

# Stealth Nucleosides

## Mode of Action and Potential Use in the Treatment of Viral Diseases

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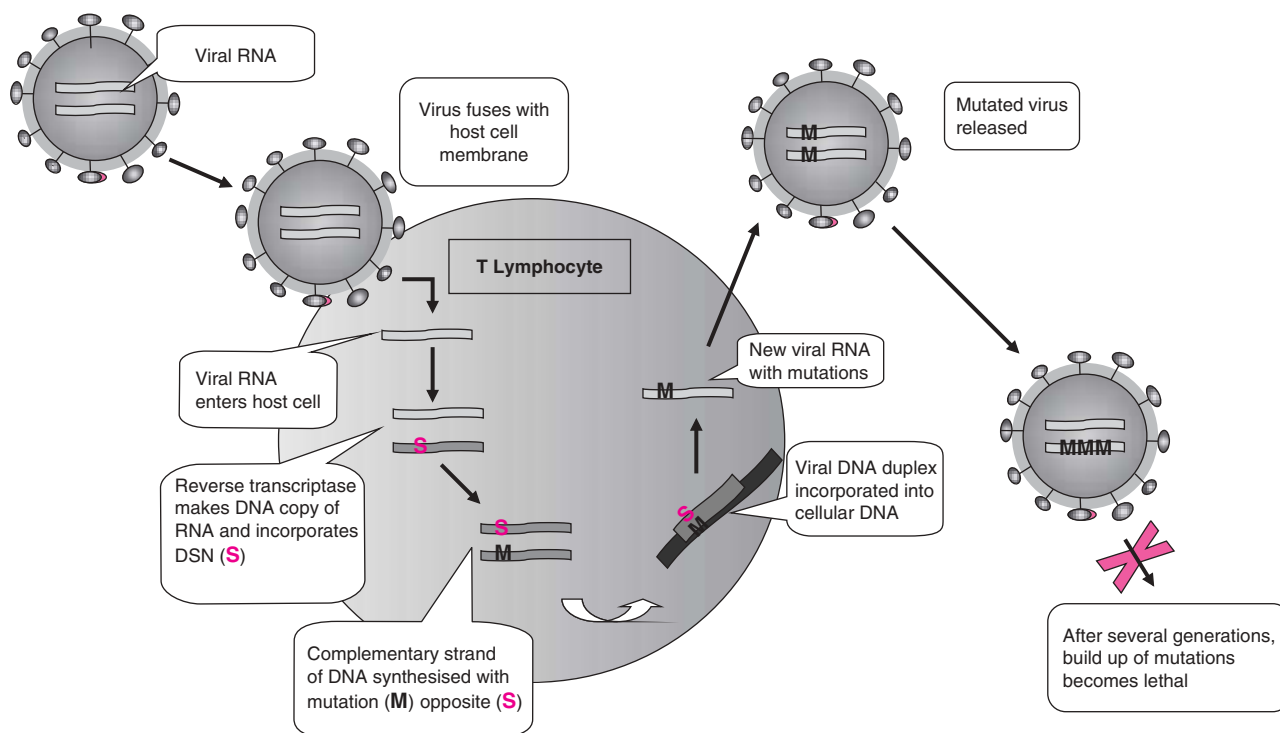
### Abstract

Riboviruses and retroviruses have been shown to spontaneously mutate at an extraordinarily high rate. While this genetic diversity allows viral subpopulations to escape conventional antivirals, it also has a cost. Indeed, this high mutation rate results in the synthesis of many defective virions. Stealth nucleosides are nucleoside analogues that are designed to increase the already high spontaneous mutation rate of viruses to the point where the virus cannot further replicate, a process known as 'lethal mutagenesis'. Rather than causing chain termination and attempting to immediately halt viral replication, as with conventional nucleoside reverse transcriptase inhibitors (NRTI), stealth nucleosides are incorporated into the viral genome during replication and, by mispairing, cause mutations to the viral genome. These mutations affect all viral proteins and cumulatively, over a number of replication cycles, are lethal to the virus. There are two distinct stealth nucleoside platforms: DNA stealth nucleosides and RNA stealth nucleosides. DNA stealth nucleosides are currently being screened for activity against HIV and may have activity against hepatitis B virus and smallpox virus, with the clinical lead DNA stealth nucleoside demonstrating activity in the low nanomolar range. In addition, DNA stealth nucleosides have been shown to be able to effectively treat NRTI-resistant HIV strains *in vitro*, which is not surprising given that the two principal modes of resistance (low affinity of reverse transcriptase for a modified sugar or pyrophosphorolysis) should not be applicable to DNA stealth nucleosides. RNA stealth nucleosides are being developed for the treatment of ribovirus infections, and particularly hepatitis C virus infection. RNA stealth nucleosides are selected for their broad spectrum of antiviral activity, and current lead RNA stealth nucleosides have potency in the same range as ribavirin.

