Viral Error Catastrophe by Mutagenic Nucleosides

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■ **Abstract**   Riboviruses and retroviruses have the highest rates of mutations of any known organism. Increasing the mutation rate of these viruses could exceed the error threshold for viability of a viral population within a host. Recent experiments with mutagenic nucleoside analogs validate this new approach to treating infection of RNA viruses. Lethal mutagenesis with HIV infected cells in culture has been documented and has been postulated to be the mechanism for treatment of hepatitis C with ribavirin. We consider the viral dynamics involved in the formation of a quasispecies, the choice of mutagenic nucleoside analogs, and the studies that have demonstrated the feasibility of lethal mutagenesis.

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INTRODUCTION

The concept of lethal mutagenesis takes root from the exceptionally high mutation rates of retroviruses and riboviruses. This high mutation rate has provided these viruses with a powerful mechanism to evade the host's immunological defenses for destroying foreign organisms. However, the same mechanism may render these viruses uniquely susceptible to killing by mutagenic nucleoside analogs. An increase in the mutation rate of these viruses may result in diminished viability on the basis of the generation of mutant-defective proteins. Further mutagenesis could force these viruses to cross the threshold for error catastrophe and obliterate the viral population (22). This review first considers the relationship between genome sizes and mutation rates and highlights the unusually high mutation rate of retroviruses and riboviruses that renders them susceptible to lethal mutagenesis. Second, we analyze mechanisms for incorporation of different mutagenic nucleoside analogs by retroviruses. Third, we summarize the initial studies demonstrating the ablation of HIV infection in cultured cells and consider current candidate nucleoside analogs. Fourth, we focus on the application of this approach to riboviruses and the status of studies with ribavirin. Last, we evaluate the potential limitations of lethal mutagenesis and the possibilities of combining a mutagenic nucleoside with other antiviral agents.

MUTATION RATE AND VIRAL FITNESS

Mutation Rate Versus Genome Size

Spontaneous mutation rates have provided unique insights into evolutionary history and mechanisms to protect the genome from environmental DNA damage. Mutations can alter fitness and facilitate selection, recombination, gene flow, and genetic drift. These changes are required for organisms to evolve in a changing environment. However, the vast majority of spontaneous mutations are deleterious in nature, reducing instead of increasing fitness (27). In an unchanging environment, there could be a progressive reduction in the mutation rate over time, decreasing the load of deleterious mutations on a species (71). In a changing environment,

however, there is a need for new mutations (5). This balancing between fitness reduction over time and adaptive response produces an overall mutation rate that is characteristic for each species.

The overall mutation rates of diverse organisms as measured by mutations per base pair per replication vary by several orders of magnitude (21). These data indicate that increasing genome size is associated with a reduction of mutation rate (Figure 1*a*, see color insert). When normalized to genome length, the mutation rate of riboviruses is approximately 1 mutation per genome per genome duplication; retroviruses have a lower mutation rate, and DNA viruses and bacteria have a much lower mutation rate, near 0.0034 (20) (Figure 1*b*). These findings imply that each class of organism, ribovirus, retrovirus, DNA virus, and bacterium has evolved to maintain a characteristic mutation rate. The observation that the mutation rate of riboviruses is five times greater than that of retroviruses is perplexing. Riboviruses and retroviruses lack a proofreading mechanism in replicating their genomes; each may require a high mutation rate to escape immune responses, and representative samples of each virus can infect the same host organism. Yet both classes of RNA virus maintain a distinct and high mutation rate compared with organisms with DNA genomes.

The mutation rate in RNA viruses is so high that the majority of viral progeny are nonviable (11). A high replication rate allows the virus to both explore sequence space and evade the immune system (35). Recombination provides a mechanism to counterbalance the detriments associated with this high mutation rate. Recombination allows highly mutated populations of viruses to generate genomes that are viable and exhibit increased fitness (49). Even with the ability to recombine genomes and produce large numbers of progeny, the mutation rates of riboviruses and retroviruses can still be only slightly increased. Riboviruses can accommodate only a modest 1.1- to 2.8-fold increase in their mutation frequency (37). The mutation frequency of retroviruses can be increased 13-fold (56). DNA-based organisms, which maintain a lower mutation rate, can accept up to an 80,000-fold increase in mutation frequency (10). These observations reinforce the fact that riboviruses maintain a mutation rate nearly five times that of retroviruses and several orders of magnitude higher than DNA-based organisms (Figure 1). There are at least three factors that contribute to the much higher mutation rate of RNA viruses compared with DNA viruses. First is the greater infidelity of RNA replicases and reverse transcriptases compared with viral DNA polymerases. Second is the lack of an error-correcting proofreading activity that appears to be an integral component of purified DNA viral polymerases. Third is the lack of known mechanisms for repair of either RNA-DNA hybrids or double-stranded (ds)RNA-replicating intermediates.

Retroviruses' and Riboviruses' Mutation Rates

RNA viruses, including riboviruses and retroviruses, are highly adaptable and contain extremely plastic genomes. Members within a viral species may contain genomic regions that vary by as much as 50% (73). These viruses achieve genetic

diversity through rapid viral production, high mutation rates, selection, and recombination. RNA viruses have in vivo mutation rates estimated to range from 10^{-3} to 10^{-5} substitutions per nucleotide copied (18). Recent studies indicate that the mutation rate of HIV-1 is 8.5×10^{-5} mutations per base pair per replication cycle (53). That this high rate of mutation is, in part, caused by infidelity of the polymerase is indicated by studies with purified reverse transcriptases that demonstrate a high level of misincorporation (3, 60, 64). By measuring mutations in retroviral long terminal repeats, O'Neil et al. (53) provided strong evidence that the major contribution to HIV-1 mutagenesis is the result of misincorporation by the reverse transcriptase and not host cell DNA or RNA polymerases. Crystal structures of viral reverse transcriptases and RNA replicases demonstrate a lack of a proofreading $3'$-$5'$ exonuclease domain (41), which is used for increasing accuracy by many cellular DNA polymerases. Furthermore, mutations produced when replicating the viral RNA are not corrected by the cellular mismatch repair pathway, which does not operate on newly produced RNA, either in the form of an RNA-DNA hybrid or in a dsRNA structure (50). RNA replicases and reverse transcriptases also exhibit frequent slippage in copying homopolymeric tracts, resulting in insertions and deletions (4, 40). Coupled with the high error rate is the high rate of replication seen in RNA viruses. Studies on the kinetics of RNA viruses have determined that as many as 10^{10} to 10^{11} new virions are produced daily within an infected individual (11, 35). RNA replicases and reverse transcriptases can also jump from one template to another during synthesis, generating recombinant species (2, 65). Recombination allows the virus to make large leaps in sequence space that would otherwise be difficult to obtain, and to rescue viral genomes from nonviable parental strains. Viral diversification through recombination is a common mechanism for positive-strand RNA viruses, including HIV and influenza virus (9, 63). It is therefore not unexpected that within a population most of the viral RNAs are either inactive or not packaged into infected virions. Nevertheless, given this high rate of mutation and replication, every single point mutation should be produced on a daily basis within an infected individual harboring a 10 Kb viral genome. This high diversity within the viral population allows for rapid adaptability in the event of environmental change and for the rapid emergence of resistance to antiviral drugs and immunological defenses (11, 43).

