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ABSTRACT

We report the activities of a novel nucleoside analog against HIV. This nucleoside (SN1212) is not a chain terminator but exerts its antiviral effects via mutagenesis of the viral genome. Serial passaging of HIV in the presence of SN1212 causes an increase in the mutation rate of the virus leading to viral ablation. Resistant strains of HIV to SN1212 have not yet been isolated and quite to the contrary, virus treated with SN1212 exhibits an increased sensitivity to not only SN1212 but to another nucleoside reverse transcriptase inhibitor (NRTI), zidovudine. HIV strains resistant to other NRTIs (e.g. zidovudine, lamivudine, stavudine, abacavir, etc) exhibit no cross-resistance towards SN1212. Multiple assays confirm that SN1212 has a favorable genotoxicity profile when compared to some approved antiviral nucleosides. In addition, SN1212 is not toxic to mitochondria nor does it show any inhibitory effects on mitochondrial DNA synthesis.

INTRODUCTION

The error-prone nature of retroviral reverse transcriptases (RT) like that of HIV allows a selective advantage for the virus to evade host immune responses and chemotherapy. Because of the error proneness of the viral RT (1, 2 & 3) the viral genome in patients does not exist as a homogenic single entity but rather as variants of nucleic acid sequences otherwise known as a quasispecies (4). The high error rate of the 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 (5). Loeb et al. (6) proposed that the mutation rate could be artificially increased by the introduction of a mutagenic nucleoside. Here we describe the activity of another mutagenic nucleoside analog, 5-Aza-5,6-dihydro-2'-deoxycytidine (Stealth Nucleoside 1212, SN1212). SN1212 can ablate HIV after 8 to 12 passages at concentrations in the nanomolar to low micromolar range. It typically increases the viral mutation rate by 50 to 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 SN1212. Rather, HIV treated with SN1212 shows increased sensitivity toward not only SN1212 but to another nucleoside analog (zidovudine or AZT) as well. SN1461 is a prodrug of SN1212 that has improved pharmacology and is being developed for oral administration for clinical trials.

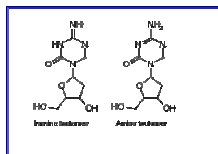


Figure 1: Immino and amino tautomers of SN1212.

MATERIALS AND METHODS

Cytotoxicity Assays: MT-2 Cells were treated with half log concentrations of SN1212 for five days at 37C. Cell viability was determined by an MTT assay. A toxic dose exhibiting 50% toxicity (ID₅₀) was determined relative to the negative drug control.

Single Passage EC₅₀: Single passage EC₅₀s were done in a 24-well plate format. MT-2 cells were infected with HIV-1 (LAI) resulting at a multiplicity of infection (MOI) of 1:5,000.

Serial Passage Experiments: The serial passage experiments were initiated in much the same manner as the single passage experiment except that the starting MOI were 1:1,000 or 1:100 and HIV NL4-3 was used.

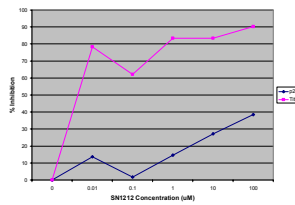


Fig. 2 Single passage inhibition of HIV growth by SN1212

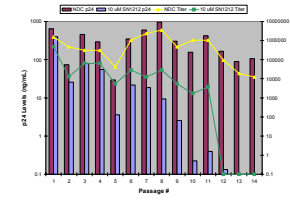


Fig. 3 Comparison of the p24 levels vs the viral titer in a serial passage study.

Cross Resistance: The cross-resistance studies were conducted in a similar manner as the single passage EC₅₀ studies described above.

Genomic DNA from HIV Viral Extinction Experiment, Passage 11															
Reverse Transcriptase Sequencing Data Summary															
	A to			C to			G to			T to			Total	Base	Mutation
	G	T	A	G	A	T	G	C	T	A	C	A	Mutations	Pairs	Rate
NDC	9	1	0	0	0	2	2	0	0	0	0	0	27	20333	0.132%
10uM SN1212	15	0	0	1	0	3	12	2	0	1	6	1	41	21355	0.192%
Δ RTIC with 1212															4.58%

Genomic DNA from HIV Viral Extinction Experiment, Passage 11															
Envelope Sequencing Data Summary															
	A to			C to			G to			T to			Total	Base	Mutation
	G	T	A	G	A	T	G	C	T	A	C	A	Mutations	Pairs	Rate
NDC	7	1	2	0	0	1	2	0	1	0	1	0	47	20384	0.22%
10uM SN1212	23	1	0	0	0	9	2	0	1	0	11	1	47	20095	0.235%
Δ RTIC with 1212															10.78%

Table 1. Sequence analysis of two HIV genes after serial passage in the presence of SN1212. A 960 bp fragment of the RT gene representing nt 2544-3439 and a 603 bp fragment of the ENV gene representing nt 6850-7452 was amplified by PCR.

