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| As Published    | http://dx.doi.org/10.1073/pnas.1013274108 |
| Publisher       | National Academy of Sciences (U.S.) |
| Version         | Final published version |
| Citable Link    | http://hdl.handle.net/1721.1/67468 |
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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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Edited by Barry S. Cooperman, University of Pennsylvania, Philadelphia, PA, and accepted by the Editorial Board May 3, 2011 (received for review September 4, 2010)

Human ribonucleotide reductases (hRNRs) catalyze the conversion of nucleotides to deoxynucleotides and are composed of α- and β-subunits that form active αβm (m = 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 triphosphohation function as an allosteric inhibitor of hRNR. Studies on the mechanism of inhibition of hRNR by CIF di- and triphosphates (CIFDP and CIFTP) are presented. CIFTP is a reversible inhibitor (K1 = 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 CIFTP suggesting its A site binding. CIFDP is a slow-binding, reversible inhibitor (K1 = 17 nM; t1/2 = 23 min). CDP protects α from its inhibition. The altered off-rate of CIFDP from E + CIFDP by CIFTP (A site) or dGTP (S site) and its inhibition of D57N-α together implicate its S site binding. Size exclusion chromatography of hRNR or α alone with CIFDP or CIFTP, 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

CIFDP (CIF, 2-chloro-2′-arabinofluoro-2′-deoxyadenosine, Clofar®, 1) is a nucleoside that is used to treat pediatric acute leukemias (1–3). Its mode of cytotoxicity is proposed to be similar, with several important distinctions, to gemcitabine (4). 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 diprophosphate (CIFDP) (the rate limiting step) (5–7) and the triphosphate (CIFTP) 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 (8, 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 CIFTP 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 CIFTP or F2C,CDP to compete effectively with the available dNTPs for incorporation into DNA. For CIFTP, the consequences are dependent on the CIFDP/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 CIFDP 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 replication/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, p53β, is induced by p53 and is proposed to be involved in supplying dNTPs for mitochondrial 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 (d)NTP (ATP, dATP, dGTP, and TTP). The latter, also called the ATP cone domain, controls NTP reduction rate: When ATP is bound, RNR is active and when dATP is bound, RNR is inactive. The β-subunit houses the essential diferric-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 αβn (n, m = 2 or 6) (16–19) and the inactive form produced by dATP binding to the A site, proposed to be αβ2 (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.
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 ClFTP 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 α responsible for inhibition. First, studies are carried out with D57N-α (altered A site), which is no longer inhibited by dATP (16, 22). They reveal that CIFTP, up to 6 μM (150 x $K_i$), 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 μM 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 [8-3H]-CIFDP+ 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 α. Size exclusion chromatography (SEC) subsequent to incubation of the hRNR or α 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 to α6βn or α6 (n ≤ 2) that 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|s,α,β, and Untagged-α.** Talo affinity chromatography replaced Ni-NTA for (His)$_2$-α purification (4) increasing yield and purity (Fig. S1). β was isolated in apo form and reconstituted in vitro giving 0.8–1.2 Y (β/2 and 3.5 Fe2+) (4). Typical specific activities (SA) for each (His)$_2$-tagged subunit, with saturating amounts of the second subunit using CDP/ATP were 650–890 nmol/[min·mg] for α and 3,000–4,100 for β. For ADP/dGTP/ATP the values were 140–160 for α and 200–220 for β. Without ATP, the values dropped to 70–80 for α and 80–95 for β. SA's of each tagged subunit for CDP or ADP reduction with 3 mM ATP remain within these ranges over 5 nm-5 μM of α (β) with 20–5-fold excess β (α). The corresponding SA of the untagged-α, under identical conditions, is increased approximately 1.5-fold relative to tagged-α.

