Clofarabine 5-di and -triphosphates inhibit human ribonucleotide reductase by altering the quaternary structure of its large subunit

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Human ribonucleotide reductases (hRNRS) catalyze the conversion of nucleotides to deoxyxynucleotides and are composed of α- and β-subunits that form active αβₙ (n = 2 or 6) complexes. α binds NDP substrates (CDP, UDP, ADP, and GDP, C site) as well as ATP and dNTPs (dATP, dGTP, TTP) allosteric effectors that control enzyme activity (A site) and substrate specificity (S site). Clofarabine (CIF), an adenosine analog, is used in the treatment of refractory leukemias. Its mode of cytotoxicity is thought to be associated in part with the triphosphate functioning as an allosteric inhibitor of hRNR. Studies on the mechanism of inhibition of hRNR by CIF di- and triphosphates (CIFDP and CIFFTP) are presented. CIFFTP is a reversible inhibitor (Kᵢ = 40 nM) that rapidly inactivates hRNR. However, with time, 50% of the activity is recovered. D57N-α, a mutant with an altered A site, prevents inhibition by CIFFTP suggesting its A site binding. CIFDP is a slow-binding, reversible inhibitor (Kᵢ = 17 nM; t₁/₂ = 23 min). CDP protects α from its inhibition. The altered off-rate of CIFDP from E × CIFDP by CIFFTP (A site) or dGTP (S site) and its inhibition of D57N-α together implicate its C site binding. Size exclusion chromatography of hRNR or α alone with CIFDP or CIFFTP, dATP or dGTP, reveals in each case that α forms a kinetically stable hexameric state. This is the first example of hexamerization of α induced by an NDP analog that reversibly binds at the active site.

allosteric regulation | oligomerization | slow-binding inhibitor

Both nucleosides enter cells via the CNT- or ENT-type transporters and their phosphorylation is required for cytotoxicity (1–4). The monophosphate of CIF (CIFMP) is generated predominantly by deoxycytidine kinase and sequentially converted to the diphosphate (CIFDP) (the rate limiting step) (5–7) and the triphosphate (CIFFTP) by MP and DP kinases, respectively (6–8). A key step in the metabolism of both CIF and F2C is proposed to be inhibition of ribonucleotide reductase (RNR) (4, 7–9), the enzyme responsible for conversion of NDPs to deoxynucleoside diphosphates (dNDPs) in all organisms. F₂C,CDP acts as a substoichiometric mechanism-based inhibitor (MBI) to inactivate human RNR (hRNR) (4, 10), whereas CIFFTP is proposed to inactivate hRNR by binding to an allosteric site that binds dATP (9). In both cases, depletion of dNDP pools results in reduction of dNTP pools, allowing CIFFTP or F₂C,CTP to compete effectively with the available dNTPs for incorporation into DNA. For CIFFTP, the consequences are dependent on the CIFFTP/dATP ratio and the inhibition of DNA polymerase α (7, 8). Chain termination of the polymerization process triggers apoptosis (5). Our recent results established that both fluoride ions (F⁻) of F₂C,CDP are eliminated during the covalent modification of RNR (4, 10, 11). We thus proposed that CIFFTP might function as an MBI with F⁻ loss, furanone formation, and covalent enzyme modification (10–14) and hence both the di- and triphosphates might play an important role in RNR inhibition. This paper reports characterization of CIFDP and -TP (Scheme S1) with the hRNR involved in DNA replication and describes an unprecedented mode of inhibition of this enzyme.