RNA Viruses Exist as a Quasispecies

RNA viruses within an infected individual exist not as a single unique variant, but as a quasispecies consisting of genetically distinct yet related genotypes (22). Initial infection of an individual may require only a few viable virions (17). Increasing viral diversity within the individual is then generated by the progressive accumulation of mutations resulting in a group of related sequences that evolve together over time, producing a quasispecies. A study by Kamp et al. (39) indicates that different RNA viral species may have achieved an optimal mutation rate. It is argued that the optimal mutation rate is 1 new mutation per time it takes the

immune system to adapt to a new viral epitope, changing the virus slightly faster than the immune system can adapt (39). An optimal mutation rate based on epitope recognition is of interest but fails to account for the differences in mutation rates between riboviruses and retroviruses with the same size genomes in the same host.

The complexity of the quasispecies increases with mutations in RNA synthesizing enzymes that render them increasingly error-prone (48). Viral diversity can also increase by recombining viral genomes within the quasispecies (65). Host-mediated immune positive and/or negative (purifying) selection can also increase viral diversity (7, 54). Positive selection occurs when an infected individual's immune system responds to specific epitopes and inactivates viruses harboring these epitopes. Variants within the viral quasispecies that are divergent enough to escape immune inactivation replicate and replenish the viral population until the immune system responds to their epitopes, repeating the cycle of selection. Purifying, or negative, selection is simultaneously taking place within the host, with a majority of mutants and recombinants producing unfit viruses that are removed from the quasispecies population (45). Because mutations in RNA viruses occur over the entire genome, some mutations are in RNA replicase or reverse transcriptase and render them error-prone and further increase the complexity of the population.

Error Tolerance for Mutations

The ability of RNA viruses to maintain high mutation rates allows them to readily acquire drug resistance. Interestingly, however, studies indicate that drug treatment is most effective when the mutation rate is either low, as in the case of bacteria, or very high (30). Furthermore, the ability to effectively combat RNA viruses may rely on the ability to initially reduce the viral population. A small population has the disadvantage of accumulating harmful mutations at an increased rate through the action of Muller's ratchet (52). Muller's ratchet is the process of acquiring mostly irreversible fitness reduction through stochastic accumulation of deleterious mutations for an asexual population of constant size or a population undergoing repetitive bottlenecks (32). It is thought that in a small population the effects of deleterious mutations will continually lower fitness unless these mutations are compensated by recombination or ameliorated by second-site mutations. However, even virus populations undergoing frequent recombination can sustain drastic fitness reduction following repeated bottleneck events (77). In small or bottlenecked populations that may or may not undergo recombination, deleterious mutations can become fixed, reducing the overall fitness. In a large population, however, these deleterious mutations are less likely to become fixed and thus do not suffer the consequences of Muller's ratchet. The mutation rate of viral populations routinely remains high and thus avoids the effects of the immune system. This high mutation rate is combined with a large population to create a viral quasispecies that can avoid the host defenses and the effects of Muller's ratchet. But, as the mutation rate increases, so do the number of deleterious mutations existing

in the persisting population, resulting in a reduced number of viable progeny and a reduction in the population base.

The mutation rate of RNA viruses is so high that it may approach the error threshold for viability of a quasispecies. The error threshold is a position in informational space where a phase transition occurs such that the genomic sequence information can no longer be perpetuated. It has been equated to the solid-to-liquid transition that occurs when ice melts (23). Although the overall mutation rate of RNA viruses is high, mutation frequencies throughout the virus genome vary with regional hot and cold spots of mutation. Genomic regions that maintain a low mutation frequency are protected by purifying selection, whereas the regions maintaining the highest mutation frequency usually emerge by positive selection. As mutations accumulate through the use of drugs or by random stochastic changes, the virus remains able to evade the host immune system but also may begin to mutate away all pathogenic information. Under natural selection, the viruses that cross this error threshold are eliminated from the population, leaving only those viruses that maintain mutation rates below this suicidal error rate.

LETHAL MUTAGENESIS

Practical Considerations

Because the mutation rate of riboviruses and retroviruses is so high, it seems feasible that a slight increase in the mutation rate might drive the viral population to exceed the error threshold for viability and obliterate the viral population within a host. We have referred to this concept as lethal mutagenesis. Among the mechanisms that increase viral mutagenesis are direct damage to genes (either DNA or RNA), incorporation of noncomplementary nucleotides when templates are copied, and incorporation of nucleotide analogs with incorrect base-pairing properties. In principle, it should be possible to selectively damage a viral genome with chemicals that specifically target viral genetic sequences. For example, one could use oligonucleotides that are complementary to viral sequences and form triple-stranded structures (74). Alternatively, alkylating agents may specifically recognize and react with viral genomes. Also, in principle, biasing nucleotide concentrations in cells would yield enhanced mutagenesis, and these could be targeted to small genomes. While these concepts are intellectually intriguing, they have not yielded new antiviral agents and are outside the scope of this review. We consider the induction of lethal mutagenesis using mutagenic nucleoside analogs that mispair at high frequency (47).

Multiple considerations govern the choice of mutagenic nucleoside analogs for lethal mutagenesis. The candidate analogs need to be transported into human cells; converted to nucleoside triphosphates by normal cellular nucleosides, nucleotide kinases, or phosphotranferases; and thereafter incorporated during viral replication. Moreover, there must be some mechanism for selective incorporation into the viral genome, for example, preferential incorporation by the viral replication

enzymes or reduced excision of the incorporated nucleotide analogs from the viral genome. Most importantly, the incorporated analogs need to form noncomplementary base pairs during viral replication. Verification of lethal mutagenesis is greatly strengthened if the mutations are random and the types of nucleotide substitutions in the viral genome are predictable from the base-pairing properties of the nucleoside analog. It would be advantageous to utilize nucleoside triphosphates, since one could circumvent the requirements for phosphorylation by nucleoside kinases; however, there is little evidence that any triphosphates are efficiently transported into eukaryotic cells. In contrast, nucleosides, particularly pyrimidines, are efficiently transported either by passive diffusion or by active transport (1). Furthermore, many pyrimidine analogs are phosphorylated by normal cellular kinases (62). As a result, pyrimidine analogs are particularly advantageous for generating nucleoside triphosphates, the immediate precursors for both RNA and DNA synthesis.

Another important consideration is the kinetics of incorporation of the nucleoside triphosphate analog by the viral polymerase and the efficiency by which it is copied when incorporated into an RNA or DNA template. Both parameters can be quantitated by in vitro assays prior to measuring effects on viral replication. Using primer extension assays, one can compare the kinetics of incorporation of a nucleoside triphosphate analog with that of the cognate nucleotide (8). Similarly, one can construct oligonucleotides containing site specifically positioned nucleotide analogs and measure the rates of incorporation of complementary and noncomplementary nucleotides (69).

