Published in final edited form as:

ACS Chem Biol. 2016 July 15; 11(7): 2021–2032. doi:10.1021/acscchembio.6b00303.

Cladribine and Fludarabine Nucleotides Induce Distinct Hexamers Defining a Common Mode of Reversible RNR Inhibition

Somsinee Wisitpitthaya[†], Yi Zhao[†], Marcus J. C. Long[†], Minxing Li[‡], Elaine A. Fletcher[†], William A. Blessing[†], Robert S. Weiss[‡], and Yimon Aye^{*,†,§}

[†]Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853, United States

[‡]Department of Biomedical Sciences, Cornell University, Ithaca, New York 14853, United States

[§]Department of Biochemistry, Weill Cornell Medicine, New York, New York 10065, United States

Abstract

The enzyme ribonucleotide reductase (RNR) is a major target of anticancer drugs. Until recently, suicide inactivation in which synthetic substrate analogs (nucleoside diphosphates) irreversibly inactivate the RNR- $\alpha_2\beta_2$ heterodimeric complex was the only clinically proven inhibition pathway. For instance, this mechanism is deployed by the multifactorial anticancer agent gemcitabine diphosphate. Recently reversible targeting of RNR- α -alone coupled with ligand-induced RNR- α -persistent hexamerization has emerged to be of clinical significance. To date, clofarabine nucleotides are the only known example of this mechanism. Herein, chemoenzymatic syntheses of the active forms of two other drugs, phosphorylated cladribine (CIA) and fludarabine (FIU), allow us to establish that reversible inhibition is common to numerous drugs in clinical use. Enzyme inhibition and fluorescence anisotropy assays show that the di- and triphosphates of the two nucleosides function as reversible (i.e., nonmechanism-based) inhibitors of RNR and interact with the catalytic (C site) and the allosteric activity (A site) sites of RNR- α , respectively. Gel filtration, protease digestion, and FRET assays demonstrate that inhibition is coupled with formation of conformationally diverse hexamers. Studies in 293T cells capable of selectively inducing either wild-type or oligomerization-defective mutant RNR- α overexpression delineate the central role of

*Corresponding Author: ya222@cornell.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchem-bio.6b00303. General materials and methods, chemoenzymatic synthesis procedures, biochemical and cell-based protocols, Figures S1–S8, and ¹H and ³¹P NMR spectra (PDF)

Author Contributions

S.W. performed nucleotide syntheses and all of the biochemical and cell-based assays except the FRET quenching experiments which were performed by Y.Z. M.J.C.L. generated the tetracycline-inducible isogenic expression Flp-In 293 T-REx cell lines stably expressing either wt- or D57N- α from humans. M.L. generated 3T3 fibroblasts stably integrated with wt- or D57N- α genes from mice with the guidance from R.S.W. E.A.F. and W.A.B. assisted with recombinant protein expression and gel filtration experiments, respectively. S.W., Y.Z., M.J.C.L., and Y.A. designed the experiments and analyzed the data. Y.A. wrote the paper, and S.W., M.J.C.L., and R.S.W. edited the paper with proofreading assistance from Y.Z., M.L., and W.A.B.

Notes

The authors declare no competing financial interest.

RNR- α oligomerization in drug activity, and highlight a potential resistance mechanism to these drugs. These data set the stage for new interventions targeting RNR oligomeric regulation.

Graphical abstract

Elucidating the basic mechanisms of clinically relevant drugs has far-reaching effects on future drug design.¹ Gemcitabine (F2C), a first-line drug for lung, pancreatic, and bladder cancers, is a suicide inactivator of the enzyme ribonucleotide reductase (RNR).² The RNR holocomplex—composed of α and β subunits—catalyzes the rate-limiting conversion of nucleoside diphosphates (NDPs) to deoxynucleoside diphosphates (dNDPs) required for *de novo* dNTP synthesis (Figure 1a).³ Because cancer cells rely on a sufficient supply of dNTPs for uncontrolled proliferation, several clinically successful nucleoside anticancer agents such as F2C (Figure 1b) target RNR as part of their cytotoxic program.⁴ During the past three decades, F2CDP has served as a mechanistic paradigm for RNR inactivation.^{2,5} F2CDP binds to the NDP-binding active site within α and inactivates the RNR $\alpha_2\beta_2$ enzymatic complex by interfering with the *conserved* proton-coupled electron transfer (PCET) catalytic pathway that supports NDP reduction (Figure S1a).² As such, α and β complex formation is a prerequisite of irreversible RNR inactivation.⁴ Until 2011, this was the only biochemically proven inhibition pathway for RNR-inhibiting nucleotides in clinical use.