RNRs supply monomeric precursors required for DNA repilication/repair and are pivotal in controlling the fidelity of these processes (15). hRNRS are composed of α- and β-subunits that form an active quaternary complex(es) whose composition(s) is (are) currently under investigation (16–19). There are two different β-subunits: One is involved in DNA replication (the focus of this paper); the other, p53δ, is induced by p53 and is proposed to be involved in supplying dNTPs for mitochondrially derived DNA replication and repair (20). α binds four substrates (UDP, CDP, ADP, and GDP, C site) and has two allosteric effector binding sites, the specificity site (S site) and the activity site (A site). The former controls which substrate is reduced by binding the appropriate dNTP (ATP, dATP, dGTP, and TTP). The latter, also called the ATP cone domain, controls NDP reduction rate: When ATP is bound, RNR is active and when dATP is bound, RNR is inactive. The β-subunit houses the essential diffric-tyrosyl radical (Y*) cofactor that initiates nucleotide reduction in the active site of α by a 35 Å proton-coupled electron transfer process (21). Interactions between α and β are weak and modulated by NDP and dNTP/ATP binding to α with the active forms of the mammalian RNR proposed to be αβₙβₘ (n = 2 or 6) (16–19) and the inactive form produced by dATP binding to the A site, proposed to be αβ₈ (17, 19).

Studies on hRNR in vitro are challenging. First, Y* in β has t₁/₂ = 25 min at 37 °C. All inhibition studies must be corrected for this inherent instability. In addition, the weak interaction of the two subunits (18) and the multiple quaternary structures of both α and the αβ-complex (4, 16–19) make the concentrations at which the inhibition studies are carried out important.

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Clofarabine 5’-di and -triphosphates inhibit human ribonucleotide reductase by altering the quaternary structure of its large subunit

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Our studies show that CIFDP is a slow-binding, reversible inhibitor with a $K_i$ of 17 nM and a $t_{1/2}$ of 23 min that is not modified by hRNR. They also show that CIFTP is a reversible inhibitor ($K_i = 40$ nM) that rapidly inactivates hRNR, but regains approximately 50% activity with time. A series of experiments was undertaken to determine the site(s) of binding of CIFDP and -TP to $\alpha$ for responsibility for inhibition. First, studies are carried out with D57N-$\alpha$ (altered A site), which is no longer inhibited by dATP (16, 22). They reveal that CIFTP, up to 6 $\mu$M (150 $\times K_i$), is unable to inhibit hRNR, suggesting that it binds only to the A site. Previous studies in crude cell extracts have shown that CIFTP at 0.3–10 $\mu$M completely inhibits CDP reductase activities (5–9).

The same mutation, however, has no effect on CIFDP inhibition, suggesting that it does not bind to the A site. Second, a series of preincubation–dilution experiments with CIFTP and either CDP (C site) or dGTP (S site) shows no effect on its inhibition, whereas similar experiments with CIFDP and CDP show mutually exclusive binding, implying CIFDP binds to the C site. Third, studies measuring CIFDP release rate from [5-$\beta^3$H]-CIFDP$\cdot$hRNR in the presence of dGTP or CIFTP show rate constants significantly different from that in their absence, providing additional support for C-site specificity.

Finally the mode of inhibition of both compounds is associated with altered quaternary structures of $\alpha$. Size exclusion chromatography (SEC) subsequent to incubation of the hRNR or $\alpha$ alone with each inhibitor in the presence or absence of effectors shows that inhibition is associated with CIFDP- or CIFTP-dependent and effector-independent protein oligomerization. In both cases, the quaternary structure changes at $\alpha\theta$ or $\alpha\theta$ ($\Delta\theta \leq 2$) is more kinetically stable than dATP-induced hexamers. These studies identify CIF nucleotides as potent reversible inhibitors of hRNR and demonstrate the previously unrecognized important role of CIFDP. Furthermore, they provide evidence that NDP's binding to the C site of hRNR large subunit can alter quaternary structure.