Theoretical Considerations

Retroviruses, and in particular HIV, present an important model for testing the validity of the concept of lethal mutagenesis. The virus replicates rapidly (57), and HIV reverse transcriptase incorporates noncomplementary nucleotides at high frequency (60). These two parameters reinforce each other and presumably account for the high mutation frequency in viral isolates. Measurements on the kinetics of viral loss after administration of protease or reverse transcriptase inhibitors to infected individuals indicate that the virus population can double every 2.6 days (76). Mathematical models predict that the production of HIV-1 can be as high as 10^{10} virions per day (51). On the basis of the mutation rate of the virus, it can be estimated that each viral genome accumulates one mutation during each replicative cycle (53, 60). Thus, within an untreated infected individual there are virions that encode every possible single, double, and triple amino acid substitution. This high rate of diversification is thought to produce drug-resistant variants prior to drug administration; after selection there would be ongoing repetitive generation of mutants with increasing drug resistance. It is not surprising that mutant viruses rapidly emerge after treatment with single or even multiple agents.

Drugs with the greatest efficacy for reducing viral loads in HIV-infected individuals include nucleoside analog chain terminators, protease inhibitors, and

non-nucleoside reverse transcriptase inhibitors (72). The rapid emergence of HIV resistance to each of these agents has limited their utility for long-term monotherapy (58). Instead, multiple drugs are administered simultaneously; as many as five drugs are given concurrently, with the expectation that the emergence of virus resistance to all five drugs is less likely than resistance to a single drug. Even this therapy is inadequate because it is frequently toxic and fails to eliminate the emergence of resistance. In contrast, it is argued that resistance to mutagenic nucleoside analogs is less likely to generate resistant strains (15).

The postulated mechanism for lethal mutagenesis of HIV is illustrated in Figure 2 (see color insert). The mutagenic deoxynucleoside analog is transported into infected cells and phosphorylated to the deoxynucleoside triphosphate by a series of cellular kinases. Unfortunately, we lack sufficient information about substrate specificities of cellular nucleotide kinases to predict the efficiency of uptake and phosphorylation of different analogs. Thereafter, the mutagenic nucleoside triphosphate is incorporated by HIV reverse transcriptase during cDNA synthesis to form an RNA-DNA hybrid. In principle, preferential incorporation into viral DNA could be achieved by utilizing mutagenic deoxynucleoside analogs that are incorporated more efficiently by HIV reverse transcriptase compared with incorporation by cellular DNA polymerases. Subsequent copying of the DNA strand results in formation of a dsDNA containing noncomplementary nucleotides opposite the template analog. Excision of the analog by cellular DNA repair enzymes would not obliterate the nucleotide substitutions. Instead, it would be necessary to also excise the normal deoxynucleotide from the second DNA strand. Incorporation of mutagenic deoxynucleotides can also occur during the synthesis of the dsDNA intermediate. Thereafter, the viral dsDNA is inserted into the host chromosome and directs the synthesis of the viral RNA containing the single-base substitutions. With iteration of this process during the HIV replication cycle, there would ensue a progressive accumulation of mutations. As a result, mutant proteins would be produced with diminished catalytic activities. Eventually, the viral genome would be irreversibly corrupted, resulting in an error catastrophe.

Mutation Induction by 5-OH-dC

The choice of nucleoside analogs for the induction of lethal mutagenesis of HIV in cultured cells was guided by the following criteria: evidence for uptake and phosphorylation in human cells; incorporation of the nucleoside triphosphate analog by HIV RT in vitro; lack of repair once incorporated into the DNA strand of an RNA-DNA hybrid; and, most importantly, evidence for mispairing during DNA synthesis. Because host cell DNA polymerases also incorporate deoxynucleoside analogs, it would also be desirable to select analogs that are efficiently removed from the host genome by DNA repair processes. Five mutagenic deoxynucleoside analogs were initially investigated (46): 1-mM 5-hydroxydeoxycytidine (5-OH-dC); 1-mM O^4 methylthymidine; 1-μM O^6-methyldeoxyguanosine; 0.5-μM

8-amino-deoxyguanosine; and 10-μM 8-oxo-deoxyguanosine. The concentration was either 1 mM or 10-fold lower than that which reduced growth of human CEM cells by $>30\%$. Following seven sequential passages, a segment of the HIV RT gene was amplified from the cellular DNA, cloned in *Escherichia coli*, and sequenced. To detect random substitutions, it is necessary to sequence DNA from individual transformed bacterial colonies. Even though the viral titer was not reduced after seven passages, there was an increase in mutations per nucleotide sequenced with both O^4-methylthymidine and 5-OH-dC. In the case of O^4-methylthymidine, multiple mutations were observed in only a few of the clones sequenced, so-called hypermutations, and viral replication was not diminished even after an additional 19 passages. With 5-OH-dC, an increased number of mutations were present in multiple clones.

Modulations of viral production in culture supernatants over the course of 18 transfers of HIV-1$_{LAI}$ in the absence or presence of 1-mM 5-OH-dC are shown in Figure 3 (46). An irreversible loss of viral titer, as measured by the p24 production, was observed after 24 sequential transfers in each of the triplicate cultures, but not in any of the triplicate controls. In this experiment (J. Zhang & L. Loeb, unpublished results), viral antigen (p24) titer was determined after each transfer, and the volume of the subsequent inoculum was adjusted so that the same amount of virus in control and 5-OH-dC-treated cells was added at each transfer step. This loss in viral titer was confirmed by infecting fresh cells with frozen supernatants obtained at the twenty-third passage: A similar loss of p24 in 5-OH-dC contained in cultures, but not in controls, was observed in subsequent passages (data not shown). In eight of nine subsequent trials, passage in 5-OH-dC abolished viral infectivity. This is in contrast to more than 50 control serially passaged cultures lacking nucleoside analogs in which there was no abolition of the virus. Sequencing
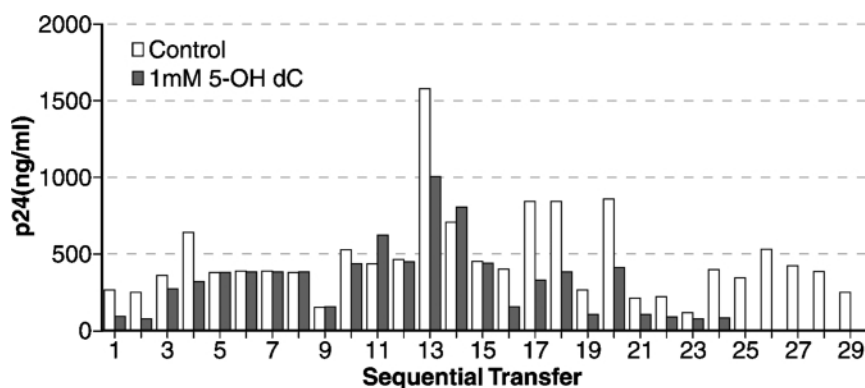
Figure 3 Sequential passage of HIV cell culture supernatants in the presence or absence of 1-mM 5-OH-dC. Results are expressed as the amount of p24 in culture supernatants.

a segment of reverse transcriptase of HIV from the penultimate passage prior to extinction in two separate experiments demonstrated a 2.6- and 5-fold increase in the frequency of A-to-G transitions (A → G). This is exactly the substitution that would be predicted if 5-OH-dC were incorporated into first-strand DNA synthesis and then base-paired with dA during second-strand synthesis. Assuming these mutations are evenly distributed, each proviral DNA would contain 20 5-OH-dC-induced mutations; this number of mutations should be sufficient to result in lethal mutagenesis.