We recently found that clofarabine (CIF), a clinically used antileukemic nucleoside (Figure 1b), functions as an allosteric RNR- α -specific activity suppressor and causes hexamerization *in vitro*^{6,7} and in cells⁸ (Figure S1b). The resultant α hexamers do not support PCET with β_2 . This discovery departed from the long-standing paradigm of nucleotide RNR inactivators that enact *irreversible* enzyme inactivation only after the reductase-active $\alpha_2\beta_2$ -holocomplex (Figure S1a) forms.^{2,4,5} To date, this new clinically relevant avenue of persistent α -hexamerization-coupled inhibition is limited to CIF, whereas the prototypic suicide inactivation mechanism by F2C holds true for many RNR-inhibiting non-natural nucleosides.^{2,4,5} This lack of evidence for generality of the drug-promoted hexamerization needs to be addressed because this mechanism promises to be a powerful avenue for the identification of small-molecule approaches to reversibly modulate an enzyme whose activity fuels cancer cell growth.⁴ We thus set about understanding RNR inhibition mechanisms of cladribine (CIA) and fludarabine (FIU), antileukemic nucleosides in clinical use with previously uncharacterized modalities of RNR inhibition.

Our data underscore the role of RNR- α hexamerization both *in vitro* and in cells. The latter for the first time offers a lens to understand the fundamental relationships between cytotoxic resistance mechanisms and RNR- α hexamerization. Chemo-enzymatic syntheses of all three phosphorylated forms of CIA and FIU enabled characterizations of their RNR- α -specific mode of inhibition, reversibility, binding-site specificities, and hexamer-inducing capabilities. These data surprisingly indicate that persistent hexamerization of RNR- α is not only a generalizable mechanism of clinical significance but can also be induced by inhibitors with a range of affinities. Beyond advancing the molecular knowledge of the active forms of antileukemic drugs, the outcomes reinforce our hypothesis that hexamerization is a broadly applicable avenue that has been serendipitously harnessed by several clinical therapies. Interestingly, oligomerization-inducing nucleotide inhibitors elicit conformationally distinct hexameric states, indicating complex regulatory behavior of these reduced-activity RNR- α -hexamers. Studies of drug toxicity in cell lines expressing wild type and oligomerization-defective mutant RNR- α under inducible promoters implicate that the loss of hexamerization capability is a mode of cytotoxic drug resistance specific to hexamer inducers. The study establishes the generality and clinical implications of RNR- α oligomerization, setting the stage for future exploitations of this novel mode of RNR regulation.

RESULTS AND DISCUSSION

CIA and FIU Inhibit Wild Type (wt)- but Not the Oligomerization-Defective Mutant-RNR- α in 3T3 Fibroblasts

CIA and FIU are metabolized to active nucleotide metabolites *in vivo*.⁹ Because phosphorylated nucleosides are noncommercial and their synthesis is challenging, we evaluated the ability of FIU and CIA to inhibit RNR in cells as a first pass. We generated NIH-3T3 cells stably overexpressing wt-mouse-(m)RNR- α . Three hours post drug treatment, RNR-subunit-specific activity was measured by the rate of [5-³H]-dCDP product formation in cell lysates. The results with CIA and FIUMP (the form of FIU used clinically)¹⁰—using concentrations similar to those used in previous pharmacological studies^{9,11,12}—showed potent RNR inhibition (Figure 1c,d).