**Results**

**Specific Activity of Human [His]$_2$$\cdot$$\alpha$-, $\beta$-, and Untagged-$\alpha$.** Talon affinity chromatography replaced Ni-NTA for (His)$_2$$\cdot$$\alpha$ purification (4) increasing yield and purity (Fig. S1). $\beta$ was isolated in apo form and reconstituted in vitro giving 0.8–1.2 Y $\cdot$ $\mu$M2 and 3.5 Fe/$\mu$M2 (4). Typical specific activities (SAs) for each (His)$_2$$\cdot$tagged subunit, with saturating amounts of the second subunit using CDP/ATP were 650–890 nmol/$\times$min mg for $\alpha$ and 3,000–4,100 for $\beta$. For ADP/dGTP/ATP the values were 140–160 for $\alpha$ and 200–220 for $\beta$. Without ATP, the values dropped to 70–80 for $\alpha$ and 80–95 for $\beta$. SAs of each tagged subunit for CDP or ADP reduction with 3 mM ATP remain within these ranges over 5 nM–5 $\mu$M of $\alpha$ ($\beta$) with 20–5-fold excess $\beta$ ($\alpha$). The corresponding SA of the untagged-$\alpha$, under identical conditions, is increased approximately 1.5-fold relative to tagged-$\alpha$.

**Time-Dependent Inactivation of hRNR by CIFDP.** Our previous studies with 2'-substituted 2'-deoxynucleotides such as 2'-ribofluo-2'-deoxythymidine 5'-diphosphate and F6CDP showed that both compounds were MBIs of bacterial and hRNRs (4, 10–14). Studies on Lactobacillus leichmannii RNR demonstrated that 2'-arabinoholonucleotides also could function as MBIs (12). Our hypothesis was that CIFDP might function similarly with removal of the 3'-hydrogen resulting in F- loss (13, 14). We initially focused on whether CIFDP could function as a time-dependent inhibitor of dNDR formation. Because CIF is an adenosine analog, dGTP and ATP were used as allosteric effectors as they maximize ADP reduction and potentially CIFDP inhibition. Incubation of one equivalent of CIFDP with hRNR/dGTP $\pm$ ATP, followed by asaying for residual activity of $\alpha$ using [5-$\beta^3$H]-CDP, ATP and saturating $\beta$, resulted in time-dependent inhibition (Fig. L4), with complete activity loss by 20 min. Many MBIs cause inactivation of RNR in part by loss of the $Y\cdot$ (14). Thus, the reaction was also assayed for $\beta$-activity (Fig. L4). Minimal loss of activity was observed. The studies show that CIFDP is a potent inhibitor of $\alpha$.

**Analysis for Chloroadenine (CIA) Release from CIFDP Incubated with hRNR.** One of the hallmarks of 2'-substituted MBIs is cleavage of the nucleobase from the sugar and loss of PP$\gamma$S, generating a furanone species that alkylates the protein (14). To determine if CIA is released, hRNR was incubated with a stoichiometric amount of CIFDP for 20 min, conditions in which complete inhibition occurred (Fig. L4). Protein was then removed by ultrafiltration and the filtrate analyzed by HPLC (Fig. S2A). No CIA was detected. To establish that the inhibitor remained unchanged, the enzyme was thermally denatured and removed by centrifugation. The supernatant was treated in two ways. First, it was incubated with alkaline phosphatase and then analyzed by HPLC. CIF was recovered quantitatively (Fig. S2B). Second, supernatant was lyophilized and then shown to inhibit the enzyme stochiometrically. The results indicate that CIFDP is not a mechanism-based, but a slow-onset, reversible inhibitor.

