Ribonucleotide reductases: radical enzymes with suicidal tendencies

Ribonucleotide reductases catalyze a key step in DNA biosynthesis, using a diverse array of unprecedented metallo-cofactors to generate a transient protein radical that initiates nucleotide reduction. The new understanding of the chemistry and biochemistry of the system has allowed rational design of inhibitors of this process, which function as antitumor and antiviral agents.

Chemistry & Biology December 1995, **2**:793–801

Ribonucleotide reductases (RNRs) catalyze the conversion of nucleotides to deoxynucleotides, providing the monomeric precursors required for DNA biosynthesis and DNA repair. These enzymes are unusual for a number of reasons. First, they have very broad substrate specificity and can catalyze the reduction of purine and pyrimidine nucleotides. Since maintenance of the relative ratios of dNTP pools is essential for the fidelity of DNA replication, they are allosterically regulated in a sophisticated and incompletely understood fashion [1,2]. Second, despite the central role of RNRs in nucleotide metabolism, implying that they should be well conserved, their primary and quaternary structures are diverse. Third, the cofactors required for the reduction process, structurally and chemically, have also not been evolutionarily conserved (Fig. 1). At present there are four classes of reductases, three of which have been and continue to be under intense investigation [3].

The RNR isolated from bacteria grown under aerobic conditions is prototypical of the class I RNRs, which also include mammalian and herpes simplex virus (HSV) RNRs. It is composed of two homodimeric subunits, R1 and R2. R2 contains the cofactor, which is composed of an unusual μ-oxo-bridged diferric cluster adjacent to a tyrosyl radical (•Tyr122 in *Escherichia coli*) [4]. The tyrosyl radical is essential for catalysis, and is generated by the diferrous form of R2 in the presence of O_2 [5,6].

The RNR isolated from *Lactobacillus leichmannii* (class II), in contrast, is a monomer that uses adenosylcobalamin (AdoCbl = coenzyme B_{12}) as a cofactor. This enzyme has been the most extensively studied of the AdoCbl-requiring RNRs [7]. Recent studies indicate that the function of AdoCbl is to generate a thiyl radical that is essential for the nucleotide reduction process [8].

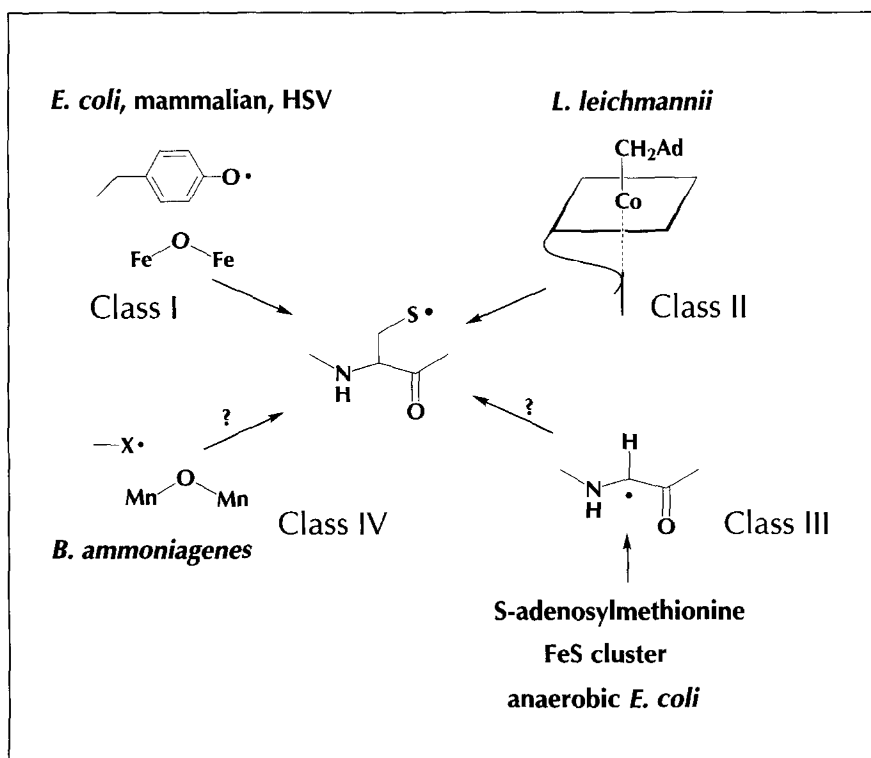

Fig. 1. RNRs use a wide range of cofactors. The cofactors used by the four major classes of RNRs are shown. Although chemically diverse, it seems likely that each of these cofactors can be used to help form a thiyl radical (center), which is essential for catalysis in at least two, and probably all four, classes of RNRs.

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The RNR isolated from *E. coli* grown under anaerobic conditions (class III) is distinct from the enzyme produced under aerobic conditions. When isolated, it proved to have an essential glycyl radical which is generated by an 'activating enzyme' [9], perhaps the second subunit of the enzyme, equivalent to R2 in the aerobic *E. coli* RNR. The activating enzyme requires S-adenosylmethionine and an iron–sulfur cluster to generate the glycyl radical via a novel mechanism [10].