We next examined the effect of these compounds on cells expressing the oligomerization-defective point mutant, D57N- α ,¹³ which is resistant to ClFTP,⁶ but not the mechanism-based inactivator F2CDP (Figure S2a–f). The functional significance of D57N- α is first briefly described. RNR- α houses three distinct nucleotide binding sites (Figure S1a).³ In addition to the catalytic C site where NDP substrate reduction occurs, the substrate specificity and overall enzyme activity are controlled by two RNR- α allosteric sites—allosteric activity (A) and specificity (S) sites. In the absence of nucleotides, α is a monomer.^{6–8,13–16} Binding of ATP, dATP, dTTP, or dGTP at the S site induces α dimerization, priming α for substrate NDP selection at the C site. Overall enzyme activity is controlled by dATP/ATP binding to the A site with increased dATP:ATP ratios associated with low reductase activity. dATP, the only natural allosteric suppressor, upon interacting with the A site, induces α hexamerization-coupled inhibition. Similar α -hexamers also assemble in crude cell extracts treated with dATP,⁸ and a recent report reveals a low-resolution (9.0 Å) crystal structure of dATP-induced human RNR- α hexamers.¹⁷ Studies of

the effects of natural nucleotide pool imbalance in mouse T-lymphosarcoma cells first identified a mutation in the A site (D57N) that disrupts dATP-mediated RNR enzyme inhibition.¹⁸ Biochemical studies subsequently established that D57N- α point mutation renders mouse RNR- α resistant to dATP-driven RNR inhibition.¹³ Consistent with the important role of oligomeric regulation, D57N- α is unable to form hexamers.^{6,13,15} The reductase activity of RNR in D57N- α -expressing mutant lines was not suppressed by either CIA or FIUMP (Figure 1c)—an inhibition signature similar to the allosteric RNR-inhibitor, CIF^{6,8} (Figures 1b–d and S1b). The D57N- α -expressing mutant lines showed resistance to CIF, whereas they were sensitive to F2C (Figure S2a–f), thereby giving us an important clue about potential links between RNR inhibition promoted by CIA/FIU nucleotides and α -hexamerization. These interesting observations with wt- and mutant- α -expressing cells are revisited further below.

Chemoenzymatic Syntheses of CIA and FIU Nucleotides

Chemoenzymatic syntheses of CIAMP, CIADP, CIATP, FIUDP, and FIUTP were accomplished (Figure 1e). Recombinant human deoxycytidine kinase (HdCK)¹⁹ efficiently catalyzed the monophosphorylation and chemical derivatizations afforded di- and triphosphates.

Determination of Active Forms in RNR Inhibition

Inhibition was assessed by measurements of CDP reduction when ATP was used as an allosteric effector at a fixed time (3 min) post incubation of the enzyme with di- and triphosphate analogs. This time period was chosen for practical reasons because the activity of human RNR is constant for only 5 min at 37 °C. Fitting the data to a tight-binding equation²⁰ revealed the apparent K_i 's of CIADP, CIATP, FIUDP, and FIUTP to be 1.4 ± 0.7 , 0.5 ± 0.1 , 9.4 ± 1.7 , and $6.8 \pm 1.3 \mu\text{M}$, respectively (Figure 2). β -specific activity remained unperturbed by these nucleotides but was suppressed by the known RNR- β -subunit-specific inhibitor, triapine (3-AP;²¹ Figure S3a). These data showed that both di- and triphosphate forms of CIA and FIU were active inhibitors and specific to α .

CIA- and FIU-Nucleotide-Induced Inhibition Is Coupled to α -Oligomerization

The RNR *catalytic mechanism for NDP reduction* at the C site is conserved from *E. coli* to *H. sapiens*.² F2CDP hijacks this conserved catalytic pathway and inactivates RNR from both bacteria and eukaryotes (Figure S1a). Notably, the only active form of F2C is F2CDP; the triphosphate (F2CTP) has no effect on RNR- α oligomeric state or holoenzyme activity.⁴ By contrast, both CIFDP and CIFTP failed to inhibit *E. coli* RNR, but target eukaryotic RNR- α independent of β by inducing RNR- α hexamerization (Figure S1b).⁴ Because F2CDP inactivation necessitates α - β complex formation (Figure S1a), and the triphosphate form, F2CTP, is not active, the inhibitory properties of CIA and FIU nucleotides we found above emulate those of CIF. We thus hypothesized that the observed inhibition is directly linked to changes in ligand-induced RNR- α oligomeric states. Two independent methods were used to test this proposal.