Stealth nucleosides are a novel class of antiviral nucleoside analogues conceived by Drs. Lawrence Loeb and James Mullins (University of Washington) and John Essigmann (Massachusetts Institute of Technology).<sup>[1,2]</sup> Stealth nucleosides function by 'lethal mutagenesis', referred to more recently as 'selective viral mutagenesis'. Nucleoside analogues developed for selective viral mutagenesis are selectively incorporated into the viral genome, causing mutations, which, over time, push the virus beyond the

threshold of viability. In contrast to currently available nucleoside chain terminators, which ultimately lose their effectiveness because of the high rate of mutation of viruses, nucleoside analogues developed for selective viral mutagenesis work with the very mechanism leading to the emergence of viral resistance.

There are two distinct classes of stealth nucleosides:



**Fig. 1.** Selective viral mutagenesis of HIV. DNA stealth nucleosides induce mutations in the viral genome of HIV until HIV is unable to further replicate. DSN = DNA stealth nucleoside.

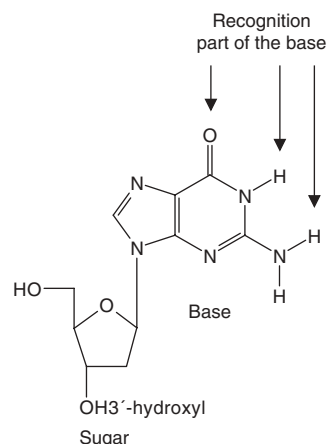
- DNA stealth nucleosides are directed at the treatment of HIV. This class of molecule may also have activity against hepatitis B virus (HBV) and smallpox virus.
- RNA stealth nucleosides have a broad spectrum of antiviral activity, including potentially all riboviruses (RNA viruses with the exception of retroviruses). RNA stealth nucleosides are being developed for the treatment of hepatitis C virus (HCV) but may have applications to the therapy of other riboviruses, such as haemorrhagic fever viruses.

### 1. The Concept of Selective Viral Mutagenesis

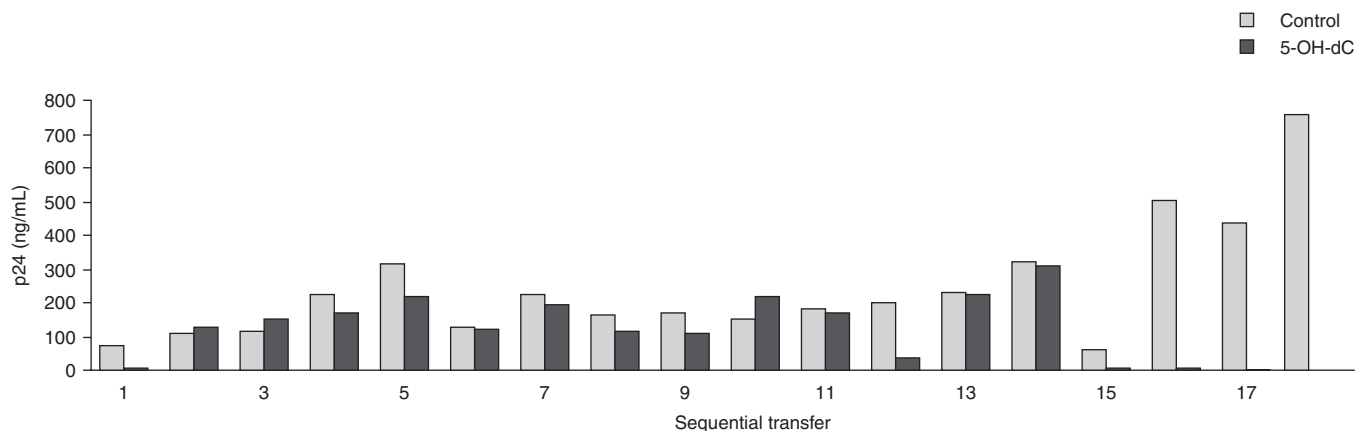
The rate of mutation of riboviruses and retroviruses is so high, and their genomes are so informationally dense, that even a modest increase in the mutation rate has been modelled to extinguish the population.<sup>[3,4]</sup> Stealth nucleosides are mutagenic nucleosides designed to further increase the already high spontaneous mutation rate of viruses. In the case of the treatment of HIV, DNA stealth nucleosides are preferentially incorporated into viral DNA by viral reverse transcriptase (HIV RT). Mutations are induced in the complementary DNA strand generated by HIV RT. During transcription of the genome of progeny viruses, these mutations are incorporated in the newly synthesised viral RNA. As a result, the mutations in the viral RNA genome accumulate with each viral replication cycle. Eventually the number of mutations in