The RNR from *Brevibacterium ammoniagenes* (which we consider as class IV) has been characterized by Follmann and coworkers [11] and is thought to possess $\alpha_2\beta$ subunit structure and a dinuclear Mn^{3+} cluster analogous to the diferric cluster of aerobic *E. coli* RNR. Although it seems plausible that this enzyme, like all the other RNRs so far investigated, uses a protein radical for catalysis, this point has not yet been established.

Despite the dramatic differences in these reductases (Fig. 1), their mechanisms for nucleotide reduction (at least for classes I–III) are similar, and unusual, involving exquisitely controlled radical-based chemistry. Each of the metallo-cofactors initiates this radical-dependent nucleotide reduction process by generating a transient protein radical. The working model at present, based on the two best characterized RNRs, those from *E. coli* and *L. leichmannii*, is that the function of both the tyrosyl radical (in *E. coli*) and the adenosylcobalamin (in *L. leichmannii*) is to generate a thiyl radical [12]. Many excellent reviews have been published on all aspects of RNRs in the past few years [2,3,5,7,13]. Here, we will focus on the new ideas on the catalytic mechanism obtained from studies using site-directed mutagenesis, mechanism-based inhibitors and structural techniques. As the diferric–tyrosyl radical requiring reductases (class I RNRs) are potentially attractive targets for the design of antitumor and antiviral agents, this new information has led to a number of different approaches to inhibitor design.

Background

The sequences of a growing number of class I RNRs are available. There are 49 residues that are completely or strongly conserved in all 23 known sequences of the 87-kDa R1 protomer, and 21 residues conserved in the 24 known sequences of the 43.5-kDa R2 protomer. The X-ray structures of R1 and R2 from *E. coli* have also been reported [14,15]. The structural studies and sequence alignments, in conjunction with chemical and biochemical studies, have provided us with a generic picture of class I RNRs (Fig. 2). The 'business end' of the molecule is the R1 subunit. It contains binding sites for the purine and pyrimidine diphosphate substrates and for the dNTPs and ATP, which act as allosteric effectors. In addition it contains five cysteines that are essential for catalysis [16]. Cysteines 225 and 462 are oxidized to form a disulfide during nucleotide reduction, thus providing the required reducing equivalents [17,18]. Cys754 and Cys759 are at the carboxy-terminal tail of R1, and are not detectable in the X-ray structure due to thermal flexibility. They shuttle reducing equivalents into and out of the active site via disulfide interchange with thioredoxin (one of several reductants present *in vivo*). The fifth cysteine, Cys439, is proposed to initiate nucleotide reduction by 3'-hydrogen atom abstraction [19].

The R2 subunit contains the diferric iron center and the tyrosyl radical essential for the reduction process [4,20]. This cofactor is buried in R2, lying 10 Å from the nearest surface [15]. Reduction of the tyrosyl radical to tyrosine results in complete loss of nucleotide reduction capabilities. It is proposed that the tyrosyl radical generates the thiyl radical on Cys439 of R1, which (based on docking of the two available structures [14]) is some 35 Å away, thus initiating the nucleotide reduction

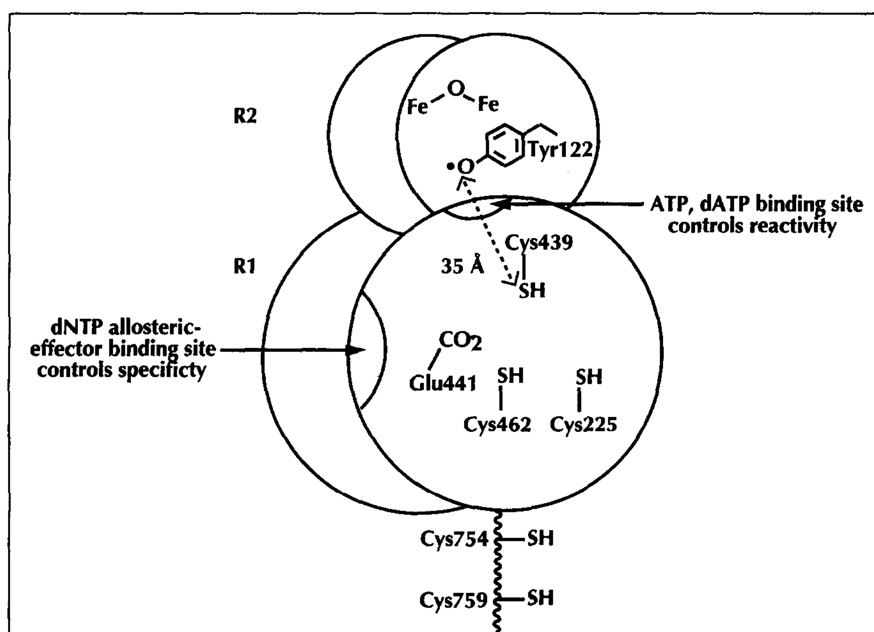

Fig. 2. Generic picture of the class I RNRs, based on extensive biochemical studies [7], and on docking of the two available X-ray crystal structures of R1 and R2 of the aerobic enzyme from *E. coli* [14,15]. The tyrosyl radical on R2 is proposed to generate a thiyl radical at Cys439, 35 Å away, via an unknown mechanism.

process. The mechanism of communication between these subunits is a major focus of investigation.