**Time-Dependent Inactivation of hRNR by CIFDP.** Our previous studies with 2'-substituted-2'-deoxynucleotides such as 2'-ribofluoro-2'-deoxyctidine 5'-diphosphate and F$_x$-CDP 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 dNMP 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/dGTPP$^±$ ATP, followed by assaying for residual activity of α using [5-3H]-CDP, ATP and saturating β, 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* (14). Thus, the reaction was also assayed for β-activity (Fig. L4). Minimal loss of activity was observed. The studies show that CIFDP is a potent inhibitor of α.

**Fig. 1.** (A) Time-dependent inhibition by CIFDP. Inhibition mixtures (IMs) contained 1.2 μM α = [β] = [CIFDP], 0.1 mM dGTP, and 0 (♦) or 3 (○) mM ATP (in assay for α); 0 (♣) or 3 (▲) mM ATP (for β). (▽) was identical to (♦) except it contained 6.0 μM CIFDP. (○) was identical to (♣) except IM contained D57N-α in place of WT-α. (●) Initial nearly complete inhibition by CIFTP and subsequent recovery of approximately 50% activity. IM contained 1.2 μM α = [β] = [CIFDP], 0.1 (●) or 1 (▲) mM ATP (in assay for α), 1.2 (◇, ○) mM CIFTP (for β), and 0 (♣, △, □) or 3 (○) mM ATP D57N-α CDP reductase activity was unaffected (▲) under identical conditions to (♦).

**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 P$_T$, 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 stoichiometrically. 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 $k_{off}$ for CIFDP from α-CIFDP was first estimated to be approximately 0.1 min$^{-1}$ by measuring recovery of α-activity upon dilution (see SI Text p. 3 and Fig. S3). Subsequently, progress curve analyses of α-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 $v_1$), and ensuing slower steady-state phase (velocity $v_2$). 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 $E$*$I$ complex, followed by a slow isomerization to a higher affinity complex, $E$*$I'$ [Scheme 1(ii)], where the rate constants $k_{2}$ and $k_2$ are both low, but $k_{2} > k_2$. 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, $k_{off}$, for onset of inhibition for each $I$. 

Aye and Stubbe
make measurements at low $[I]$ (CIFDP). This value, however, lies within the range of the upper limit for $k_{\text{off}}$ of approximately 0.1 min$^{-1}$ measured above. From $k_{-2}$, the $\alpha$-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 $\cdot$ I$^*$ to E $\cdot$ I, the true affinity of CIFDP or overall dissociation constant of E $\cdot$ I$^*$ ($K_1^*$) can be calculated using Eq. 3 (23–25):

$$K_1^* = \frac{K_{\text{app}}}{1 + (k_2/k_{-2})}. \quad [3]$$

The value of $17 \pm 7 \text{ nM}$ 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.

Characterization of hRNR Inhibition Mechanism by CIFTP: A Rapid Reversible Inhibitor. Previous studies in cultured KS62 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 CIFTP (approximately 1:7) (8), altered dNTP pools (6–9), and inhibition of NDPreductase activity in the crude cell extracts by CIFTP (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 CIFTP over RNR, inactivation of $\alpha$ was complete within the first time point. The inhibited hRNR•CIFTP complex was then analyzed using Sephadex-G25 chromatography or ultrafiltration (Fig. S2C, •, •). In both cases, 100% activity was recovered demonstrating that CIFTP is a rapidly reversible inhibitor, an observation in contrast to CIFDP (a). As with CIFDP, a similar inhibition experiment probing the remaining $\beta$-activity showed that CIFTP also primarily targets $\alpha$ (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

Fig. 2. CIFDP is a C-site-directed, slow-binding reversible inhibitor of hRNR. (A) Progress curves for ADP reduction. [CIFDP] was varied from (•) 0, (♦) 1.5, (●) 3.0, (●) 6.0, (●) 9.0 to (▼) 12.0 μM. The solid curves are the best nonlinear fits of the data to Eq. 1. The dotted line is the uninhibited progress curve. (Inset) A replot of $k_{\text{obs}}$ for the progress curves as a function of [CIFDP]. The hyperbolic curve is the best nonlinear fit of the data to Eq. 2. (B) Perturbations of $[\text{d}^3\text{H}]$-CIFDP residence $t_{1/2}$ in response to ternary complex formation. Release buffer contained: 0.1 mM dGTP (A); 0 or 5 μM CIFDP (B); and 5 μM CIFTP = CIFDP (C). (C) CDP protects $\alpha$ from CIFDP inhibition. The pre-incubation mixture contained hRNR (5 μM) and either CDP alone (0.5 (●) or 3 (●) mM), or CIFDP alone (10 μM (●)) or both (10 μM CIFDP and 0.5 (●) or 3 (●) mM CDP).