each viral genome is so large that active viral proteins are no longer produced (figure 1).

As shown in figure 2, a deoxyribonucleoside consists of a nucleobase linked to a deoxyribose sugar. The challenge of designing DNA stealth nucleosides is that they require modification to the recognition part of the base in order to induce mutations by mispairing with incoming nucleotides. This differs from almost all approved antiviral nucleoside reverse transcriptase inhibitor (NRTI) chain terminators (e.g. didanosine, lamivudine, zalcitabine), where the only modification is to the sugar moiety. At a minimum,



**Fig. 2.** Structure of deoxyribonucleoside.



**Fig. 3.** Mean levels of HIV p24 antigen in 5-hydroxy-2'-deoxycytidine (5-OH-dC [1 mmol/L]) treated versus control medium as a function of number of sequential infections by HIV in tissue culture. The experiment was performed in triplicate.

an NRTI has a modification to the 3'-hydroxyl to preclude DNA chain extension.

The first analogue to validate the principle of selective viral mutagenesis was 5-hydroxy-2'-deoxycytidine (5-OH-dC).<sup>[1]</sup> While sequential passaging of HIV in the presence of drug has been used to select for resistant variants,<sup>[5]</sup> a similar experimental scheme was used in the laboratories of Drs Loeb and Mullins to demonstrate activity of 5-OH-dC. After each cycle of infection, replication of HIV was assessed by measuring the presence of a viral protein (p24), after growth of cells for 4 days, in the presence or absence of 5-OH-dC. The results of these experiments are shown in figure 3. After 16 passages of HIV into fresh cells in the presence of 5-OH-dC, an irreversible loss in viral levels was observed and no detectable p24 protein was observed after the 17th passage. In fact, nested polymerase chain reaction (PCR) performed at passage 23 could not detect the presence of viral RNA.

The graph in figure 3 represents the means of triplicates of the first experiment carried out with 5-OH-dC. Since then, this experiment has been done in triplicate a total of three times. In eight of nine trials, 5-OH-dC led to passage-dependent changes in the virus population, resulting in loss of infectivity by passage 30.

A second principle underlying the concept of selective viral mutagenesis is that the types of mutations caused by a given nucleoside analogue in the HIV genome should be in accord with the base-pairing properties of that nucleoside analogue. Sequencing of a segment of reverse transcriptase obtained from HIV in the presence of 5-OH-dC at the penultimate passage prior to extinction (passage 16) confirms this principle. The 5-OH-dC caused a 5.6-fold increase in the frequency of the substitution of adenosine for guanine that would be predicted on the basis of the chemical structure of this nucleoside analogue.

A third principle of selective viral mutagenesis is that while a DNA stealth nucleoside is poorly repaired in an RNA•DNA hybrid, such as that produced by HIV RT,<sup>[6]</sup> it is subject to facile excision by DNA repair enzymes should it be incorporated in a DNA•DNA duplex. Cellular DNA is subjected to continual attack and there are at least 130 DNA repair genes.<sup>[7]</sup> In contrast, cellular repair of incorporated NRTI may be relatively inefficient. This has been shown to be the case for the 3'-5' exonuclease active site of mitochondrial polymerase. Because of the general role of the 3'-OH in the exonucleolytic catalysis, the inefficient excision of chain terminators by polymerases with intrinsic exonucleases may be a general phenomenon. Indeed, nuclear polymerase  $\delta$  and  $\epsilon$  have also been shown to remove zidovudine-monophosphate inefficiently from the 3'-termini.<sup>[8]</sup>