The most recent proposal [16] for a generic nucleotide reduction mechanism is shown in Figure 3. The key steps involve cofactor-mediated formation of a transient thiyl radical which initiates the nucleotide reduction process by abstracting the 3'-hydrogen atom. After loss of H_2O, the two cysteines on the α-face of the nucleotide (Cys462, Cys225) deliver the required reducing equivalents, generating a 3'-ketodeoxynucleotide and a disulfide radical anion. This intermediate is subsequently reduced to give dNDP and a disulfide, regenerating the thiyl radical. The essential metallo-cofactor, the interactions between the two subunits of the class I RNRs and the unusual radical intermediates in the nucleotide reduction process have all provided proven targets for design of inhibitors of this essential enzyme.

Inhibitors of diferric–tyrosyl radical dependent RNRs

Three potentially complementary approaches have successfully targeted RNR *in vivo*, based on the detailed understanding of the biochemistry of this system and of nucleotide metabolism. One involves nucleotide analogs as mechanism-based inhibitors. A second involves reduction of the essential tyrosyl radical required for nucleotide reduction. The third focuses on inhibition of the interaction of the R1 and R2 subunits, both of which are required for nucleotide reduction. All three approaches have led to compounds used clinically or in clinical trials.

Nucleotide inhibitors requiring 3'-carbon–hydrogen bond cleavage

The first approach, using nucleotide analogs as inhibitors, has provided considerable insight into the catalytic capabilities of RNRs and has also shown promise in generation of antitumor agents that may be clinically useful [21]. Both gemcitabine (2',2"-difluorodeoxycytidine, dFdC) [22,23] and (*E*)-2'-methylenefluoro-2'-deoxycytidine ((*E*)-FMC) [24,25] are now in clinical trials for the treatment of solid tumors of the breast and prostate. The requirement that the RNR inhibitor must be a nucleotide, but the therapeutically administered drug must be a nucleoside (because nucleotides are unable to penetrate the cell membrane) combined with the complexity of nucleotide metabolism raises the concern that drugs of the this kind could interfere in a multitude of metabolic pathways and lead to unwanted cytotoxic effects. Despite the potential problems, these compounds appear to be promising.

Inhibitors of RNR can be used synergistically with DNA chain terminators targeting DNA polymerase [26]. This is easy to rationalize; inhibition of RNR decreases the amounts of dNDPs produced, and therefore decreases the amounts of dNTPs (the substrates for the polymerase) available (Fig. 4). Nucleotide chain terminators (NTP analogs) will then be used more often by the polymerase, because of the decreased competition from the normal substrates. For cytidine nucleotide analogs, a second pathway may further potentiate the inhibition of DNA synthesis. DeoxyCTP has been reported to be an end-product inhibitor of deoxycytidine kinase (dCK), although its potency *in vivo* has been the subject of some debate [27–29]. Decreases in levels of dCTP should therefore increase the activity of the kinase, thus increasing the conversion of cytidine analogs into the active di- and tri- phosphate forms.

In 1976 Thelander, Eckstein and coworkers [30] reported that 2'-chloro-2'-deoxynucleotides such as ClCDP and

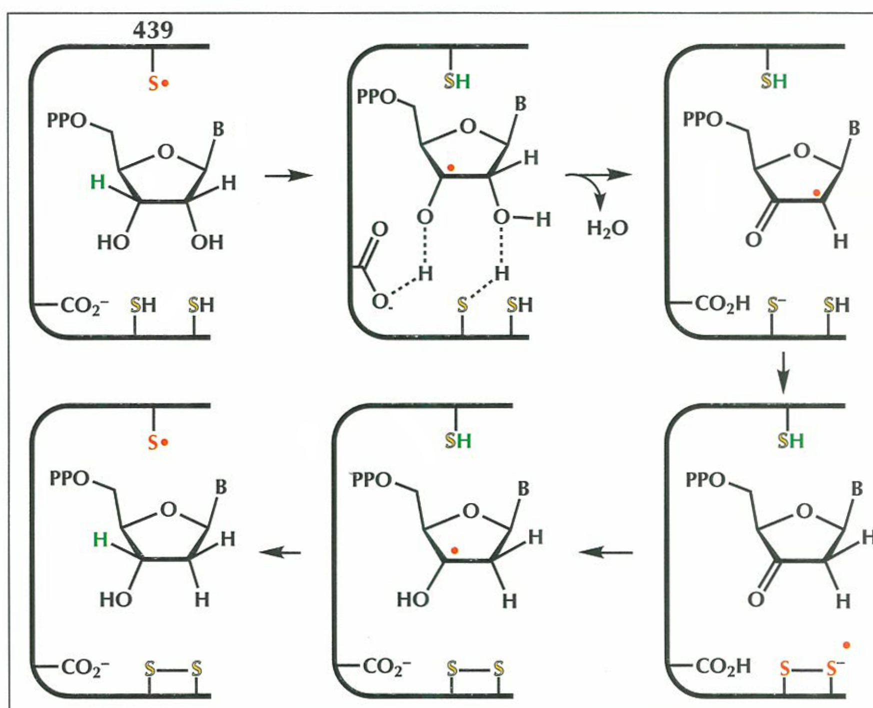

Fig. 3. Proposed mechanism for nucleotide reduction by RNRs. A transient thiyl radical (red) initiates the nucleotide reduction process by abstracting the 3'-hydrogen atom (green) from the nucleoside diphosphate. H_2O is lost, and the two cysteines on the α-face of the nucleotide then deliver the required reducing equivalents, generating a 3'-ketodeoxynucleotide and a disulfide radical anion. This intermediate is subsequently reduced to give dNDP and a disulfide and to regenerate the thiyl radical. B, base.