First, we took advantage of our recently developed FRET reporter platform that directly reports on ligand-driven α -oligomerization changes⁷ (Figure 2e). Each of the inhibitors in

varying concentrations (or buffer alone as control) was incubated with a solution comprising a 1:1 mol/mol mixture of fluorescein (F) and tetramethylrhodamine (T)-labeled α in DTT. The labeled proteins were functionally validated to be capable of hexamerization by gel filtration analysis in the same way as the wild type. Evaluation of the inhibitor-orchestrated dose-dependent FRET quenching revealed that EC_{50} 's of oligomerization induced by CIADP, CIATP, FIUDP, and FIUTP were 1.7 ± 0.5 , 0.5 ± 0.1 , 5.2 ± 1.2 , and $4.6 \pm 0.8 \mu\text{M}$, respectively (Figure 2f). These EC_{50} values lie within the range of K_i 's measured above (Table 1) although the conditions for these two assays are different and cannot necessarily be compared directly. Notably as with K_i 's, the EC_{50} 's for FIU nucleotides were also higher than those of CIA. These data suggest that inhibition potencies are positively correlated with the capabilities of the inhibitors to drive α -oligomerization, and thus inhibition is likely coupled to oligomerization. In addition, time-dependent FRET analysis showed that oligomerization was complete in much less than 5 min for CIADP, FIUDP, and FIUTP (Figure S3b). Interestingly, time-dependent inhibition analysis also revealed similar time scales for enzyme inhibition (Figure S3c). Despite the differences in assay settings, the two data sets correlate both in terms of kinetics and thermodynamics of inhibition properties, further underscoring the central role of α -hexamerization in RNR inhibition.

Our second method, gel filtration analysis, revealed that 3-min incubation with saturating amounts of CIADP and CIATP gave persistent hexamers (Figure 3a–d). FIUTP behaved similarly (Figure 3f). Unexpectedly, pretreatment of α with *saturating* FIUDP was insufficient to yield hexamers by gel filtration when there was no FIUDP in the running buffer (Figure 3e). The eluted α in this case was only a monomer. When $20 \mu\text{M}$ FIUDP was included in the running buffer, these conditions yielded exclusively α_6 . This unexpected result was consistently obtained using two different batches of FIUDP as well as two different gel-filtration columns. The capability of all four nucleotides to promote α_6 unequivocally expanded the generality of the novel ligand-driven hexamerization beyond the sole example, CIF.^{4,6,8} However, given the behavior of FIUDP-induced hexamers, it is surprising that the affinity of FIUDP is not drastically different from its analog, FIUTP (Figure 2c,d, Table 1). Since CIFD(T)P-induced hexamers are kinetically stable beyond complete inhibitor dissociation,⁸ whereas hexamers induced by FIUDP unexpectedly are of low kinetic stability, it can be inferred that RNR- α forms different hexamers in the presence of different nucleotide drugs.

CIADP and FIUDP Exhibit Reversible RNR Inhibition

The diphosphates of CIA and FIU are anticipated to bind the catalytic (C) site on α as previously demonstrated for CIFDP⁶ and F2CDP.² CIADP and FIUDP could engage in chemical reaction with the transient thiyl radical in the C site formed during the RNR catalytic cycle, similarly to F2CDP and many other inactivators^{2,5} (Figure S1a). Alternatively, they could bind at the C site without being chemically modified, like CIFDP.⁶ First, we asked to what extent CIADP and FIUDP could be recovered intact post enzyme inhibition. To an assay mixture containing 1:1 RNR- α and RNR- β under turnover conditions was added a molar equivalent of CLADP to initiate enzyme inhibition. After heat-inactivation, denatured enzymes were removed, and the small-molecule fraction was

analyzed by HPLC. Greater than 98% of CIADP was recovered (Figure 3g). The analogous assessment of FIUDP was precluded due to instability under HPLC elution conditions.