Evidence indicates that 5-OH-dC is not toxic to the host genome (L. Loeb & R. Daifuku, unpublished results). First, an electrochemical assay was established to detect 5-OH-dC; fewer than 1 residue was found per 7×10^5 nucleotides in cellular DNA after culturing cells for 6 days in the presence of 1-mM 5-OH-dC. Second, incubation of transformed human lymphoblasts in 4-mM 5-OH-dC did not increase the frequency of host cell–encoded hypoxanthine-guanine phosphoribosyltransferase (*hgprt*) mutations. Third, incorporation by cellular DNA polymerases has been a major source of toxicity with chain-terminating deoxynucleotides that are incorporated by the mitochondrial DNA polymerase and result in diminished mitochondrial function (38). There was no decrease in mitochondrial DNA after culturing human T-cell line (CEM) initials of patient lymphoblasts in 2-mM 5-OH-dC for 8 days. Control cell cultures grown in 1.0-μM dideoxycytidine (ddC or zalcitabine) result in a 99% reduction in mitochondrial DNA.

Mutation Induction by the Deoxycytidine Analog SN1212

5-OH-dC provided a proof of principle for the lethal mutagenesis of HIV. However, the concentration of 5-OH-dC required for abolition of viral infection (0.5 to 1.0 mM) in vivo is likely to be prohibitive. As a result of extensive synthesizing and screening potential mutagenic nucleoside analogs, a deoxycytidine analog, SN1212 (5,6-dihydro-5-aza-2′-deoxycytidine), was selected for clinical development. SN1212 has an EC_{50} (effective concentration inhibiting 50% of viral replication) of 10 nM and a TD_{50} (toxic dose inhibiting 50% of cellular proliferation) of $\geq$1 mM. Passaging experiments have been performed for SN1212 that were similar to those reported with 5-OH-dC at drug concentrations ranging in 10-fold increments from 10 to 100 μM. The amount of p24 was permanently reduced to less than the limit of detection (4 ng/ml) by passage 8, and no infectious HIV was recovered after passage 12 in cells incubated with 100-nM SN1212 (Figure 4, see color insert). In fact, by passage 14, infectious virus could not be recovered at concentrations ranging from 100 to 100 μM (K. Harris & R. Daifuku, unpublished results). The discordance demonstrated in Figure 4 between viral infectivity and conventional surrogate markers of viral load, such as p24, is not surprising, as it has also been observed in vitro by others (14, 55), and presumably reflects the increased proportion of noninfectious viral particles in the presence of a viral mutagen, in this case, SN1212.

Cloning and sequencing segments of HIV RT and the V3 loop of the envelope gene from the eleventh passage (the penultimate passage prior to extinction) indicate that 10-μM SN1212 enhances the mutation frequency by 1.5- and 1.9-fold ($p < 0.05$), respectively. The most frequent mutations are A $\rightarrow$ G transitions (enhanced 1.7- and 3.3-fold) and C $\rightarrow$ T transitions (enhanced 1.5- and 9-fold), though all transitions are increased. These transitions are consistent with the formation of both an amino (pairing with guanine) and an immino (pairing with adenine) tautomer of SN1212, and incorporation of SN1212 during reverse transcription and DNA duplex formation. In the case of both 5-OH-dC and SN1212, nearly all mutations were single-base substitutions; only a two- to threefold increase in the frequency of random mutations was sufficient to obliterate viral infection. The findings that only a modest increase in mutation frequency is adequate to abolish viral infection substantiate the concept that the mutation frequency of HIV is close to the error threshold for viability of the quasispecies (24).

Studies on cellular genotoxicity suggest that if SN1212 is incorporated into host cell nuclear DNA, it is rapidly excised by DNA repair processes. This may be the result of its nonplanar base. SN1212 does not increase above the spontaneous background of the mutation frequency of a cellular gene, *hgprt*, in both Chinese hamster ovary cells (up to 1 mM) and male lymphoblasts (up to 3 mM). In addition, SN1212 does not demonstrate evidence of mitochondrial toxicity by either an increase in lactate production or an inhibition of mitochondrial DNA synthesis at the highest dose tested, 320 μM.

LETHAL MUTAGENESIS OF RNA VIRUSES

Riboviruses contain an RNA genome. They encode an RNA replicase that utilizes ribonucleoside triphosphates for the synthesis of progeny RNAs. These unique replicating enzymes and the high mutation rate of these viruses have stimulated interest in the use of mutagenic ribonucleotide analogs to induce lethal mutagenesis (Figure 5, see color insert).

Prescient Studies with QB Phage

In the 1960s, Spiegelman et al. (70) published a series of elegant papers demonstrating the evolution of QB RNA using a cell free system. QB RNA is a small single-stranded (ss)RNA that replicates through a dsRNA intermediate. Biologically active QB RNA was replicated autocatalytically; the nature of the newly synthesized product depends on the fidelity of QB replicase, nucleotide pools, metal cations, temperature, and speed of replication (33, 34). Moreover, they demonstrated that one could alter the complexity of the quasispecies, select for specific drug-resistant mutants, and select for replicases with altered fidelities. These prescient papers set the stage for both theoretical (24–26) and experimental (19) studies demonstrating the quasispecies nature of RNA viruses. Mutator ribonucleoside triphosphate analogs could be preferentially incorporated by the viral replicase and

enhance viral mutagenesis. Moreover, these analogs would be incorporated into a dsRNA, which is resistant to excision by any known repair mechanism.

Poliovirus and Vesicular Stomatitis Virus

Initial studies on the induction of mutagenesis in riboviruses were carried out by Holland and coworkers (36, 37). They exposed poliovirus and vesicular stomatitis virus to chemicals that damage nucleic acids, and to ribonucleoside analogs that can be incorporated by the viral replicase and subsequently miscode. Incubation of both viruses with ethyl methanesulfonate, nitrous acid, 5-azacytidine, and 5-fluorouracil (5-FU) resulted in a 1.1- to 2.8-fold enhancement in mutation frequency at single sites on the genome. This modest increase in mutagenesis was accompanied by a greater than 100-fold decrease in viral titer. Thus, as in the case of HIV, a small increase in mutation frequency is associated with a large reduction in viral titer.