**CIFDP is a Slow-Binding, Reversible Inhibitor.** The upper limit for $k_{\text{off}}$ for CIFDP from $\alpha$$\cdot$CIFDP was first estimated to be approximately 0.1 min$^{-1}$ by measuring recovery of $\alpha$-activity upon dilution (see SI Text p. 3 and Fig. S3). Subsequently, progress curve analyses of $\alpha$-activity in the presence of 3 mM [8-14C]-ADP and variable [CIFDP] were carried out (Fig. 2A). In the absence of inhibitor, dADP formation is linear over 15 min (with adjustments to account for enzyme instability) at 37°C (controls A and B, SI Text). In the presence of CIFDP, kinetics are nonlinear: with an initial rapid phase (velocity $\nu_1$), and ensuing slower steady-state phase (velocity $\nu_2$). This biphasic profile is commonly observed with nonclassical slow-onset reversible inhibitors (23–25). This type of inhibition can be described as a single-step mechanism (Scheme 1(i)), where the rate constant for association ($k_1$) or dissociation ($k_{-1}$) or both are inherently slow. Alternatively it can be described as a two-step mechanism involving rapid equilibrium binding of I to E giving an E$\cdot$I complex, followed by a slow isomerization to a higher affinity complex, E$\cdot$I* [Scheme 1(ii)], where the rate constants $k_{-2}$ and $k_2$ are both low, but $k_{-2} \ll k_2$. To distinguish between (i) and (ii), a nonlinear regression analysis of the progress curves in Fig. 2A was performed using Eq. 1 to obtain the best fit values for the observed rate constant, $k_{\text{obs}}$, for onset of inhibition for each I.
Characterization of hRNR Inhibition Mechanism by ClFTP: A Rapid Reversible Inhibitor. Previous studies in cultured K562 and CEM cells suggested that RNR is a target responsible in part for CIF’s cytotoxicity. Their measurements of the relative concentrations of CIFDP and ClFTP (approximately 1:7) (8), altered dNTP pools (6–9), and inhibition of NDP reductase activity in the crude cell extracts by ClFTP (7, 8) led to the suggestion that the triphosphate was responsible for RNR inhibition. To test this proposal in vitro with purified hRNR, time-dependent inhibition studies were carried out (Fig. 1B). They showed that with a 5-fold excess of CIFDP over RNR, inactivation of α was complete within the first time point. The inhibited hRNR•ClFTP complex was then analyzed using Sephade-G25 chromatography or ultrafiltration (Fig. S2C, m, •). In both cases, 100% activity was recovered demonstrating that ClFTP is a rapidly reversible inhibitor, an observation in contrast to CIFDP (A). As with CIFDP, a similar inhibition experiment probing the remaining β-activity showed that ClFTP also primarily targets α (Fig. 1B).

Rapid reversible interaction between hRNR and CIFTP was also apparent from the linearity of the progress curves at [CIFTP] between 0 to 300 nM and [E] = 300 nM (Fig. 3A). However, with 600 to 1,500 nM CIFTP, a lag phase was observed, with no additional increase in inhibition above 600 nM (Fig. 3A). This unusual make measurements at low [I] (CIFDP). This value, however, lies within the range of the upper limit for $k_{off}$ of approximately 0.1 min$^{-1}$ measured above. From $k_{-2}$, the α•CIFDP complex $t_{1/2} = 23 \pm 3.8$ min (23–25).

Assuming that the rate limiting step in (ii) (Scheme 1) is associated with the breakdown of E•I$^*$ to E•I, the true affinity of CIFDP or overall dissociation constant of E•I$^*$ ($K^*$) can be calculated using Eq. 3 (23–25):

$$K^* = \frac{K_{aapp}}{1 + (k_{-2}/k_{-1})}.$$  

[3]

The value of 17 ± 7 nM obtained is within the range required to observe CIFDP release in the off-rate experiment above. Thus two independent approaches support the slow-binding inhibitor model (ii) (Scheme 1) and provide a binding constant of 17 nM.

**Scheme 1.** Two possible kinetic mechanisms that describe slow-binding inhibition.
behavior was more apparent in a replot of fractional activity of RNR vs. [I] (Fig. 3A, Inset), where maximum inhibition is approximately 50%. This interesting behavior will be discussed subsequently. A $K_i$ of 40 nM was extracted from the data in Fig. 3A, Inset using Eq. 4, which imposes no restrictions about [I] relative to [E] (23–26).

\[
Y = \frac{50}{[E]} \left( [[E] + [I] + K_i] - ([[E] + [I] + K_i]^2 - 4[E][I])^{1/2} \right). \quad [4]
\]

In this equation, Y is the percent of fractional inhibition, [E] and [I] are the total concentrations of α and CIFTP, respectively, and $K_i$ is the dissociation constant.