2'-azido-2'-deoxynucleotides, such as N_3CDP, were potent inactivators of *E. coli* RNR. A detailed understanding of the mechanism by which these compounds inactivate the enzyme has evolved since their discovery [7]. It was the insights gained from these studies that led McCarthy and coworkers [25,31] to design (*E*)-FMC as a potent, specific inhibitor of RNR.

A generalized view of the mechanism responsible for enzyme inactivation by nucleotide analogs is shown in Figure 5. The initial step, as in the case of NDP reduction, is 3'-hydrogen atom abstraction by a thiyl radical. Deprotonation of the 3'-hydroxyl concomitant with removal of the leaving group, X, results in formation of a 2'-deoxy-3'-ketonucleotide radical which can then be reduced from the top face (β-face) or the bottom face (α-face) of the inhibitor. Reduction from the bottom face results ultimately in the inability to regenerate the thiyl radical on Cys439 and hence, in the case of the *E. coli* RNR, the tyrosyl radical cannot be re-formed. A 3'-ketodeoxynucleotide is also generated; its fate will be discussed below. Since the tyrosyl radical is essential for catalysis, inactivation of R2 is a major mechanism of RNR inhibition by many nucleotide mechanism-based inhibitors. If reduction occurs from the top face, however, the thiyl radical on Cys439, and hence the tyrosyl radical, is regenerated along with production of the same putative 3'-ketodeoxynucleotide. The subtle differences in protonation state of the cysteines in the active site during inactivation by these inhibitors, compared to their state during the normal reduction process (compare Fig. 3 with Fig. 5) causes the 3'-ketodeoxynucleotide to dissociate into solution. This compound then chemically decomposes to generate PPi, free nucleic-acid base and 2-methylene 3(2H) furanone [7]. The last of these inactivates RNR by nonspecific alkylation of the R1 subunit. Incubation of [2'-^{3}H] and [3'-^{3}H]-2'-chlorodeoxynucleotides with RNRs, followed by trapping of the intermediate 3'-ketodeoxynucleotide with $NaBH_4$ and analysis of the stereochemistry of the products at C2', has established that reduction can occur from either face of the nucleotide [7,32]. These results showed that a given nucleotide analog inhibitor can use two different mechanisms for enzyme inactivation. The partitioning between R1 and R2 inactivation depends on the nucleotide analog, the allosteric effector and the reducing system present in a subtle way, which is incompletely understood. The results from studies with a variety of mechanism-based inhibitors can be accounted for by this paradigm (Fig. 5), accompanied, as outlined below, by a few additional twists that have interesting mechanistic implications (Fig. 6).

Understanding the mechanism of inhibition

Inactivating one molecule of *E. coli* RNR requires 1.5 equivalents of N_3UDP. Enzyme inactivation is accompanied by rapid loss of the essential tyrosyl radical on R2 and production of N_2 (Fig. 6a), initiated by 3'-hydrogen atom abstraction [30,33]. Loss of the essential tyrosyl radical occurs concomitant with rapid formation of a new nitrogen-centered radical [34,35], which recent studies have shown to be covalently attached to Cys225 and probably to the 3'-ketodeoxynucleotide generated during the inactivation event [36]. Ultimately the nitrogen-centered radical disappears (with a $t_{1/2}$ of 20 min) and is accompanied by the generation of pyrophosphate, nucleic acid base, and the furanone (compound **1**, Fig. 6), which stoichiometrically alkylates R1 [33]. The evidence that HN_3 is released before the formation of N_2 and the nitrogen-centered radical