In the case of 5-OH-dC, an electrochemical assay demonstrated that 5-OH-dC was not incorporated into cellular DNA at the limit of sensitivity of six molecules of 5-OH-dC per  $10^6$  cytidine. This lack of stable incorporation of 5-OH-dC was confirmed by the fact that 5-OH-dC at a concentration of 4 mmol/L does not increase the mutation frequency at the hypoxanthine phosphoribosyltransferase (*HPRTI*) locus above background in transformed human lymphoblasts. Finally, there is no evidence of mitochondrial toxicity, as measured by mitochondrial DNA production, after culturing CEM lymphoblasts in the presence of 0.5–2.0 mmol/L 5-OH-dC for 8 days.

## 2. DNA Stealth Nucleosides for the Treatment of HIV

As of 2001, 16 antiviral agents were approved in the US for the treatment of HIV infection. Seven are nucleoside/nucleotide analogue chain terminators (NRTI), six are protease inhibitors and three are non-nucleoside reverse transcriptase inhibitors (NNRTI).

Until recently, zidovudine remained the mainstay of anti-HIV drugs. The administration of zidovudine to patients with advanced

HIV disease has been shown to prolong survival, improve neurological function, transiently improve CD4+ lymphocyte counts, and decrease the rate of antigenaemia. However, the short-term benefits observed with zidovudine monotherapy, together with the emergence of zidovudine resistance during long-term treatment, suggested that combination chemotherapy would be required for prolonged control of HIV infection.<sup>[9,10]</sup>

In 1996, clinical trial results demonstrated that protease inhibitors could dramatically reduce the amount of HIV in a patient's blood and in combination therapy regimens could, in some cases, result in undetectable viral RNA by PCR. A clinical trial of saquinavir, zidovudine and zalcitabine combination chemotherapy demonstrated increased CD4+ counts and decreased viral burden that were significantly greater than with a two-drug regimen.<sup>[11]</sup> However, as with NRTI, there is evidence that cross-resistance develops to protease inhibitors.<sup>[12]</sup> In fact, simultaneous mutations of the HIV genome coding for resistance to protease inhibitors and NRTI have been described.<sup>[13]</sup> Unfortunately, resistance has been found to develop rapidly after initiation of the most recently approved class of antiretrovirals, NNRTI, with cross-resistance among agents of this class nearly universal.<sup>[14]</sup> Of note, combination therapy regimens (highly active antiretroviral therapy; HAART), typically initiated with triple-drug therapy, are expensive and because of their complexity and side effects adversely affect patients' quality of life. Full therapeutic benefit may require near-perfect adherence to the dosage, frequency, timing and dietary restrictions of many agents.<sup>[15]</sup> Furthermore, if virological, immunological or clinical failure develops during triple therapy, a regimen of five or more drugs may be necessary, so-called mega-HAART.<sup>[16]</sup> These complex regimens also induce important morphological or metabolic changes, namely alterations in body-fat distribution, dyslipidaemia and lactic acidosis.<sup>[17]</sup> Not surprisingly, resistant strains are being transmitted to new patients with increasing frequency. For example, in San Francisco, between 1996 and 2001, the proportion of newly infected patients with virus resistant to any drug class increased from 16.7% to 27.6%.<sup>[18]</sup>

Activity of both purine and pyrimidine DNA stealth nucleosides has been demonstrated; an example of pyrimidine DNA

stealth nucleosides is 5-formyl-2'-deoxycytidine (5-fo-dC). This DNA stealth nucleoside is a sufficiently potent viral mutagen that is capable of eradicating HIV in a single passage, as opposed to the multiple passages required for 5-OH-dC, although a single passage does not preclude multiple cycles of viral replication.