Foot-and-Mouth Disease Virus

Foot-and-mouth disease virus (FMDV) is a picornavirus that causes an economically detrimental disease in farm animals and provides a productive experimental model for exploring the relationships between viral fitness, mutagenesis, and extinction. Repetitive passage of FMDV in cell culture in the presence of 5-FU or 5-azacytidine resulted in up to a $\leq$6.4-fold increase in mutation frequency and in the occasional extinction of the viral population (66a). Pariente et al. (55) established a series of mutant viruses exhibiting differences in replicative fitness and measured the effects of mutation induction of these viruses. Low replicative fitness favored viral extinction by mutagenesis. Extinction of intermediate fitness virus required a combination of 5-FU and the antiviral inhibitor guanidinine HCl but was not observed with the mutagen or inhibitor alone. Extinction of high fitness virus required the combination of 5-FU and two antiviral inhibitors, guanidinine HCl and heparin. Extinction of each of these viral populations was accompanied by increased mutagenesis. These studies establish the potential extinction of the viral population by combining mutagenic nucleoside analogs with chemicals that inhibit synthesis of viral genomes.

Lymphocytic Choriomeningitis Virus

Lymphocytic choriomeningitis virus (LCMV) is an arenavirus and a natural pathogen of wild mice. It contains a ssRNA genome. Passage of LCMV in the presence of 5-FU resulted in a progressive loss of viral infectivity, and at a concentration of 25 μg/ml viral extinction was observed (31). 5-FU-mediated extinction of LCMV is associated with 3.6- to 10-fold increases in the mutation frequencies of three viral encoded genes, NP, GP, and L-polymerase. A larger increase in mutation frequency might have occurred, but these highly mutated sequences might not be amenable to PCR amplification owing to loss of primer hybridization. Thus, it may not have been feasible to demonstrate the mutation rate necessary for a meltdown

of viral genetic information. Because 5-FU could mediate extinction of LCMV in vitro, it was also tested in a mouse model. Because infection in the mouse is controlled by a vigorous T-cell response that eventually leads to viral clearance, RAG2-KO mice were used, which lack mature T and B cells. LCMV infection in these mice results in the establishment of a persistent infection. After 12 days, the 4 control mice had high titers of LCMV in serum and spleen, whereas 3 out of 4 mice treated with 5-FU did not have detectable virus in serum and spleen (66). These studies extend the types of viruses that are amenable to lethal mutagenesis, and the studies in mice substantiate the feasibility of in vivo lethal mutagenesis as a therapeutic approach.

Hantaviruses

Hantaviruses are negative-sense ssRNA viruses. They are transmitted from rodent hosts to humans and cause two greatly feared illnesses, hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Except for ribavirin, no other antiviral drugs for the treatment of hantaviral diseases have been identified. In two separate studies, ribavirin was demonstrated to inhibit the synthesis of viral RNA and proteins (68). The ablation of viral synthesis was accompanied by an 8.6-fold increase in mutation frequency. Considering that these results are similar to the effects of ribavirin on hepatitis C, Severson et al. (68) proposed that ribavirin induced an error catastrophe on the basis of incorporation of ribavirin-triphosphate by the viral polymerase.

Viral Hepatitis C and Ribavirin

The medically most important ribovirus inducing a chronic viral disease is the one responsible for hepatitis C. Crotty and coworkers (13, 14) proposed that ribavirin, one of the few effective therapeutics for hepatitis C virus (HCV) infection, functions as a viral mutagen. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a ribonucleoside analog that resembles guanosine and exhibits activity against a wide spectrum of RNA viruses (16). In the absence of facile systems for serial passage of HCV, studies on ribavirin have been limited to incorporation of ribavirin-triphosphate by the viral replicase in vitro or measurement of mutation induction by related viruses. This impediment has to some degree recently been resolved by the development of subgenomic HCV RNA molecules that replicate autonomously in transfected cells. Contreras el al. (12) demonstrated that ribavirin acted as a mutagen in a full-length HCV replicon system. Ribavirin broadly increased error generation, especially in otherwise invariant regions of the genome.

The conjecture that ribavirin induces lethal mutagenesis (13) was based in part on reports that poliovirus polymerase incorporates ribavirin-triphosphate. Using primer extension assays, Arnold et al. demonstrated that ribavirin-triphosphate is incorporated by the RNA-dependent RNA polymerase and that incorporation occurs opposite both template cytidine and uridine template residues (2). In cell

culture in the presence of 1-mM ribavirin, the frequency of mutation is 1 per genome per replication cycle with poliovirus (14), which represents an increase of approximately 100-fold. That the loss of viral titer occurs by an error catastrophe is suggested by studies that compare mutation frequency with viral titer (13). A 4-fold enhancement in mutation frequency was coincident with a 10-fold decrease in viral RNA; a 10-fold increase in mutation frequency nearly obliterated the viral population.

Initial experiments to demonstrate that a viral mutagen might be used for the treatment of HCV were performed with bovine viral diarrhea virus (BVDV). Because of the difficulty in growing HCV in vitro, a related virus, BVDV, has been proposed as a surrogate for HCV. One analog, 5-formyluridine, has been observed to enhance viral mutagenesis and ablate BVDV replication in vitro (L. Li & R. Daifuku, unpublished results). The corresponding deoxynucleoside analog is produced by exposure of DNA to reactive oxygen species and is believed to base-pair with all three bases, C, T, and G, in addition to its cognate base, A (6). Culturing of BVDV in 5-formyluridine results in a 3.8-fold enhancement in mutation frequency, which is similar to that reported for ribavirin (44).

In addition to lethal mutagenesis, other mechanisms have been proposed to account for the therapeutic effect of ribavirin (ribavirin does not effect measurably HCV viral loads in vivo) (78). However, because of the inability to culture HCV, the effect of ribavirin on viral infectivity is difficult to assess. Ribavirin enhances the helper T-cell response; these cells could target the virus. Ribavirin inhibits cellular inosine-5′-monophosphate dehydrogenase activity (IMPDH), thereby depleting the intracellular pool of GTP, which could indirectly result in mutagenesis. However, inhibition of IMPDH may not by itself account for the effectiveness of ribavirin in ameliorating infections with riboviruses. Ribavirin has a greater action against FMDV than does mycophenolic acid, even though both inhibit IMPDH and produce similar reductions in intracellular GTP pools. Inhibition of IMPDH could operate in concert with lethal mutagenesis; reduction in GTP pools would enhance incorporation of ribavirin-triphosphate by the viral polymerase. IMPDH inhibition alone was not sufficient to suppress the quantity and quality of replicon RNA. Ribavirin, however, reduced HCV replicon colony-forming efficiency in a dose-dependent fashion and was mutagenic (78).

Not all studies have shown ribavirin to be a viral mutagen. Although ribavirin demonstrated an antiviral effect against lymphocyctic choriomeningitis virus, this effect did not appear to be mediated by viral mutagenesis. Rather, it appeared to be mediated through inhibition of the viral genome synthesis by the replicase (66). Perhaps more importantly, patients on ribavirin monotherapy for the treatment of HCV did not show significant mutations in the viral genes HVR1, NS5A, and NS5B over the course of at least 12 months of therapy (61).