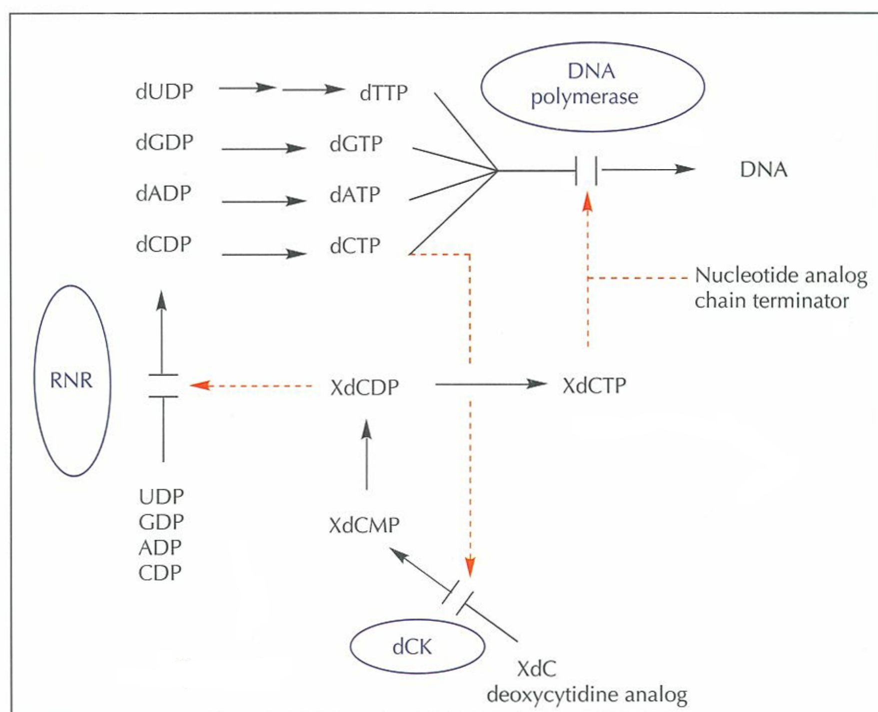

Fig. 4. Summary of nucleoside metabolism pathways. Nucleoside diphosphates are reduced by RNRs to deoxynucleoside diphosphates, then phosphorylated to the corresponding triphosphates, which are used as substrates for DNA polymerase. Inhibitors of RNRs reduce the available pool of dNTPs, decreasing the competition for nucleotide analog chain terminators and increasing their effectiveness. Inhibitors of dCTP production may also decrease feedback inhibition of deoxycytidine kinase (dCK). This increases phosphorylation of deoxycytidine analogs, leading to increased production of the triphosphate, which inhibits DNA polymerase. There are thus two mechanisms for RNR inhibitors to increase the effectiveness of deoxycytosine-based DNA polymerase inhibitors.

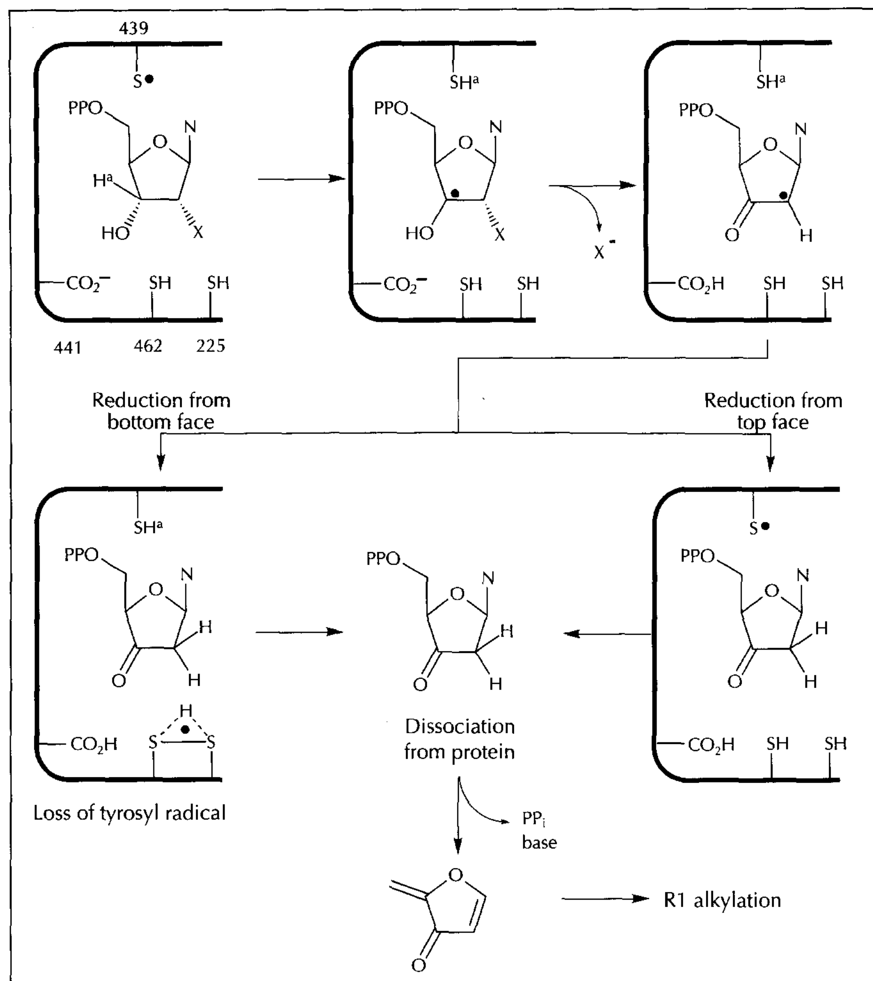

Fig. 5. Mechanism of inhibition of an RNR by a nucleotide analog. After abstraction of the 3'-hydrogen atom by the thiyl radical, the leaving group, X^-, is lost generating a 3'-keto-2'-deoxynucleotide radical. This species can be reduced from either the β-face (top) or the α-face (bottom). If reduction occurs from the α-face, the thiyl radical cannot be regenerated and the enzyme is inactivated. Reduction from the β-face results in a 3'-ketodeoxynucleotide which dissociates from the active site and decomposes to yield the 2-methylene-3(2H)-furanone, which non-specifically alkylates the R1 subunit of the enzyme, inactivating it (this can be detected by absorbance at $\lambda \approx 320$ nm). The factors that determine whether the reduction occurs from the top face or the bottom face are incompletely understood. B, base; PPi, pyrophosphate.

is based on studies with a mutant R1, in which Cys225 is changed to a serine. With this mutant, tyrosyl radical is not lost and no nitrogen-centered radical is generated [37]. Only azide is detected. These observations provide support for the hypothesis that Cys225 is central in the conversion of the hydrazoic acid to N_2 and the covalently bound nitrogen radical species. The studies with this inhibitor support the proposal that reduction from the α-face of the nucleotide can proceed by an electron-transfer mechanism and that RNR catalyzes radical-dependent reactions.