5-fo-dC can be produced endogenously in cells. DNA containing 5-methyl-2'-deoxycytidine can in the presence of radiation lead to the formation of 5-fo-dC, which has been postulated to be one cause of C→T transition mutations frequently found in cytosine methylation sites. The presence of an intramolecular hydrogen bond between the 5-formyl group of 5-fo-dC and the 4-amino function may affect the equilibrium between the amino- and imino-tautomers and thus induce the transition mutation C•G→T•A and the transversion mutation C•G→A•T.<sup>[19,20]</sup> It is unclear whether mammalian cells possess repair enzymes for 5-fo-dC.<sup>[19]</sup>

When tested against the laboratory-adapted strain HIV LAI, 5-fo-dC has an EC<sub>50</sub> (effective concentration preventing 50% of viral replication) of 3 μmol/L, while the IC<sub>50</sub> (concentration that inhibits 50% of cellular viability) is >1 mmol/L. 5-fo-dC increases the mutation rate at least 2.4-fold above control values, as the rate in the untreated control may have been overestimated (table I). This is consistent with data generated for 5-OH-dC (see above) or reported in the literature for other analogues,<sup>[21,22]</sup> as leading to viral extinction *in vitro*.

5-fo-dC represents an example of a DNA stealth nucleoside capable of eradicating HIV in a single passage, but its relative lack of potency makes it a questionable candidate for further development. By way of comparison, our clinical lead candidate, SN1212, has an EC<sub>50</sub> of 10 nmol/L and an IC<sub>50</sub> of >1 mmol/L. SN1212 is currently undergoing testing for animal toxicology and pharmacology, as a prelude to clinical trials.

While routine screening of candidate DNA stealth nucleosides is performed against HIV LAI, the more active candidates are also screened for activity against strains of HIV with pre-existing resistance to NRTI. Resistance toward NRTI is achieved via two distinct and generally exclusive mechanisms. Efficiency of discrimination or excision by pyrophosphorolysis in the presence of

**Table I.** Mutation rate in a fragment of HIV reverse transcriptase at third passage in tissue culture in the presence of 5-formyl-2'-deoxycytidine (5-fo-dC) or in the absence of drug (control)

Analogue	Mutations/nucleotide	%	G→A	T→C	A→T	A→C	G→T	C→G
			A→G	C→T	T→A	C→A	T→G	G→C
5-fo-dC <sup>a</sup>	16/14 170	0.113	6	9	1			
Control <sup>b</sup>	4/8352	0.047	1		1	1		1

a 78 μmol/L.

b The mutation rate in the control may be an overestimate because it was not confirmed in both directions.

**A** = adenine; **C** = cytosine; **G** = guanine; **T** = thymine.

nucleotides of a given NRTI is a key determinant in the emergence of one or the other resistance pathway.<sup>[23]</sup> Decrease in affinity of HIV RT for an NRTI usually involves alterations in the sugar moiety of an analogue, e.g. mutations M184V or Q151M.<sup>[24]</sup> Alternatively, chain terminators may be removed by pyrophosphorolysis, or reverse nucleotide polymerisation, where pyrophosphate acts as acceptor molecule for the removal of the chain terminator. Removal of the chain terminator frees RT to incorporate the natural nucleotide substrate and rescue viral replication. ATP has also been proposed as an acceptor molecule for the removal of chain terminators and is referred to as primer unblocking.<sup>[25]</sup>

The following is a summary of reasons why viral resistance is less likely to emerge to DNA stealth nucleosides than to NRTI:

- DNA stealth nucleosides apply less selective pressure to a viral population for emergence of resistant variants than approved antivirals, which attempt to immediately halt viral replication.
- DNA stealth nucleosides adversely affect all viral proteins.
- Decreased affinity of HIV RT for a modified nucleoside sugar is one mechanism of viral resistance. For example, it has been shown that RT may recognise the absence of a 3'-OH group, resulting in cross-resistance among chain terminators.<sup>[26]</sup> DNA stealth nucleosides, like natural nucleosides, have a 3'-OH group.
- Pyrophosphorolysis, the other principal mechanism of viral resistance to conventional nucleoside analogues, is unlikely to be applicable to DNA stealth nucleosides. Pyrophosphorolysis results in the excision by RT of a chain terminator preventing DNA chain elongation.