Thus, although mutagenesis has been demonstrated by ribavirin in a number of viral systems, it has not been shown in all settings. Demonstration of the mutagenic role of ribavirin may be affected by the following. (*a*) Specific mechanisms of action may dominate in specific experimental settings. (*b*) Concentrations

necessary for activity of ribavirin-triphosphate as a mutagen may not be achievable in target cells. (*c*) Selected clones for sequencing are likely to under-represent the mutagenic effect of a viral mutagen because of negative selection. Thus, sequenced clones do not reflect the mutational load induced by a viral mutagen associated with a lack of infectivity. (*d*) Measuring a surrogate marker of viral infectivity may underestimate the treatment effect. For example, quantitation of a fragment of viral RNA or a viral protein may not reflect the effect of a viral mutagen on viral infectivity because of the generation of noninfectious virions.

OTHER VIRUSES

Viral Hepatitis B

Hepatitis B virus (HBV) is a DNA virus that replicates through an RNA intermediate. HBV is a member of the *Hepadnaviridae* family and copies the DNA to form a messenger mRNA that is reversed transcribed to produce the viral DNA. It offers two pathways for the evolvement of lethal mutagenesis on the basis of the incorporation of either deoxyribonucleotide or ribonucleotide analogs. Opportunity exists for the incorporation of ribonucleotide analogs during viral mRNA synthesis by human RNA polymerase II and of deoxyribonucleotide analogs during reverse transcription or double-strand synthesis by the viral polymerase. The lack of an efficient cell culture system for the continuous passage of HBV has hampered the screening of nucleoside analogs for the lethal mutagenesis of hepatitis B.

There are two approved classes of drugs for the treatment of HBV: interferon and nucleoside analogs. The fact that nucleoside analog chain terminators are effective serves as a prognosticator for the possible utility of mutagenic nucleosides. The two approved nucleoside analogs are lamivudine and adefovir. Lamivudine is an $2',3'$-dideoxynucleoside analog of cytidine that inhibits DNA synthesis by terminating the nascent proviral chain. Three Phase III studies of lamivudine demonstrated that 15 to 17% of patients on lamivudine compared with 4 to 13% of patients on placebo converted from being hepatitis Be antigen positive (HBeAg) to HBeAg negative with undetectable HBV DNA. It is noteworthy that resistance to lamivudine in the course of therapy has been described (75). Adefovir has more recently been approved for use in the United States; its major advantage over lamivudine appears to be a slower emergence of resistance (75).

There is evidence that ribavirin is active against HBV. A six-month course of ribavirin at doses of 800 to 1200 mg/day resulted in inhibition of viral replication. Mean HBV DNA decreased from 162.7 to 114.3 pg/ml at week 20 (28). More recently, a small placebo-controlled trial demonstrated benefits. HBeAg seroconversion occurred in 50% of the patients in the ribavirin group as opposed to 6.6% in the placebo group (29). Because ribavirin is a viral mutagen, it is possible that ribavirin is incorporated at the transcriptional level, as has been hypothesized for the treatment of HIV by mutagenic ribonucleoside analogs (47).

Emerging Viral Infections

Novel viruses continue to emerge or spread their geographic range. These are most commonly riboviruses, the most recent being the SARS virus. While the clinical benefit of ribavirin against SARS remains unclear, it has been the treatment of choice throughout the epidemic (42). Treatment of other emergent viral threats benefit from antivirals with a broad spectrum of activity, such as viral mutagens, rather than antivirals targeting a specific viral enzyme.

ADVANTAGES OF MUTAGENIC NUCLEOSIDE ANALOGS

Potential for Resistance to Mutagenic Deoxynucleoside Analogs

Resistance to all four currently approved classes of HIV antivirals does develop. In tissue culture, resistant virus emerges after 2 to 3 passages in the presence of a non-nucleoside reverse transcriptase inhibitor such as nevirapine, and emergence of resistance to chain terminators such as AZT (azidothymidine) has been noted after 12 passages. In fact, while passaging has been used to demonstrate activity of viral mutagens, it has also been used to select for viruses resistant to conventional antivirals, albeit under somewhat different tissue culture conditions. For example, Larder et al. (42a) elicit resistance by testing high viral inocula with increasing drug concentrations, whereas passaging experiments for mutagenic nucleoside analogs typically use fixed concentrations at lower inocula.

The following are reasons why viral resistance to mutagenic nucleoside analogs is likely to emerge than viral resistance to conventional therapeutics. (*a*) Substituents that alter base-pairing properties are facing the incoming nucleotide and do not appear to coordinate with amino acid residues on the viral polymerase. (*b*) Viral mutagens apply less selective pressure to a viral population for emergence of resistant variants than do antivirals that immediately terminate viral replication. (*c*) Viral mutagenic nucleosides cause mutations in all encoded viral proteins. (*d*) Decreased utilization of modified nucleoside sugars by HIV RT is a major mechanism of viral resistance to chain terminating nucleoside analogs. Viral mutagens do not require sugar modification for activity.

However, it remains possible that selection will occur for viral polymerases that recognize base modifications present in viral mutagens and that these viral polymerases may attempt to exclude nucleotides with such bases from incorporation into the viral genome.

Potential for Resistance to Mutagenic Ribonucleoside Analogs

Resistance of riboviruses to mutagenic ribonucleoside analogs could involve mutations in the viral-encoded polymerase. Resistance to ribavirin has been reported rarely in the literature. One such report (67) observed that Sindbis virus

mutants selected for resistance to mycophenolic acid also demonstrated resistance
to ribavirin. Recently though, a single mutation in the poliovirus RNA polymerase
was reported to confer resistance to ribavirin by increased fidelity of replication.
Whereas resistance could not be elicited by passaging in the presence 400-μM rib-
avirin, less stringent conditions, 100 μM followed by 400 μM, led to the emergence
of resistant virus. A single mutation in the RNA polymerase, G64S, decreased the
mutation frequency by approximately threefold, as measured by guanidine resis-
tance assay (59). If confirmed, this observation would raise the possibility that viral
mutagens may decrease the fitness of viruses by increasing their fidelity of repli-
cation. Viruses can evolve by selection for error-prone polymerases in response to
environmental pressures. However, we still lack studies that directly indicate that
the emergence of a more accurate polymerase is associated with a decrease in the
emergence of drug-resistant variants. While such an improvement in fidelity might
decrease the production of noninfectious virions, the cost to the viral population
might be a lesser chance of emergence of genetic variants resistant to conventional
antivirals.

In the case of the treatment of retroviruses or hepadnaviruses by mutagenic
ribonucleoside analogs, development of resistance would presumably require mu-
tations to occur in the host cell RNA polymerase. This enzyme is encoded by
the host genome, and mutations that reduce incorporation of the ribonucleotide
analogs would not accumulate within the viral genome. Thus, lethal mutagene-
sis of HIV and hepatitis B, utilizing ribonucleoside analogs, is unlikely to result
in the emergence of resistant viral mutants. A major impediment for developing
mutagenic ribonucleotide analogs incorporated by the host RNA polymerase is
the presence of cognate ribonucleoside triphosphates present in cells in millimolar
concentrations.