A second intriguing observation has been made with this same site-directed mutant of R1, Cys225→SerR1. This mutant uncouples 3'-carbon–hydrogen bond cleavage from the nucleotide-reduction process. Incubation of CDP with Cys225→SerR1 and R2 resulted in loss of the tyrosyl radical, and release of cytosine and pyrophosphate ([38]; Fig. 6b). The inability to complete the nucleotide-reduction process, due to the mutation of Cys225, leads to cleavage of the R1 polypeptide into two pieces. The cleavage is stoichiometric with tyrosyl radical loss, suggesting that the fragmentation is a radical-based reaction. Recently, the site of cleavage has been established by mass spectroscopic analysis to be between Ser224 and Ser225 of the mutant enzyme (W.A. van der D., C. Zeng, K. Biemann, J.S., A. Hanlon, & J.E. Kyte, unpublished data). The Ser224 fragment does not have a carboxylate carboxyl terminus, but a carboxamide. Furthermore, Val226 has a formyl group on its nitrogen (Fig. 6b). The fate of the remaining two carbons of Ser225 has not been defined. Although the chemistry of this process is unknown, these studies provide the first unambiguous identification of the active site of RNR. Moreover, the uncoupling of the nucleotide-reduction process from the 3'-hydrogen atom abstraction and the use of [3'-^{2}H]-NDP have established that there is an isotope effect on tyrosyl radical reduction [38]. These studies provide the first direct evidence that the tyrosyl radical on R2 is involved in hydrogen-atom abstraction from the NDP on R1, some 35 Å away.

A detailed understanding of the modes of inactivation of wild type RNR by ClNDP, N_3NDP and of Cys225→SerR1 by CDP has led to the design of a variety of mechanism-based inhibitors. One of the designed inhibitors, (*E*)-FMCDP (Fig. 6c), is now in clinical trials [24,25]. Its mode of inactivation is similar to that described above with an interesting twist and a mechanistic bonus (W.A. van der D. *et al.*, & E. Wagner, unpublished data). One and a half equivalents of FMCDP is sufficient for enzyme inactivation, which results from a partitioning between both R1 and R2