**Isolation/Characterization of (His)$_6$-D57N-α: A Probe for CIFTP Site Specificity.** RNR has two well-characterized effector binding sites: the A site governing activity and the S site controlling specificity (15). CIFTP can potentially bind to either or both sites, if it is a dATP analog as initially proposed (9). dATP binding is associated with changes in the quaternary structure of mouse RNR to an aggregate proposed to be α6 or α6β2 in the absence or presence of β2, respectively (17, 18). D57 resides in the A site (22) and the mutation of D57 to N, in the mouse D57N-M1 substantially decreases the dATP binding affinity to this mutant, essentially removing its ability to inhibit RNR (16, 22). We hypothesized that the human D57N mutant would have the same phenotype as mouse and that CIFTP under conditions in which the WT α is 100% inhibited, would no longer inhibit α.

To investigate this proposal, the human D57N-α was overexpressed in *Escherichia coli* in soluble form only with growth at 18 °C. The mutant was purified to >95% homogeneity (Table S1 and Fig. S1) with a typical yield of 100% inhibited, would no longer inhibit α.

We initially tested the preincubation and dilution inhibition of α followed by approximately 50% recovery of activity. Time-dependent inactivation with 5-fold molar excess CIFTP-α, analyzed over 20 min conditions where D57N-α remains uninhibited (Fig. 3B), revealed rapid loss of >90% activity followed by recovery of activity to approximately half the maximum (Fig. 1B). These results may provide an explanation for the lag phase associated with the initial phases of the progress curves (Fig. 3A). Our hypothesis is that the lag phase observed in the first 5 min corresponds to the transient, >90% inactivated α (Fig. 1B) associated with binding of one CIFTP/α and that with time, the enzyme changes conformation such that the inhibitor no longer binds to all available A sites, giving α with approximately 50% activity (Fig. 1B). In principle, CIFTP could bind to both S and A sites (2 CIFTP/α) with subsequent loss from the S site. However, the observation that no initial inhibition occurs upon incubation of D57N-α with CIFTP (Fig. 1B, △) under identical conditions to WT-α (Fig. 1B, ○) suggests that if CIFTP does bind to the S site, it is not inhibitory. Titration experiments monitoring dCDP production under initial and final conditions (2 vs. 20-min incubation times, respectively) provide compelling evidence for two different inhibited states of α by CIFTP (Fig. S4C). Binding studies with isotopically labeled CIFTP are required for detailed understanding of stoichiometry associated with these unusual results.

**Inhibition of (His)$_6$-D57N-α by CIFTP.** The diphosphate is expected to bind to the C site. Nevertheless, it has been reported that natural dNDPs at low mM concentrations can act as allosteric effectors (27). In addition, the presence of F and Cl could enhance affinity for the A site, despite the weakened binding anticipated by replacing the triphosphate (dATP and ATP normal effectors) with the diphosphate. The results shown in Fig. 4 (○) reveal that unlike CIFTP, CIFTP inactivates D57N-α similarly to WT-α. The data thus suggest that CIFTP does not bind to the A site.

**Perturbation of $k_{on}$ of [8-3H]-CIFTP from E • I’ (Scheme 1) by dGTP and CIFTP.** To further confirm CIFTP binding to the C site, hRNR and [8-3H]-CIFTP ([E] = [I] = 2 μM) were preincubated to form E • I’ complex (Scheme 1), which was then rapidly diluted 400- or 40-fold into the release buffer (RB) containing 0 or 5 μM (approximately 300 × $K_i$) CIFTP, respectively, and the $k_{on}$ (s) of [8-3H]-CIFTP measured. The presence of CIFTP in RB minimizes [8-3H]-CIFTP reassociation with enzyme. The measurements were then repeated with either saturating dGTP (0.1 mM, approximately 50 × $K_i$ (16, 28)), or CIFTP (5 μM, 125 × $K_i$) in addition to CIFTP in the RB. Any alterations in $k_{on}$ within each pair are most reasonably attributed to ternary complex wherein CIFTP or dGTP binds simultaneously to [8-3H]-CIFTP+E [E • I’, Scheme 1 (i)].