Combination Therapy

Clinical studies with mutagenic nucleoside analogs are likely to involve patients
who have developed resistance to conventional therapies. A major use is likely to
be in combination with other approved antivirals. There are few empirical studies
specifically designed to evaluate the combination of conventional antiviral therapy
with nucleoside analogs that enhance viral mutagenesis. As discussed above, incu-
bation of infected cell cultures of FMDV with 5-FU resulted in viral extinction only
after the addition of one or two additional antiviral inhibitors (55). However, 5-FU is
not a potent viral mutant and also acts to terminate DNA synthesis. Although this re-
search was performed before ribavirin was documented as a viral mutagen, it should
be noted that ribavirin is approved for the treatment of HCV in combination with
interferon-α. The combination of a viral mutagen with an immunomodulator yields
greater sustained virologic responses than does administration of either drug alone.

Only a limited number of studies measuring the combined effects of 5-OH-
dC with chain-terminating nucleosides have been carried out. In one experi-
ment, HIV-1-infected cells were treated for five passages in 5-OH-dC (1 mM).

Subsequently, passaged and unpassaged cells were treated with low-dose AZT (1.6 nM) (J. Zhang & L. Loeb, unpublished results). In another experiment, HIV passaged in the presence of 5-OH-dC was extinguished after five passages in low-dose AZT, whereas unpassaged virus was still present after eight passages in AZT. In another experiment, HIV was passaged in the presence of AZT (1.6 nM), 5-OH-dC (0.5 mM), or a combination of AZT and 5-OH-dC. Marked viral suppression was found at passage 5 for the combination therapy and viral extinction occurred by passage 9, whereas no significant effect was demonstrated by either antiviral alone.

It is unclear how to best administer a viral mutagen in combination with conventional antivirals. On the basis of modeling, Gerrish & Garcia-Lerma (30) have proposed that it would be most efficacious to administer the viral mutagen before the start of conventional antiviral therapy, allowing sufficient time for mutation selection within the population. Their studies indicate that if the mutagen is discontinued soon after initiation of antiviral treatment, it has the advantage of slowing compensatory evolution and thus maintaining viral suppression for a longer period. Viral mutagenesis may also be administered following the initial reduction of the viral population, allowing the effects of Muller's ratchet to reduce the fitness of the smaller viral population.

PERSPECTIVES

The concept of lethal mutagenesis offers a new opportunity for the design of antiviral agents. This concept is particularly attractive for therapies directed against RNA viruses and viruses that utilize RNA as an intermediate in their replication. The high rates of mutagenesis of these viruses suggest that their genomes are maintained at a fidelity that is close to the error threshold for viability. Experiments on divergent viruses indicate that a slight increase in mutation rate can push the genome over a crevice and induce an error catastrophe with ablation of viral populations. Evidence to support virus killing by lethal mutagenesis include the following. (*a*) Increases in mutation frequency in the viral progeny promote viral extinction. (*b*) The corresponding nucleoside triphosphate is incorporated by the viral polymerase or replicase. (*c*) The type of mutation can be predicted by the base-pairing properties of the candidate analog. (*d*) Mutation rates in tissue-culture-infected cells can be decreased or increased by increasing or decreasing the concentration of the cognate nucleotide, respectively. (*e*) Preferential mutagenesis of the viral genome can be achieved either by enhanced incorporation of the analog by the viral enzyme or by decreased removal of the mutagenic nucleotide when present in an RNA-DNA duplex or in dsRNA. (*f*) Ablation of the viral population depends on viral load; reduction in viral load by other agents would enhance the effectiveness of the mutagenic nucleotide.

Therapy based on mutagenic nucleoside analogs will likely require continuous long-term drug administration, especially for viruses that integrate into the human genome or reside in protective cellular environments. Last, the idea of

error tolerance in RNA viruses is similar to that of error tolerance in cancer cells. Both maintain a hypermutable state that can readily acquire drug resistance and escape from the host's defenses. In cancer, a mutator phenotype may exist, which accounts for the marked heterogeneity seen in solid tumors. Thus, many of the chemotherapeutics that are effective in cancer therapy are highly mutagenic and should be considered as prototypes for designing mutagenic nucleoside analogs directed against viral infections.