$$[P] = \nu_4 t + \frac{(\nu_4 - \nu_2)(1 - \gamma)}{k_{\text{obs}}t} \ln \frac{1 - \gamma(e^{-k_{\text{app}}t})}{(1 - \gamma)},$$

where

$$\gamma = \frac{[E]}{[I]} \left(1 - \frac{\nu_2}{\nu_4}\right)^2. \quad [1]$$

In Eq. 1, $[P]$ is [dADP], [E] is the total enzyme concentration, and $t$ is time. Tight-binding inhibition under these conditions is accounted for by the $\gamma$-term of Eq. 1, which is required when $[I]$ is similar to [E] (Fig. 2A) (23–25). A plot of $k_{\text{obs}}$ vs [I] gives the results in Fig. 2A, Inset. The hyperbolic relationship suggests that CIFDP inhibits hRNR via mechanism $(ii)$, Scheme 1. Subsequent analysis using Eq. 2 (23–25) gives $k_2$, $k_{-2}$, and $k_{\text{app}}$, where $k_{\text{app}}$ is an apparent $K_1$ for the initial collision complex E $\cdot$ I (or $k_{-2}/k_1$):

$$k_{\text{obs}} = k_{-2} + \left[\frac{k_2[I]}{K_{\text{app}}[I]} + 1\right]. \quad [2]$$

The best fit values of $k_2$ and $k_{\text{app}}$ are 0.58 ± 0.01 min$^{-1}$ and 0.40 ± 0.06 μM, respectively. The value for $k_{-2}$ (0.03± 0.01 min$^{-1}$) is obtained from the y intercept (Fig. 2A, Inset) and is associated with substantial error due to the inability to
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).

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

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

Isolation/Characterization of (His)$_g$D57N-$\alpha$: 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 $\alpha 6$ or $\alpha 6\beta$ in the absence or presence of $\beta 2$, respectively (17, 18). D57 resides in the A site (22) and the mutation of D57 to N, in the mouse (27). In addition, the presence of F and Cl could enhance affinity for the A site, despite the weakened binding anticipated by replacing the triphosphates (dATP and ATP normal effectors) with the diphosphate. The results shown in Fig. 1A (○) reveal that unlike CIFTP, CIFDP inactivates D57N-$\alpha$ similarly to WT-$\alpha$. The data thus suggest that CIFTP does not bind to the A site.

Perturbation of $k_{\text{off}}$ 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 (1), which was then rapidly diluted 400- or 40-fold into the release buffer (RB) containing 0 or 5 μM (approximately 300 $\times K_i$) CIFTP, respectively, and the $k_{\text{off}}$ (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 $\times K_i$) and CIFTP (5 μM, 125 $\times K_i$) in addition to CIFTP, in the RB. Any alterations in measured $k_{\text{off}}$ 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 (ii)].

The $k_{\text{off}}$ for [8-3H]-CIFTP were first measured ± CIFTP in the RB and found to be similar: 0.026 vs. 0.034 min$^{-1}$ with $t_{1/2}$, 26 vs. 21 min [Fig. 2B, RB = B, and Fig. S5A (● 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 $k_{\text{off}}$ value is also within range of that extracted from progress curve analysis of CIFTP (Fig. 2C). 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, increases in $k_{\text{off}}$ of 6- and 3.5-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 $\alpha$: 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, when high substrate concentrations render traditional substrate protection assays ineffective due to substrate inhibition (29). For hRNR the SA of $\alpha$ 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. S6A and B).