The $k_{on}$ for [8-3H]-CIFTP were first measured ± CIFTP in the RB and found to be similar: 0.026 vs. 0.034 min$^{-1}$ with $t_i$, 26 vs. 21 min [Fig. 2B, RB = B, and Fig. S5 A (●) and B (▼)], consistent with CIFTP binding exclusively to the C site, because binding of CIFTP to other sites (S or A site) might have altered the [8-3H]-CIFTP release rate. The $k_{on}$ value is also within range of that extracted from progress curve analysis of CIFTP (Fig. 2A). In contrast to these results, when a saturating amount of dGTP (S site), or CIFTP (A site) in addition to CIFTP, was placed in the RB (Scheme 1), which was then rapidly diluted 400- or 40-fold, respectively, were observed [Fig. 2B, RB = A and C vs. B, and Fig. S5, (●) vs. (▼)], and (●) vs. (△)]. Thus CIFTP binds at a site unique from these two nucleotides, supporting C-site binding.

**CDP Protection Against CIFTP Inhibition of α: Preincubation-Dilution Experiments.** In a third set of experiments to support CIFTP binding to the C site, a series of pairwise preincubation-dilution assays was undertaken. These experiments were modeled after those described by Nakamura and Abeles to determine the site specificity of slow-onset and slow-release reversible inhibitors such as compactin for HMGCoA reductase, where high substrate concentrations render traditional substrate protection assays ineffective due to substrate inhibition (29). For hRNR the SA of α at 0.5 mM CDP, 15 mM Mg$^{2+}$, and 3 mM ATP is reduced by half when [CDP] is increased to 10 mM, and increasing [Mg$^{2+}$] does not prevent the observed substrate inhibition.

We initially tested the preincubation-dilution assays with CIFTP against CDP and dGTP. As expected for a rapid reversible inhibitor, dilution of the preincubation mixture and analysis for dCDP formation showed that neither CDP (C site) nor dGTP (A site) had any effect on inhibition by CIFTP (Fig. S6 A and B).

We then studied the effect of CDP on the slow-onset inhibition of CIFTP (Fig. 2C). α (5 μM) was preincubated with CIFTP (10 μM − 600 × $K_i$) alone, with CDP (0.5 or 3.0 mM) alone, or with CIFTP (10 μM) and CDP (0.5 or 3.0 mM). CDP thus protects α from CIFTP, consistent with the expected competition between CDP and CIFTP for binding to the C site.

**Quaternary Structure of hRNR Subsequent to Inactivation by CIFTP, CIFTP or CFDIP and CIFTP.** Previous studies have shown that dATP
inactivates eukaryotic class Ia RNRs by altering the quaternary structures of α and the holocomplex: in the former case to an αβ state and in the latter case to an α6β2 state (17, 19). Given that CIFTP may be an analog of dATP, it is conceivable that its mechanism of RNR inhibition is associated with alteration of its quaternary structure. In addition, recent studies with F2CDP indicate that this analog not only modifies α covalently but also alters its quaternary structure (4).

SEC with a General Electric (GE) Superdex™ 200 10/300 GL column has been used to determine if inhibition of RNR by CIFTP or CIFDP is associated with an altered quaternary structure (Table 1 and Fig. S7 A–Q). Experiments were carried out with various combinations of α or holoenzyme, effectors (dATP, dGTP, and ATP), and CIF nucleotides. The elution buffer contained 150 mM NaCl and either 0.5 mM ATP (entries 1–14), 20 μM dATP (entry 20), or no nucleotides (entries 15–19). Without effectors, α is a monomer (19) with retention time (RT) of 26 min (Table 1, entry 18, and Fig. S7). β migrates as a dimer with a RT of 26 min. When α alone or holoenzyme ± effectors (dGTP or ATP) were injected onto the column and eluted with ATP the protein eluted in broad peaks over approximately 10 min with variable RTs and maximum absorbance from 22–25 min, indicative of interconverting species (Table 1 and Fig. S7 F–J).