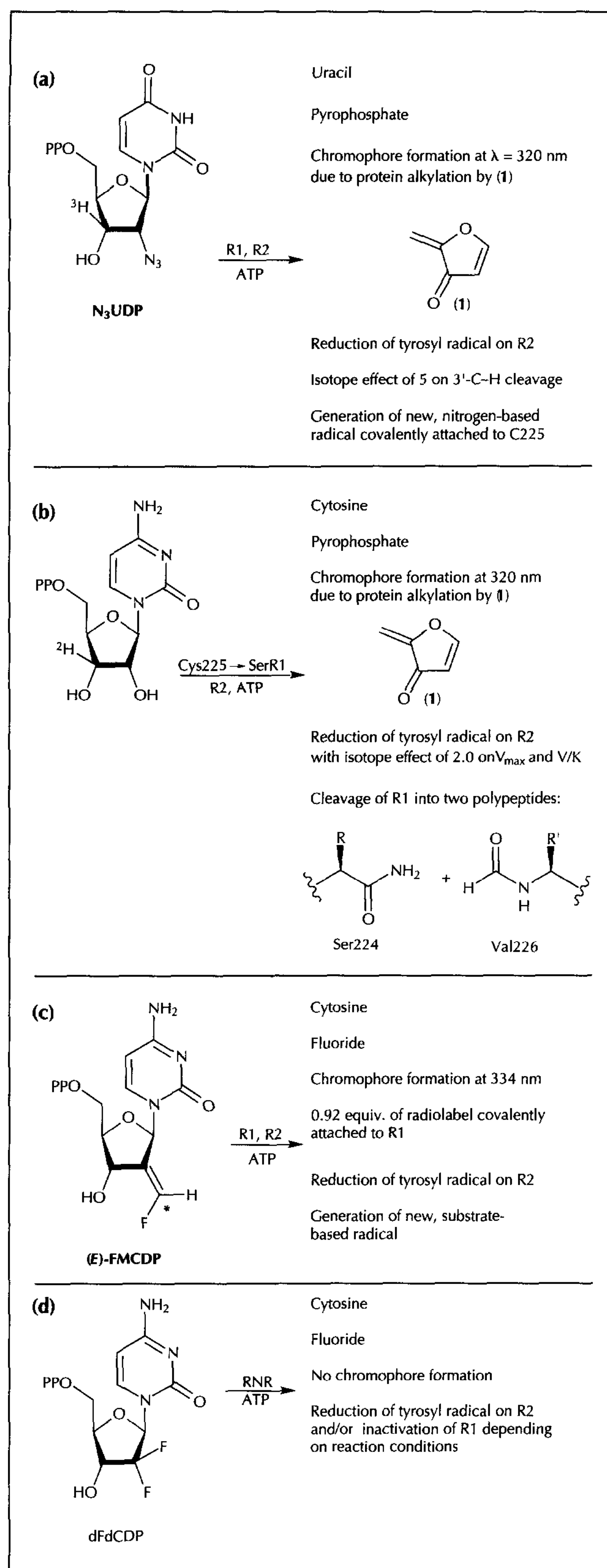

Fig. 6. Reactions of nucleotide analog inhibitors with RNR and RNR mutants. Brief descriptions are given of the reaction of **(a)** N_3UDP with the wild-type aerobic *E. coli* enzyme R1R2; **(b)** CDP with the Cys225→Ser R1 mutant and R2; **(c)** (*E*)-FMCDP with the wild-type R1R2 enzyme (* indicates ^{14}C label); and **(d)** dFdCDP with the wild-type enzyme. These reactions are further described in the text.

inactivation. The tyrosyl radical is readily lost (Fig. 6c), a chromophore associated with R1, detectable at wavelength 334 nm, rapidly appears, cytosine and fluoride ion are released and R1 is stoichiometrically labeled. Studies with a variety of site-directed R1 mutants suggest that the covalently modified residue is either Cys439 or Glu441. Efforts to identify a labeled peptide have been unsuccessful, but solid state NMR experiments are now in progress using [6'-^{13}C]-FMCDP to determine whether an oxygen or a sulfur atom is attached to the labeled carbon.

As in the cases of inactivation of wild type R1 by N_3UDP and Cys225→SerR1 by CDP, loss of the tyrosyl radical is accompanied by formation of a second radical. Recent electron paramagnetic resonance spectroscopy studies of this reaction using [6'-^{2}H]-(*E*)-FMCDP have demonstrated that the second radical is substrate-derived. A mechanism that accommodates all of the available data is shown in Figure 7. Preliminary results suggest that the stabilized 3'-radical intermediate (compound **2**, Fig. 7) can be directly observed using this inhibitor, which would provide the first direct evidence for 3'-hydrogen atom abstraction from a nucleotide.

Gemcitabine (dFdC) is another nucleoside analog which is presently being evaluated for clinical use against leukemias and solid tumors [22,23]. Studies on the metabolism of dFdC have indicated the possibility of self-potentiation via the synergistic mechanisms described above (Fig. 4) [39]. *In vitro* about four equivalents of dFdCDP are required to completely inactivate RNR from *E. coli*. The mode of inhibition is strongly dependent on the reaction conditions, and, although similar to the paradigm shown in Figure 5 (Fig. 6d), its mechanistic details have not been elucidated (R. Sanchez, G. Yu, W.A. van der D. and J.A.S, unpublished data). It is interesting that dFdCDP is the only mechanism-based inhibitor studied to date which does not produce an observable chromophore on R1 concomitant with R1 inactivation.

Tyrosyl radical reduction

The second approach to inhibitor design of Class I RNRs uses compounds such as hydroxyurea (HU) which has been shown to reduce the essential tyrosyl radical on R2, generating an oxidized HU radical [40]. HU does not bind to R2 and the X-ray structure of R2 from *E. coli* has revealed that Tyr122, which is the residue that is ultimately reduced, is 10 Å from the closest surface [15]. The mechanism by which this reduction occurs therefore remains a mystery. It is intriguing that the rate of HU-mediated reduction of the tyrosyl radical is potentiated by the R1 subunit in the presence of certain substrates/or effectors [41]. Since the communication between R2 and R1, separated by ~35 Å, has been proposed to occur via a coupled electron and proton transfer process, it could be that HU is intercepting an intermediate radical generated during this process.