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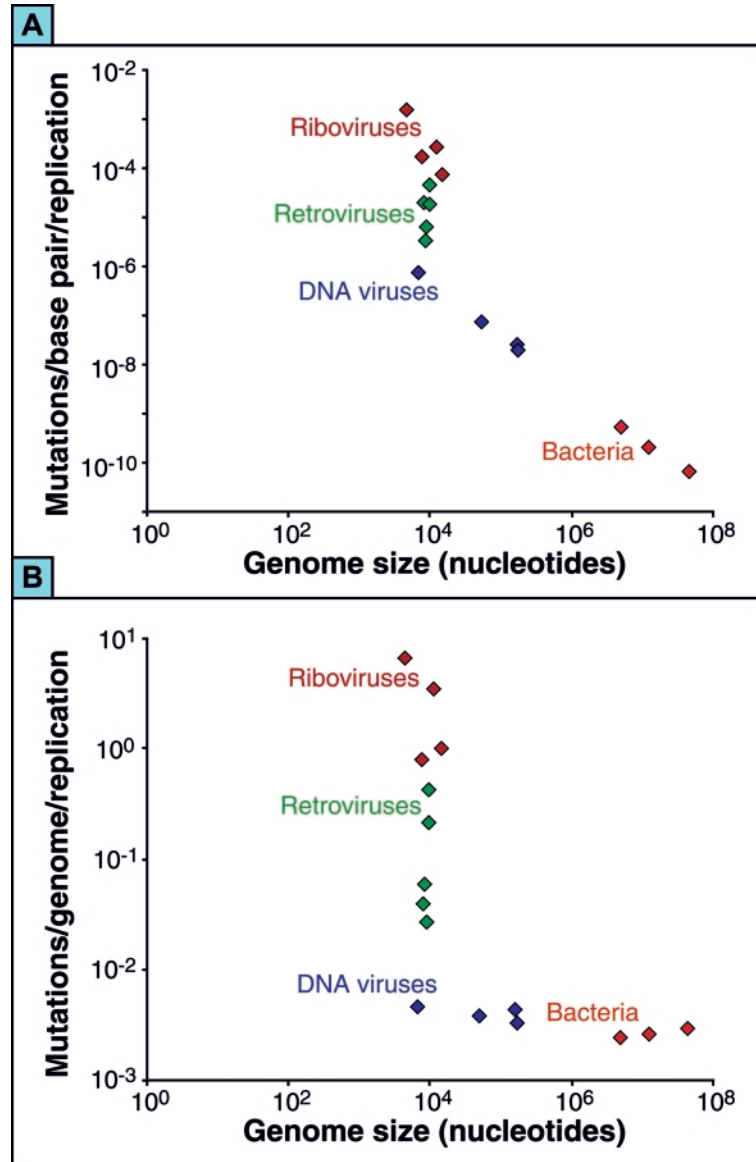
Figure 1   Relationship between genome size and mutation rate. The mutation rate for several classes of species including riboviruses (QB, poliovirus, VSV, influenza A), retroviruses (SNV, MLV, RSV, BLV, HIV-1), DNA viruses (M13, λ, T2, T4), and bacteria (*Escherichia coli*, *Saccharomyces cerevisiae*, *Neurospora crassa*) is indicated as either (*a*) mutations per base pair per replication or (*b*) mutations per genome per replication. Each class of species is indicated with a different color. Data are from Reference 20.
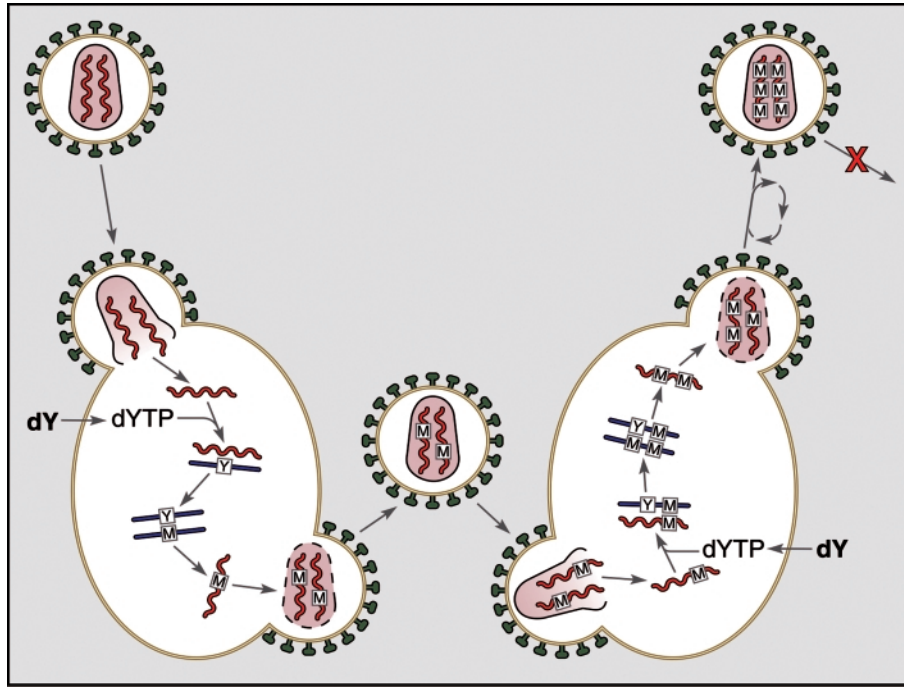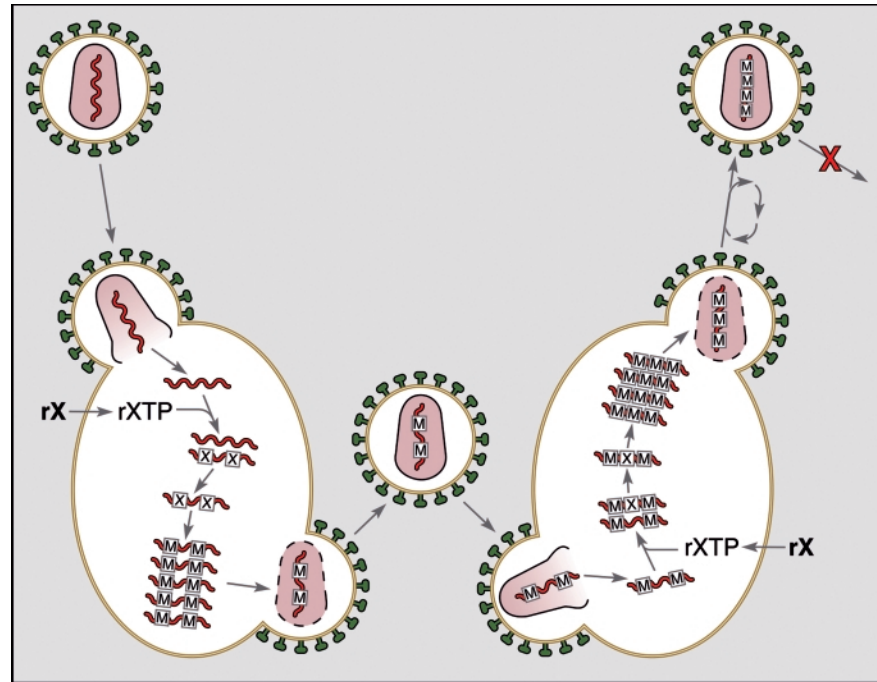
Figure 2   Progressive accumulation of mutations in an HIV genome during multiple rounds of infection. dY and dYTP refer to nucleoside and nucleoside triphosphate analogs, respectively. Y represents an analog and M represents an analog-induced mutation present in the HIV genome. Red and blue lines represent viral RNA and DNA, respectively.
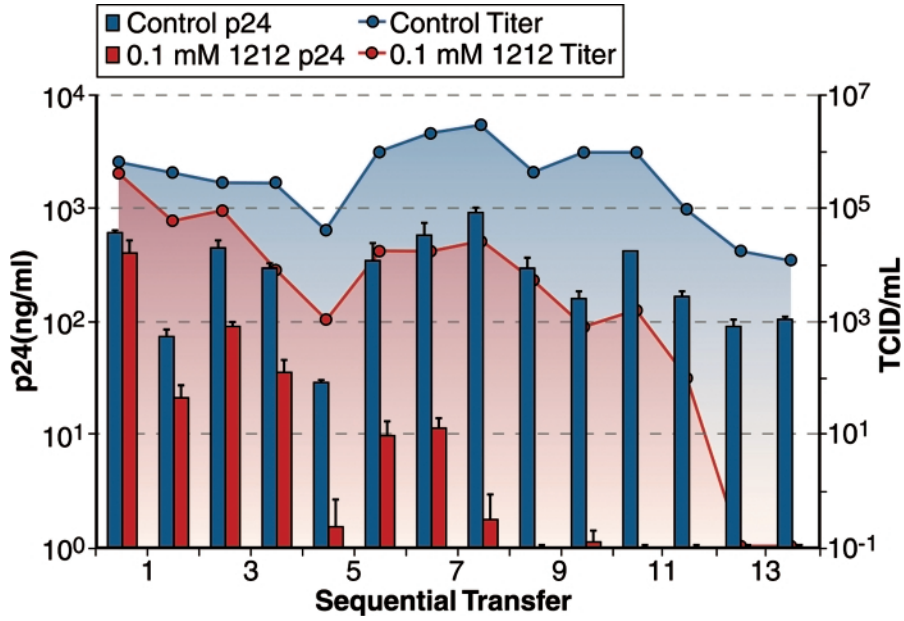
Figure 4   Sequential passaging of HIV in the absence (no drug control) or presence of SN1212 (100 nM). Comparison of p24 antigen performed in quadruplicate cultures to viral infectivity, measured by $TCID_{50}$. The multiplicity of infection of viral inoculum at passage 1 is 1:100. (Note different scale for p24 and $TCID_{50}$.)

Figure 5 Progressive accumulation of mutations in a ribovirus genome during multiple rounds of infection. rX and rXTP refer to ribonucleoside and ribonucleoside triphosphate analogs, respectively. X represents an analog and M represents an analog-induced mutation present in the HIV genome. Red lines represent viral RNA.