Sequencing: Two regions of the HIV genome were targeted for sequencing; the reverse transcriptase (RT) gene because it is relatively conserved, the V3 loop of the envelope (ENV) gene because of its sequence heterogeneity.

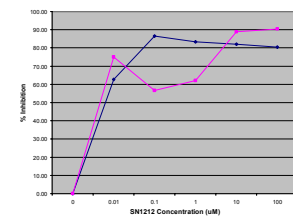


Fig. 4 SN1212 cross-resistance studies. RTIC is an AZT-resistant strain containing the following resistant mutations: 67N, 70R, 215F and 219Q. 1617 is a multidrug resistant (ABC, DDI, 3TC, D4T, TDF, DDC and AZT) containing the following resistant mutations: 69K, 70G, 75I, 77L, 116Y, 151M and 184V.

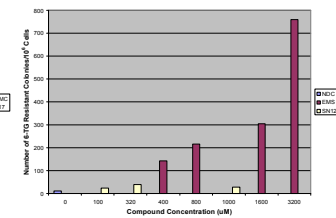


Fig. 5 SN1212 genotoxicity studies. CHO cells were treated with SN1212 and a known mutagen EMS. The hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene was used to measure genotoxic effects.

Genotoxic studies of SN1212. Detection of mutations in the HGPRT gene in CHO cells and in male lymphoblasts was used as an indicator for possible mutagenicity of SN1212 upon host genomes.

DISCUSSION

SN1212 demonstrates a favorable therapeutic index (ID₅₀/EC₅₀), in the range of 100,000, in various cell types. It should be noted that EC₅₀ values for viral mutagens may have a different significance than EC₅₀ values for currently approved antiviral agents. The discordance demonstrated in Figure 2 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 (8, 9), and presumably reflects the increased proportion of non-infectious viral particles in the presence of a viral mutagen, in this case, SN1212. Furthermore, the EC₅₀ of SN1212 has been shown to be very dependent on the MOI. The viral inhibition curve presented in Figure 2 can be achieved with MOI's in the range of 1:1,000 to 1:5,000 and over a range of such experiments, the EC₅₀ 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 EC₅₀, presumably reflecting fewer rounds of viral replication and less opportunity for incorporation of the mutagenic analog. Reproducible EC₅₀'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. Passaging of a virus with a higher MOI should be "equivalent" to infecting cells with a lower MOI. To achieve viral eradication *in vitro*, passaging is necessary. 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 viral mutation rate caused by SN1212. The majority of the mutations were G→A and A→G transition mutations. There was a slight increase in the number of C→T and T→C transitions as well. This enrichment in G→A or C→T transitions would be expected, based on the assumption that SN1212 forms both immino and amino tautomers (Fig. 1). The amino tautomer would pair with guanine, while the immino tautomer would pair with adenine. Though SN1212 is a nucleoside, it differs from currently approved antiretroviral nucleosides in having an unmodified sugar moiety. Thus not surprisingly, HIV strains resistant to NRTIs like AZT and 3TC are as sensitive to SN1212 as wild type virus. To date, it has not been possible to isolate strains of HIV resistant to SN1212, nor to demonstrate cross-resistance to strains of HIV resistant to conventional NRTIs. Quite to the contrary, virus which has been exposed to SN1212 is more sensitive to SN1212 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 SN1212 but was also demonstrated for AZT.

Increased Sensitivity of SN1212 Passaged Virus to SN1212 and AZT		
	SN1212 EC ₅₀	AZT EC ₅₀
SN1212 Naïve Virus	10 nM	8.7 nM
10 uM SN1212 Passaged Virus	1.4 nM	0.62 nM

Table 2. Increased sensitivity of SN1212-passaged virus towards SN1212 and AZT. Naïve virus and virus exposed to SN1212 were used to determine the respective EC₅₀s for SN1212 and AZT.

A possible explanation for this phenomenon is that the genomes of the virus exposed to SN1212 have accumulated drug induced 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 SN1212, the virus is less fit to deal with another bottleneck brought about by the addition of a new drug (NRTI, NNRTI or PI).

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