The first two propositions have been demonstrated to be true by passaging experiments (e.g. those performed with 5-OH-dC, see above) and by sequencing fragments of the viral genome. The latter two hypotheses were tested by assembling a panel of three NRTI-resistant HIV strains (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), where resistance is achieved by pyrophosphorolysis or enhanced RT discrimination. These strains have most of the mutations in susceptibility to NRTI present in routine clinical samples,<sup>[27]</sup> namely:

- HIV-1 LAI-M184V: M184V mutation with resistance to lamivudine (3TC). M184V decreases the likelihood of incorporation of lamivudine 5'-triphosphate (3TC-TP) by interaction with the sulphur of the oxathiolane ring, but interestingly also enhances sensitivity to zidovudine, perhaps by reducing pyrophosphorolytic activity.<sup>[28]</sup>
- HIV-1 RTMDR1: 74V, 41L, 106A and 215Y mutations with resistance to zidovudine, didanosine, nevirapine and other NNRTI. Template/primer repositioning may play a role in the decreased DNA synthesis processivity associated with the 74V

mutation for didanosine. Resistance mutations 41L and 215Y enhance pyrophosphorolysis.<sup>[24]</sup>

- HIV-1 RTMC: 67N, 70R, 215F and 219Q with resistance to zidovudine. All of these mutations enhance pyrophosphorolysis.<sup>[24]</sup>

When tested against these three strains, the EC<sub>50</sub> of SN1212 remained essentially unchanged, confirming the lack of cross-resistance between DNA stealth nucleosides and NRTI.

### 3. RNA Stealth Nucleosides for the Treatment of Riboviruses

RNA stealth nucleosides are being developed for the treatment of ribovirus infections, with a particular focus on HCV infection. Until recently, interferon- $\alpha$  was the only drug approved as a single therapy for HCV in Europe and North America. In the short term, about 50% of patients have a response to interferon, but the long-term results have been disappointing. Recently approved, peg-interferon- $\alpha$  has reduced systemic clearance, which enables once-weekly administration while maintaining the same adverse-effect profile. Sustained virological responses (SVR; defined as undetectable HCV RNA at 24 weeks follow-up after 48 weeks of treatment) were 18–25% for peginterferon- $\alpha$  versus 12% for interferon- $\alpha$ . Among patients with viral genotype 1 (the majority of patients infected with HCV in North America), the SVR was 14%, while other viral genotypes had a 45% response rate.<sup>[29]</sup> The addition of ribavirin to peginterferon can increase SVR to 34–42% in patients with viral genotype 1.<sup>[30]</sup>

The applicability of interferon-based therapies to a metropolitan clinic population infected with HCV was recently assessed.<sup>[31]</sup> Only a minority of patients (28%) were ultimately treated. Of the untreated patients, 34% were not treated because of contraindications to interferon therapy. Thus, the paucity of effective treatments for HCV suggests that novel therapies are required in the medical marketplace.

RNA stealth nucleosides are designed to exploit the inherent differences in the fate of viral RNA and of host cell messenger RNA (mRNA) and are preferentially incorporated into viral RNAs by viral RNA replicases. During synthesis of progeny virions, the mutagenic nucleoside analogues in the viral RNA miscode at high frequency and cause mutations in the newly synthesised viral RNA. With each viral replication cycle, mutations accumulate in the viral RNA genome. Eventually, the number of mutations in each viral genome is so large that no active viral proteins are produced. In human cells the genetic material is DNA and is replicated during each cellular division by DNA polymerases that catalyse the polymerisation of deoxyribonucleotide triphosphates.