Finally, a sample containing α with 0.6 mM dATP, where both the S and A sites are saturated (16, 28) and approximately 90% enzyme activity is lost (Fig. S4A), also elutes with broad features suggesting an equilibrium mixture of α oligomers (entries 11 and 19, Fig. S7H). Only when dATP (20 μM) is included in the elution buffer is the recently reported dATP-induced hexamerization of hRNR α detected (Fig. S7N) (19).

The results with CIFDP and CIFTP under various conditions with α alone or holoenzyme are in stark contrast. In all cases, the protein(s) elute(s) as a sharp peak and the RTs in general vary from 17.1 to 17.7 min (Table 1). The RTs are nearly independent of the presence of dGTP or ATP in the sample or the elution buffer. They are also nearly the same with α or holoenzyme in the sample, although they are slightly longer in the former case. Molecular weight (MW) standards (Fig. S7, Inset) give apparent MWs from 540 to 620 kDa within the range of α6 to α6β2 where n = 0–2 (Table 1), respectively. SDS-PAGE analysis of protein-containing fractions eluted from the column (corresponding to entries 1–4, Table 1) relative to standards having different ratios of α and β, demonstrated that the 17-min peak constitutes an α6 complex with variable amounts of β2 (n ≤ 2). Thus neither β nor any effector (at S or A site) is required for hexamerization. Two points are of interest. First, dGTP binding to the S site, which enhances α dimerization (16), is not a prerequisite for hexamerization caused by CIFDP (Fig. S7 J vs. K). Second, unlike dATP-induced hexamerization, which is observed only by SEC with 20 μM dATP in the elution buffer (entries 11 and 19 vs. 20) (16, 19), CIFTP, which binds to the same site, yields a unique form of α6 that is much more kinetically stable (Fig. S7 E and L vs. H and N).

**Discussion**

Active and inactive quaternary structures of mouse and *Saccharomyces cerevisiae* RNR have recently received much attention (16–19). The αn-subunit has been shown to exist in multiple oligomeric states (n = 1, 2, and 6). Similarly, the RNR complex, αβ6α, can also exist in multiple states. As thoughtfully described by Kashlan and Cooperman and subsequently by Rofougaran et al., the distribution of states will depend minimally on the concentration of the subunits, their localization, and the concentrations of ATP, dNTPs, and Mg2+ (16, 17). Thus reported differences in quaternary structure are in part related to the conditions under which the measurements are made and their resolution.

Studies using gas-phase electrophoretic-mobility macromolecule analysis, electrospray ionization mass spectrometry, analytical ultracentrifugation (AUC), SEC (17, 18), and recent structural analyses (19) have all suggested mouse α can generate a hexamic, inactive structure.

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**Table 1. CIFDP and CIFTP-induced hRNR oligomerization analyzed by SEC**

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<th>Entry</th>
<th>Protein (effector/inhibitor)* ‡∥</th>
<th>Retention time, min‡∥</th>
<th>Apparent mass, kDa∥</th>
<th>Fig. S7†</th>
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<td>621</td>
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<td>17.7</td>
<td>541</td>
<td>K</td>
</tr>
<tr>
<td>17</td>
<td>α (CIFTP)</td>
<td>17.5</td>
<td>567</td>
<td>L</td>
</tr>
<tr>
<td>18</td>
<td>α</td>
<td>26.0</td>
<td>78</td>
<td>M</td>
</tr>
<tr>
<td>19</td>
<td>α (dATP)</td>
<td>23.3</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*ClFDP SEC studies describe a reversible NDP inhibitor binding at the active site causing α-hexamerization. The studies collectively suggest that inhibition by CIFDP or CIFTP is related to conversion of α to αβ2, which can then bind β subunit(s). The results are intriguing as during the SEC analysis, neither inhibitor is present in the elution buffer and CIFTP has likely dissociated and CDPF partially dissociated from RNR subsequent to sample injection. Inhibition by CIP nucleotides thus results in different types of hexameric states of the hRNR large subunit that may preclude β from binding or binding in an active form.

