strain containing the following resistant mutations: 67N, 70R, 215F and 219Q. 1617 is a multi-drug resistant strain (ABC, DDI, 3TC, D4T, TDF, DDC and AZT) containing the following resistant mutations: 69K, 70G, 75I, 77L, 116Y, 151M and 184V.

sis were performed. KP-1212 did not cause a significant increase in lactic acid levels at doses up to 1 mM, whereas ddC, a known mitochondrial toxin which inhibits mtDNA synthesis via chain termination, demonstrated a dose-dependent increase in lactate levels (Fig. 4b). Similarly, DNA Southern dot blots indicated that there was a 6% decrease of mitochondrial DNA (mtDNA) when cells were exposed to 320 μM KP-1212. In contrast, cells treated with a one hundred fold lower dose of ddC (3.2 μM) exhibited a 51% decrease in mtDNA relative to the negative drug control (data not shown). In another report (Murakami et al., 2005), the enzymology of KP-1212 interaction with HIV RT, DNA pol β and DNA pol γ have been studied in greater detail.

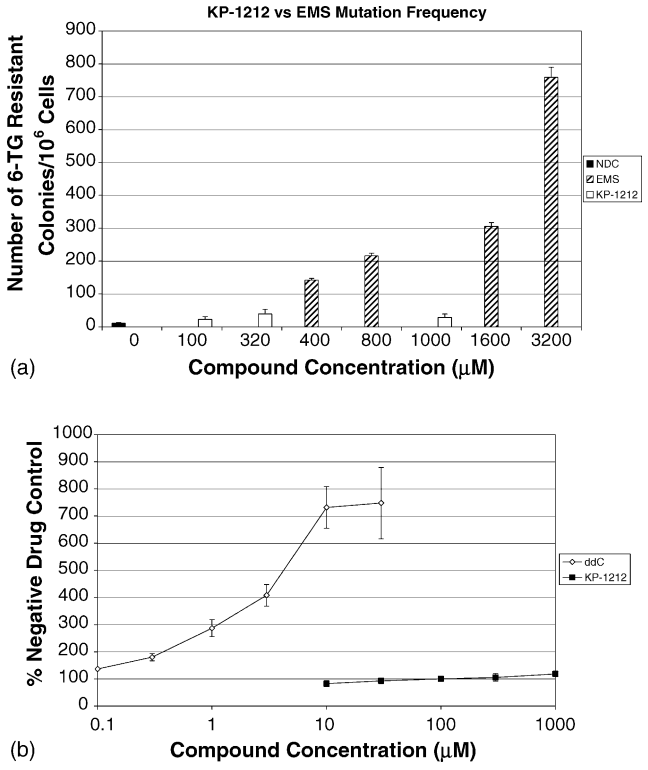

Fig. 4. (a) KP-1212 genotoxicity studies. CHO cells were treated with KP-1212 or a known mutagen, EMS. The hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene was used to measure genotoxic effects. Cells with a defective HGPRT gene are resistant to 6-thioguanine. (b) Mitochondrial toxicity studies. CEM cells treated with either ddC or KP-1212 were assayed for the accumulation of lactic acid over a 9-day-period. The results were then compared to the levels of the negative drug control.

4. Discussion

KP-1212 demonstrates a favorable therapeutic index (CC_{50}/EC_{50}), in the range of 100,000, using a variety of cell lines culture. The viral inhibition curve in Fig. 1b was obtained with HIV LAI, and similar EC_{50}'s were obtained with NL4-3. For many of our screening assays, NL4-3 is the preferred strain because its relative genetic homogeneity facilitates the detection of mutations induced by KP-1212. It should be noted that EC_{50} values for viral mutagens may have a different significance than EC_{50} values for currently approved antiviral agents. The discordance demonstrated in Fig. 1b between viral infectivity and conventional surrogate markers of viral load, such as p24, is not surprising, as it has also been observed in the context of in vitro treatment of riboviruses by mutagenic RNA nucleosides (Crotty et al., 2000, 2001; Pariente et al., 2001) and presumably reflects the increased proportion of non-infectious viral particles in the presence of a viral mutagen, in this case, KP-1212. Evidence indicates that ribavirin increases the mutation frequency of poliovirus by 10-fold at a concentration of 1 mM (Crotty et al., 2001). The non-infectious viruses can still produce the marker of interest, e.g. p24 or HIV RNA, but are not capable of viral propagation. Furthermore, the EC_{50} of KP-1212 has been shown to be very dependent on the MOI. The viral inhibition curve presented in Fig. 1b can be achieved with MOI's in the range of 1:1000 to 1:5000 and after several such experiments, the EC_{50} is considered to be approximately 10 nM. A higher MOI leads to the integration of relatively unaffected viral genomes in a larger proportion of cells. Thus, a higher MOI leads to higher EC_{50}, presumably reflecting fewer rounds of viral replication and less opportunity for incorporation of the mutagenic analog. Reproducible EC_{50}'s cannot be reliably achieved with an MOI of 1:300 or greater. It is also noteworthy that in a single passage experiment, viral inhibition beyond 80–90% cannot be achieved. This presumably reflects the difficulty in incorporating a sufficient number of mutations that are lethal to all of the viral population in a small number of cycles of viral replication.