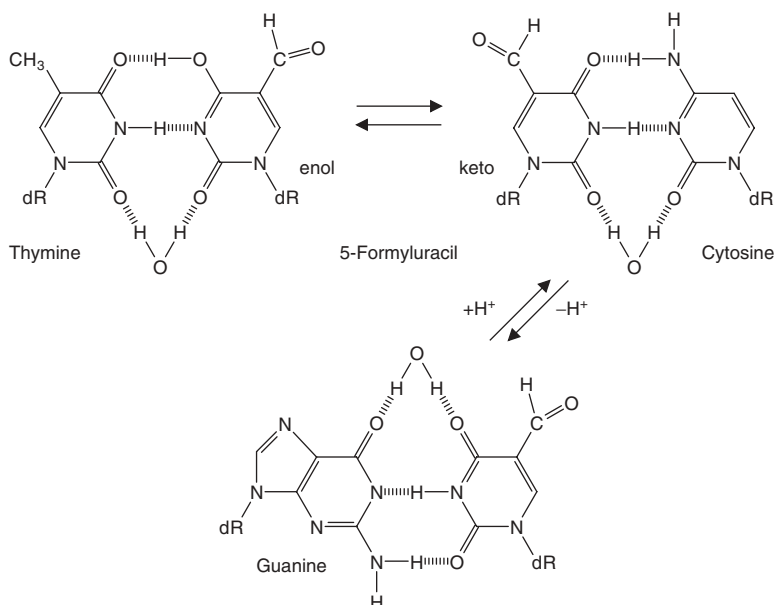
The information that DNA contains is transcribed into RNA by RNA polymerase, which polymerises ribonucleotide triphosphates. The newly synthesised RNA guides the synthesis of proteins that carry out cellular functions. The information in the cellular RNA is not incorporated into the cellular genome and is not passed on to subsequent generations of new cells. Even if the host cell RNA should contain the nucleoside analogue, and in such instance mispair with transfer RNAs to yield cellular proteins with altered amino acid sequences, it will be a transitory phenomenon. Miscoding by RNA is unlikely to be detrimental to host cell functions. These altered RNAs have a short lifetime in cells, generally less than a day,<sup>[32]</sup> and are not incorporated into the genome of progeny cells. Furthermore, studies on the insertion of random sequences into plasmid-encoded genes indicate that the encoded proteins tolerate a wide variety of mutations, even within the catalytic site of enzymes.<sup>[33]</sup>

Finally, there is a low risk of genotoxicity from RNA stealth nucleosides. Ribonucleoside reductase (RR) is responsible for the reduction of the diphosphate of ribose to deoxyribose, thus providing a potential pathway for RNA stealth nucleosides to become integrated into genomic DNA. Fortunately, experimental data suggest that RR is exquisitely sensitive to substrate modification,<sup>[34]</sup> and RNA stealth nucleosides are selected on the basis of experience with DNA stealth nucleosides such that, should the RNA stealth nucleosides be reduced to a deoxyribose and incorporated into DNA, it would be as readily repaired as a DNA stealth nucleoside.

Screening of candidate RNA stealth nucleosides was initiated against measles virus (MV) [Nagahata strain] and bovine viral diarrhoea virus (BVDV) [Singer strain]. MV is a member of the family Paramyxoviridae and BVDV is a nonhuman pathogen member of the family Flaviviridae that is distantly related to HCV. Because of the difficulty of growing HCV *in vitro*, BVDV has been used to screen drugs for activity.<sup>[35]</sup> The medical relevance of Flaviviridae is further highlighted by two recently re-emerging infections that are causing considerable concern in the US, dengue and West Nile fever, while two other emergent viral pathogens, Nipah and Hendra viruses, are Paramyxoviridae.<sup>[36]</sup>

A number of analogues were found to prevent BVDV replication in one passage; one such discovered early was 5-formyluridine (5-fo-U)<sup>[37]</sup>. The deoxy derivative, 5-formyldeoxyuridine (5-fo-dU), has been identified as a thymine lesion in DNA. It is one of the most abundant pyrimidine products formed by ionising radiation in the presence of oxygen. A potential mutagenic effect of 5-fo-dU was initially suggested because 5-fo-dU is removed efficiently by DNA glycosylase, including human cell free extracts.<sup>[38]</sup> In *Escherichia coli* a number of proteins are involved in the repair pathways for 5-formyluracil, including AlkA, Nth, Nei and MutM.<sup>[39]</sup> In an *E. coli* system, 5-fo-dU was able to mispair with guanine, cytosine and thymine during replication in addition to pairing with its cognate base, adenine<sup>[38]</sup> (figure 4).

The EC<sub>50</sub> of 5-fo-U against BVDV is 150 µmol/L and against MV is 140 µmol/L, while the IC<sub>50</sub> is 1 mmol/L. By way of



**Fig. 4.** Possible based pairing of 5-formyluracil, in addition to pairing with cognate base, adenine (reproduced from Bjelland S, Ånensen H, Knaevelsrud I, et al. Cellular effects of 5-formyluracil in DNA. *Mutat Res* 2001; 486: 147-54.<sup>[38]</sup> Copyright (2001), with permission from Elsevier Science). **dR** = deoxyribose.