†Of major peak. Differences (if any) between duplicate runs are less than 0.05 min. In cases with CIFTP, protein elution and RTs are identical between 2 and 20 min incubation.

‡Determined by comparison with GE MW standards (Fig. S7, Inset); provided only for single, well-defined sharp peaks (see Fig. S7 A–Q).

∥Theoretical mass: un (n, kDa): (1, 92); (2, 184); (4, 368); (6, 533); (7, 645), αβ6α (m, kDa): (1, 600); (2, 647); (4, 740); (6, 834). Untagged-α (n, kDa): (1, 90); (2, 180); (4, 360); (6, 540); (7, 630).

**Respective elution profiles in Fig. S7 A–Q.**

**Profile of entry 3 is identical to that of entry 2 (Fig. S7B).**

**Profile of entry 5 looks identical to that of entry 2 (Fig. S7B).**

**Profile of entry 19 looks identical to that of entry 11 (Fig. S7H) except RT at maximum absorbance is shifted.**
The relationship between quaternary structure and holoenzyme activity has also been investigated, and a model based on kinetic analysis under conditions similar to dynamic light scattering and AUC measurements has been proposed (16, 17). There is a consensus that α2β2 is the minimal complex required for active hRNR (16–19). However, additional complexes found under physiological conditions (α6β6, α6β2) have also been suggested to be active (16, 17). Finally, the importance of quaternary structure to the inhibited state of hRNR inactivated with F2-CDP has also been reported (4). Using SEC, α6β6 was suggested to be the inactivated state.

In light of these studies and our results with CIFDP and CIFTP, the quaternary structure again moves front and center as a unique biochemical analysis under conditions similar to dynamic light scattering and AUC measurements has been proposed (16, 17). There is a consensus that α2β2 is the minimal complex required for active hRNR (16–19). However, additional complexes found under physiological conditions (α6β6, α6β2) have also been suggested to be active (16, 17). Finally, the importance of quaternary structure to the inhibited state of hRNR inactivated with F2-CDP has also been reported (4). Using SEC, α6β6 was suggested to be the inactivated state.

What are the implications of these studies for hRNR as a therapeutic target? Although it is still early, a few observations can be made. First the ratio of the CIF metabolites and their stabilities will likely play a key role in the duration and extent of RNR inhibition. In peripheral mononuclear cells isolated from chronic lymphocytic leukemia and acute myeloid leukemia patients, and in cultured CEM cells under certain conditions, CIFMP levels are elevated and present for extended times and can serve as a reservoir for di- and triphosphate states of the drug (6–9). Whether the observed characteristics including the relative ratios of CIFDP: CIFTP hold for other cell lines will play an important role in understanding hRNR inhibition.

Our results in vitro indicate that both CIFDP and CIFTP reversibly inhibit the enzyme and that the α•CIFDP* tight complex once formed prevails for some time (also see Fig. 4A and SI Text, p. 4). Thus it is possible, given prolonged retention times of all CIF nucleotides in the cell (8), that even if the triphosphate is present at much higher concentrations than the diphosphate, the diphosphate will eventually be converted into an inhibited state [E • I, Scheme 1 (ii)] with a moderately long half-life.

Our studies implicate the importance of CIFDP in RNR inhibition. They further indicate the importance of quaternary structure of α alone for targeting inhibitors. Detailed structural analysis of the different inhibited states and screening methods with fluorescent probes to determine formation of these states (30) could lead to yet unidentified compounds that alter RNR’s quaternary structure to cause its inhibition.

Materials and Methods

See SI Text for details. Syntheses of (8-β-H)- CIFDP and CIFTP were carried out as described in SI Text. In all assays, dCDP:dADP production was analyzed according to the method of Steeper and Steuart (for dC) (31) or Cory et al. (for dA) (32), respectively, subsequent to dephosphorylation using alkaline phosphatase.

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