Serial passaging a virus with a higher MOI might be "equivalent" to infecting cells with a lower MOI for a single passage. To achieve viral eradication in vitro, passaging is necessary. Viral extinction can be demonstrated by passaging the virus in the presence of KP-1212. Not surprisingly, the data suggest that with a higher initial MOI, more passages are

necessary for viral ablation. Based on sequencing analysis of genomes from treated versus untreated virus, it is reasonable to conclude that viral ablation is achieved by the increase in number of mutations per viral genome caused by KP-1212. This mutagenic nucleoside was selectively incorporated into the HIV genome by the HIV RT enzyme thus pushing the viral genome past the threshold for error catastrophe. The majority of the mutations were G to A and A to G transitions. There was a slight increase in the number of C to T and T to C transitions as well. This enrichment in transitions would be expected, based on the assumption that KP-1212 forms both imino and amino tautomers (see Fig. 1a). The amino tautomer would pair with guanine, while the imino tautomer would pair with adenine. This pattern of mutations is markedly different from that reported by enhancing the nucleotide pool in deoxycytidine where the predominant mutational flow is A to G (Balzarini et al., 2001) and suggests that an imbalance in nucleotide pools induced by KP-1212 is unlikely to be the principal mechanism of action.

Though KP-1212 is a nucleoside, it differs from currently approved antiretroviral nucleosides in having an unmodified sugar moiety. It has been suggested that one mechanism of resistance to NRTI is achieved by pyrophosphorolysis, by which RT excises a chain terminator preventing DNA chain elongation, or by enhanced RT discrimination. The possibility of resistance to KP-1212 via pyrophosphorolysis is remote since KP-1212 is not a chain terminator. Resistance due to enhanced RT discrimination, such as the M184V mutation, which decreases the likelihood of incorporation of 3TC-TP by interaction of RT with the sulfur of the oxathiolane ring of lamivudine, is also unlikely since KP-1212 has an unmodified sugar (Daifuku, 2003). Thus, not surprisingly, HIV strains resistant to NRTIs like AZT and 3TC are as sensitive to KP-1212 as wild type virus. Similarly, passage of HIV in the presence of KP-1212 does not select for mutants resistant to known NRTIs, non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PI) (Virologic Inc., data not shown). Attempts to isolate a strain resistant to KP-1212 by previously published methods (Larder et al., 1990, 1991) have not been successful (data not shown). Quite to the contrary, virus which has been exposed to KP-1212 is more sensitive to KP-1212 than naïve virus. This is in contrast to what is seen with other drugs, where passaging in the presence of a particular drug usually leads to the selection of a drug-resistant variant. This increased sensitivity is not limited to KP-1212 but was also demonstrated for AZT. A possible explanation for this phenomenon is that the genomes of the virus exposed to KP-1212 have accumulated random mutations throughout the viral genome. As the vast majority of mutations are harmful, reversions or compensatory mutations necessary to mitigate the damage are unlikely to occur at a sufficient frequency. Thus, after several rounds of viral replication in the presence of KP-1212, the virus is less fit to deal with another bottleneck brought about by the addition of a new drug (Gerrish and Garcia-Lerma, 2003; Nowak and Schuster, 1989).