**Table II.** Mutation rate of a fragment of polymerase and a fragment of envelope, in bovine viral diarrhoea virus treated with RNA stealth nucleosides compared with untreated control

Analogue	Mutations/nucleotide	%	G→A	T→C	A→T	A→C	G→T	C→G
			A→G	C→T	T→A	C→A	T→G	G→C
5-fo-U <sup>a</sup>	19/12 660	0.147	3	8	2	2	2	2
Control	8/19 160	0.038	1	4	2	1		

a 5-formyl uridine (5-fo-U) concentration is 2 mmol/L.

**A** = adenine; **C** = cytosine; **G** = guanine; **T** = thymine.

comparison, the EC<sub>50</sub> of ribavirin in our assays is 15 µmol/L and 10 µmol/L, respectively. More recently, a number of RNA stealth nucleosides have been identified with potency in the same range as ribavirin.

The mutation frequency induced by 5-fo-U to the BVDV genome was calculated and compared with untreated control virus (table II). 5-fo-U has been shown to increase the mutation rate of the viral genome of BVDV 3.9-fold above background.

#### 4. Ribavirin and Other Mutagenic Ribonucleoside Analogues

A number of investigators have demonstrated the possibility of using mutagenic ribonucleoside analogues to treat riboviruses. The dominant mechanism of action of ribavirin was recently demonstrated to be viral mutagenesis.<sup>[21,40]</sup> Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a ribonucleoside analogue that resembles guanosine and inosine. Until recently, the mode of action of ribavirin was unknown, although several theories were proposed to explain its antiviral activity, two most prominently:<sup>[41]</sup>

- inhibition of cellular inosine-5'-monophosphate dehydrogenase activity (IMPDH), thereby depleting intracellular pool of guanosine triphosphate (GTP);
- immunomodulation, with enhancement of helper T-cell response and suppression of B-cell proliferation.

Ribavirin was most commonly believed to be an IMPDH inhibitor. However, strengthening the argument that ribavirin's primary mode of action is through mutagenesis of the viral genome is the finding that: (i) carbocyclic analogues of ribavirin, which are potent inhibitors of IMPDH, do not possess antiviral activity; and (ii) a ribavirin derivative (4-fluoro-1-β-D-ribofuranosyl-1H-pyrazole-3-carboxamide) with enhanced antiviral activity proved to be a less potent inhibitor of IMPDH than ribavirin.<sup>[42]</sup>

Crotty et al.<sup>[21,40]</sup> measured the mutagenic potential of ribavirin on poliovirus and found that it templates incorporation of cytidine and uridine with equal efficiency. An increase in the mutation rate of 2- to 6-fold above background produced a substantial antiviral

effect, similar to data generated by us (see table II). In fact, the antiviral activity of ribavirin was found to correlate directly with its mutagenic activity. More recently, studies have been performed to determine the mode of action of ribavirin against HCV. A deletion derivative of HCV RNA-dependent RNA polymerase (RDRP) [NS5B] was used to test the hypothesis that the antiviral activity of ribavirin against HCV can be mediated at least in part by the RDRP. In this assay, ribavirin was found to template opposite cytidine and uridine with equal efficiency, but also caused a substantial block to RNA elongation of nascent RNA when it was present in the template.<sup>[43]</sup>

Other confirmation of the therapeutic potential of mutagenic ribonucleoside analogues was recently provided by treating foot-and-mouth disease virus with a weakly mutagenic nucleobase (fluorouracil) *in vitro*.<sup>[22,44]</sup> Finally, an *in vitro* transcription model has demonstrated the theoretical feasibility of using a mutagenic ribonucleoside analogue for the treatment of HIV.<sup>[45]</sup>

#### 5. Conclusion

DNA and RNA stealth nucleosides are novel classes of nucleoside analogues designed to function with the very mechanism, lack of fidelity during viral replication, that defeats current therapeutics. They differ from conventional NRTI by their incorporation in a growing chain of DNA or RNA and their ability to mispair with incoming bases. Functionally, rather than outright attempting to halt viral replication, they are designed to further increase the already high mutation rate of retroviruses and riboviruses to the point where the virus cannot synthesise functional proteins and replicate. The benefits of this approach are the low likelihood of viral resistance and, for RNA stealth nucleosides, the broad spectrum of antiviral activity. The recent finding that the predominant mechanism of action of the approved therapeutic ribavirin is selective viral mutagenesis validates the approach. Having synthesised a number of stealth nucleosides with the requisite *in vitro* activity, the challenge now is to further develop these stealth nucleosides and demonstrate that they are safe and effective *in vivo*.

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