We then studied the effect of CDP on the slow-onset inhibition of CIFTP (Fig. 2C). $\alpha$ (5 μM) was preincubated with CIFTP (10 μM $\times 600 \times K_i$) 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 $\alpha$ 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 CIFTP and CIFTP. Previous studies have shown that dATP

...
inactivates eukaryotic class Ia RNRs by altering the quaternary structures of α: the holocomplex: in the former case to an α6 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).

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, min</th>
<th>Apparent mass, kDa</th>
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<td>2</td>
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<td>621 B</td>
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<tr>
<td>3</td>
<td>α, β (CIFTP)</td>
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<td>621 —**</td>
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</table>

For entry 20, elution buffer contained 20 μM dATP; α (dATP) 17.9 516 N

*Elution buffer: 150 mM NaCl, 15 mM MgCl2 in 50 mM Hepes (pH 7.6) ± specified effectors. Flow rate = 0.5 ml/min.

††Incubation sample contained [His]β-α = [His]β-α = 15 mM; [ntagged-α] = 15 μM; [CIFDP] = [CIFTP] = 75 μM; [dGTP] = 0.1 mM; [ATP] = 3 mM; and [dATP] = 0.6 mM (entry 11) or 20 μM (entry 19). Parentheses specify nucleotides in each sample.

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

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

‡‡‡Theoretical mass: αn (n, kDa): (1, 92); (2, 184); (4, 368); (6, 553); (7, 645). αβm (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. S7).

***Profile of entry 5 looks identical to that of entry 2 (Fig. S7).

****Profile of entry 9 looks identical to that of entry 11 (Fig. S7H) except RT at maximum absorbance is shifted.

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, Insert) 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 βα (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 α6-subunit has been shown to exist in multiple oligomeric states (n = 1, 2, and 6). Similarly, the RNR complex, αβαβα, 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 concentrations 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.

Finally, because α-hexameration is thought to involve its N terminus (the ATP cone domain), the (His)6 tag and linker were cleaved with thrombin to generate “untagged”-α with an additional Gly-Ser unit. A number of experiments were repeated (entries 12–14, Table 1) with untagged-α, and the RTs remain unaltered after taking into account the change in MW.

The CIFDP SEC studies describe a reversible NDP inhibitor binding at the active site causing α-hexameration. The studies collectively suggest that inhibition by CIFDP or CIFTP is related to conversion of α to α6, 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 CIFDP partially dissociated from RNR subsequent to sample injection. Inhibition by CIF nucleotides thus results in different forms of hexameric states of the hRNR large subunit that may preclude β from binding or binding in an active form.

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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 RNR (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 F2CDP 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 CIFTF, the quaternary structure again moves front and center as a unique mode of RNR inhibition. Both nucleotides cause aggregation to α6 and to α6β6 (α ≤ 2) when 1:1 α:β is present. In the case of CIFDP, the initial slow binding and associated conformational change lead to an inactive state with a dissociative t1/2 of 23 min. In the case of CIFTF, the rapidly formed inactivated complex undergoes a conformational transition(s) on a minute time scale to recover approximately 50% of the activity. The molecular and structural basis of these changes are actively being investigated by cryoelectron microscopy, crystallography, and additional biochemical experiments.

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: CIFTF hold for other cell lines will play an important role in understanding hRNR inhibition.

Our results in vitro indicate that both CIFDP and CIFTF reversibly inhibit the enzyme and that the αCIFTF* tight complex once formed prevails for some time (also see Fig. 4D 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 CIFTF were carried out as described in SI Text. In all assays, dCDP:dADP production was analyzed according to the method of Steeper and Steuert (for dCD) (31) or Cory et al. (for dA) (32), respectively, subsequent to dephosphorylation using alkaline phosphatase.

ACKNOWLEDGMENTS. We thank the reviewers for insightful comments. This research was supported by National Institutes of Health Grant GM29595 (to J.S.) and Damon Runyon Cancer Research Fellowship DRG2015-09 (to Y.A.).