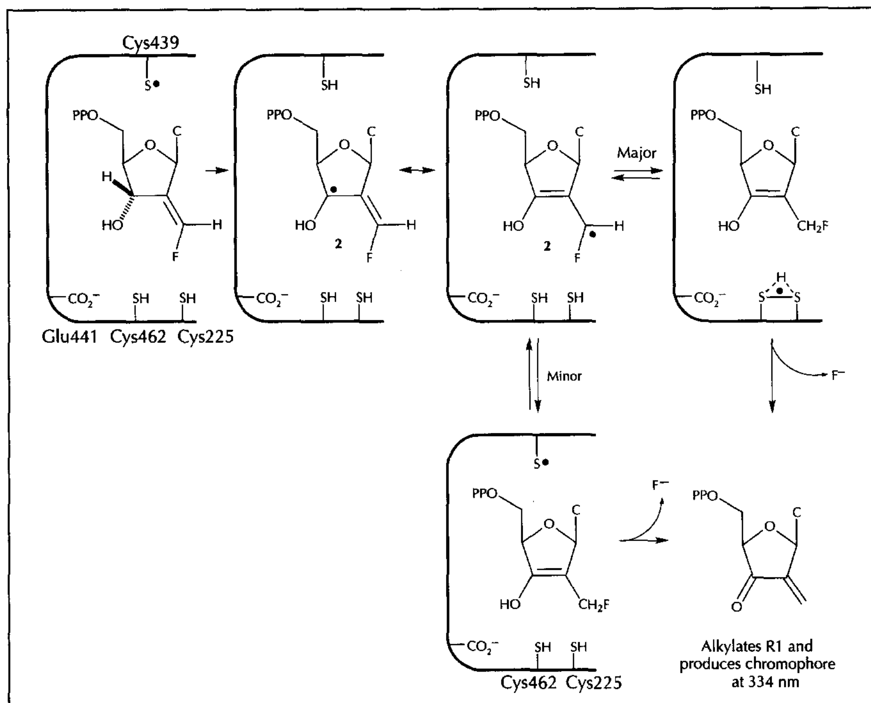

Fig. 7. Proposed mechanism for RNR inhibition by (*E*)-FMCDP. Hydrogen abstraction from the 3' carbon of the inhibitor proceeds normally. The radical intermediate (**2**) can be reduced from the bottom face (major pathway) or top face (minor pathway). In both cases a reactive enol is generated that can rapidly lose fluoride to generate an α,β-unsaturated ketone activated towards nucleophilic attack. Because reduction occurs from the bottom face in the major pathway, the predominant mode of inactivation is from loss of the tyrosyl radical. C, cytosine.

HU has recently been claimed to potentiate the effect of AZT, the triphosphate of which inhibits the reverse transcriptase enzyme of human immunodeficiency virus, in the treatment of AIDS [42]. This is reminiscient of the effect of the nucleotide mechanism-based inhibitors of RNR on inhibitors of DNA polymerase, and the mechanism is probably much the same. Although HU can clearly inhibit class I RNRs by reducing their essential tyrosyl radical, caution is warranted when considering this as a therapeutic target. Studies in *E. coli* by Reichard, Fontecave and coworkers [5,43] have shown that a flavodoxin reductase and a unknown factor, in the presence of O_2, can regenerate the tyrosyl radical after it has been reduced. It is not yet clear whether such a metabolic pathway exists in eukaryotic systems; it might, for example, regulate nucleotide reduction as a function of the cell cycle. It is possible, however, that efficient inactivation of RNR might require inhibition of a putative regeneration pathway as well. On the other hand, incomplete inhibition may be ideal, since the activity of the mammalian enzyme is required for normal cell replication and survival.

Thiosemicarbazones [44] are also known to destroy the tyrosyl radical on R2 and may be placed in the same category of inhibitors with HU, although their mechanism of inactivation remains to be established. These compounds have recently been developed in conjunction with acyclovir (9-[(2-hydroxyethoxy)methyl]guanine, the triphosphate of which inhibits HSV polymerase) as topically applicable HSV inhibitors [45,46].

Inhibition of subunit interactions

Biochemical studies have established that the R1 subunit of RNR can be easily separated from the R2 subunit and more quantitative studies have revealed the K_d for interaction of the subunits to be ~0.18 μM for *E. coli* [47], and ~0.17 μM for mouse RNR [48]. This weak interaction provides a third approach to the design of inhibitors of RNRs. Dutia *et al.* [49] and Cohen *et al.* [50] observed with RNR from HSV-1 that the nine amino acids at the carboxy-terminal end of the R2 subunit of this enzyme are the predominant source of binding energy between the two subunits. Studies on the mammalian [51] and *E. coli* RNRs [47] suggest that the carboxy-terminal tails of their respective R2 subunits provide the predominant source of interaction energy between subunits as well. Since the carboxy-terminal tails of all of these subunits have unique sequences, it might be possible to design specific inhibitors [52]. The largest effort in drug design using this approach is from the group at Bio-méga, targeting HSV-RNRs. They have recently designed a peptidomimetic of the carboxy-terminal tail of HSV R2, which inhibits nucleotide reduction both *in vitro* and *in vivo* [53].

Despite the solution of the X-ray structure of *E. coli* R2, the conformation of the carboxy-terminal tail has been elusive as the X-ray structure did not detect the terminal 30 amino acids. NMR analysis of mammalian R2 [54], HSV R2 (S.R. LaPlante, N. Aubry, N. Moss & M. Liuzzi, abstract, Keystone Symposium on Molecular and Cellular Biology, March, 1993) and, more recently, *E. coli* R2 [55] indicate that the carboxy-terminal tail is detectable using this method, as it tumbles at a rate different from the overall rate of globular R2, indicating that it is not folded as part of the globular domain but behaves as a distinct entity. A peptide corresponding to the carboxy-terminal tail of the *E. coli* R2 subunit allowed the successful crystallization of the R1 subunit, but only 8 of the 20 amino acids are apparently detectable [14]. Thus, although this

region of R2 is clearly important in interactions between the two subunits, more work will be required before the molecular details of the contacts between the subunits are understood. Design of inhibitors of essential subunit interactions could provide a new paradigm in drug design. Whether this approach is applicable to situations in which the inter-subunit interactions have an affinity higher than the sub-micromolar affinity of R1 for R2 remains to be established, however.

Summary

Since the discoveries of RNRs in the 1960s, a wealth of knowledge has been obtained about the chemistry and biochemistry of these unusual metallo-proteins. The new frontiers will involve understanding the assembly and disassembly of the metallo-cofactors, the mechanism(s) of allosteric regulation which govern specificity and turnover, and the regulation at the transcriptional [56] and translational levels and discovering how these proteins fit into the machinery involved in DNA replication and repair.

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