Cellular DNA is subjected to continual attack and there are at least 130 DNA repair genes (Wang et al., 1998; Wood et al., 2001). It is possible that KP-1212 will be efficiently repaired should it be incorporated into cellular DNA because of the non-aromatic (non-planar) nature of the base moiety. Base-excision repair has been demonstrated for oxidized non-aromatic pyrimidines, such as deoxyuridine glycol and thymidine glycol (Wang et al., 1998). Based on experiments with DHAC, there is evidence that KP-1212 is a substrate for DNA repair enzymes (Covey et al., 1986). To investigate whether KP-1212 could be incorporated into the host nuclear DNA, we first studied the effects of KP-1212 on the hypoxanthine guanine phosphoribosyl transferase (HGPRT) gene in CHO cells and human male lymphoblasts. At concentrations of 1–3 mM, KP-1212 did not exhibit a significant increase in HGPRT mutants when compared to the negative drug control. This is most likely due to enhanced discrimination and proofreading capability of host DNA polymerases and host DNA repair enzymes, neither of which HIV possesses. Studies performed on KP-1212 by BioReliance (Rockville, MD) confirm these findings (data not shown). KP-1212 was not found to be mutagenic in the Ames test with or without metabolic activation. In the mutagenicity assay in L5187Y/tk$^{+/-}$ mouse lymphoma cells, KP-1212 was positive only in one of two replicates at the lowest dose tested (500 μg/ml) at 24 h and not at higher doses (up to 2500 μg/ml). At the highest dose tested (2000 mg/kg of KP-1461) in the rat erythrocyte micronucleus test, there was an increase noted in micronuclei in males only, and the test was reported as weakly positive. Based on published studies, there is no evidence that KP-1212 is more genotoxic than approved NRTIs. For example, zidovudine has been reported to be moderately mutagenic in the absence of metabolic activation at 24 h in the mouse lymphoma assay at all doses up to 600 μg/ml (Ayers et al., 1996).

Many NRTIs are toxic to mitochondria (Birkus et al., 2002; Feng et al., 2001; Johnson et al., 2001). One of the hallmarks of mitochondrial toxicity is lactic acidosis due to shut down of the electron transport chain. Human CEM cells treated with KP-1212 over a prolonged time do not exhibit an elevation in lactic acid, whereas ddC caused a dose-dependent increase in lactate levels. KP-1212 was not found to significantly inhibit mitochondrial DNA synthesis. Cells treated with 320 μM KP-1212 exhibited a 6% decrease in mtDNA versus a 51% reduction in mtDNA observed with cells treated with ddC at 3.2 μM. These results are not surprising, as a recent publication suggests that incorporation of nucleoside chain terminators in mtDNA by polymerase γ and subsequent lack of repair by exonuclease is due to the absence of a 3′-OH group, and that this may be a general phenomenon extending as well to polymerases δ and ε (Feng et al., 2001). A detailed study of the enzymology of KP-1212's interaction with the nuclear polymerase β, the mitochondrial polymerase γ and HIV-1 RT are described by Murakami et al. (2005). Mitochondrial DNA is prone to oxidative injury due to its proximity to the electron transport chain. Damage to mtDNA

may be in the form of base modifications, abasic sites and various other types of lesions. Older literature suggests that mitochondria lack repair mechanisms because of a study that showed UV-induced pyrimidine dimers were not removed from mtDNA. However, subsequent research has shown that mitochondria have the capacity to remove oxidative damage, although they lack any enzymatic machinery for the removal of bulky lesions from their DNA. In vitro repair studies have shown that mitochondria contain all the enzymes required for base excision repair (BER) and that this process is very similar in both mitochondria and nuclei (Mandavilli et al., 2002).

KP-1212 is a novel nucleoside analog for the treatment of HIV. Instead of halting viral replication by DNA chain termination, it mutates the viral genome of HIV without demonstrating significant genotoxicity or mitochondrial toxicity. To date, it has not been possible to isolate strains of HIV resistant to KP-1212, nor to demonstrate cross-resistance to strains of HIV resistant to conventional NRTIs. DHAC, the ribonucleoside analog of KP-1212 has been administered to patients parenterally at doses of up to 7 g/m^2/day (Curt et al., 1985). It has demonstrated its safety in Phase II oncology clinical trials where the most significant side effects were transient pleuritic chest pain and nausea and vomiting at parenteral doses of 1.5–5 g/m^2/day (Creagan et al., 1993; Samuels et al., 1998). It is noteworthy that DHAC has been shown to be metabolized to KP-1212 by ribonucleotide reductase (Kees and Avramis, 1995).

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