To validate that FIUDP-induced inhibition proceeds reversibly, we measured the off-rate of FIUDP dissociation by monitoring recovery of enzyme activity upon dilution. The measurements were also extended to include FIUTP, CIADP, and CIATP. The intrinsically low activity and stability of the mammalian enzyme and low assay sensitivity set practical limitations on the fold dilution possible, and thus complete dissociation of E•I complex, and hence full activity regain, was thus not achieved post dilution. Nevertheless, a time-dependent regain of activity was observed for each of the four inhibitors subsequent to dilution, and the half-lives of dissociation were all around 1–2 min (Figure S4). This value likely reflects an off-rate of the inhibitor ligand *rather than intrinsic stability of the hexamer*, because hexamers induced by CIF nucleotides persist beyond inhibitor dissociation in the absence of other competitor nucleotides.⁸ Since the ligand off-rate is 5–10-fold shorter than the retention time of the hexameric state by gel filtration analysis, these data add further credence to the assertion that the (lack of) stability of different hexamers on gel filtration reflects inherently different α_6 states as opposed to different ligand residence times.

Determination of Binding Site Specificities of CIATP and FIUTP

1. By Inhibition Assays—On the basis of the enzymatic activity of human RNR as an NDP reductase, binding of NTPs to the C site is not possible. RNR- α possesses two triphosphate-binding allosteric sites, S and A (Figure S1a). We resolved which of these two sites is accountable for enzyme inhibition induced by CIATP and FIUTP by first exploiting the previously characterized D57N-human RNR- α mutant. The D57N point mutation renders, for instance, ~300-fold reduced affinity to dATP binding at the A site relative to the wt, when measured using CDP as a substrate.^{13,22} The mutant retains close to wt activity in the presence of ATP and is as responsive to S-site effector (dGTP) binding as wt.⁶ Under conditions in which wt-RNR- α was fully inhibited, the D57N point mutant was unresponsive to either CIATP or FIUTP up to at least 10-fold above the measured K_i 's for wt under analogous conditions (Figure S5). These data indicate that the triphosphates of CIA and FIU promote inhibition *via* A site binding, similar to ClFTP.

2. By fluorescence anisotropy—To provide independent support for A-site binding, we performed a series of fluorescence anisotropy (FA) assays to measure binding of Texas Red-5-dATP (T*-dATP hereafter) to RNR- α and how this binding is affected by the ligands under study. CIATP was thus used as a representative example because the lower K_i value of CIATP compared to that of FIUTP (Table 1) renders the experimental conditions more amenable. The introduction of wild type RNR- α to a solution of free T*-dATP resulted in a significant increase in FA, consistent with the formation of a ligand–protein complex (Figure S6a). Under these conditions in which there are no competing ligands, T*-dATP binds (independently) to both S and A sites on RNR- α . Exclusive binding of the nucleotide effectors to the S-site causes RNR- α dimerization. A-site binding (irrespective of S-site occupancy) results in hexamerization. We note that titration of unlabeled dATP to a solution of 6 μ M wt- or D57N- α and 1.2 μ M T*-dATP showed that not all of the label was displaced, presumably due to nonspecific binding of T*-dATP (Figure S6b). However, because these

experiments assay specific release of the dye from specific sites upon the addition of a competing ligand, any nonspecific binding will not affect the conclusion. In the first experiment, we introduced saturating amounts of dTTP (Figure S6c), an S site-exclusive binder, to RNR- α treated with T*-dATP (equilibrium mixture of A- and S-site-bound forms). We observed a $49 \pm 1\%$ drop in FA, which must represent displacement of T*-dATP (bound at the S site) by dTTP because the S site and A site are uncoupled (there is the possibility of compensatory binding to the A site; Figure 4a). Subsequent addition of CIATP in a saturating amount (Figure S6d) to this mixture resulted in a further $20 \pm 1\%$ decrease in FA (Figure 4a). The simplest explanation for the observed drop is due to CIATP displacing some of the bound T*-dATP at the A site.

In the second set of experiments, we made use of both wild type and D57N RNR- α . First, the A-site mutant D57N RNR- α ($6 \mu\text{M}$) was incubated with T*-dATP ($1.2 \mu\text{M}$), leading to exclusive S site occupation. The addition of S-site-only binding ligand dTTP (Figure S6c) resulted in a $39 \pm 1\%$ drop in FA, dislodging T*-dATP (Figure 4b). The addition of an alternative S-site-only binding ligand dGTP (Figure S6e) also yielded a similar level of percent reduction in FA (Figure 4b). By contrast, the addition of CIATP in place of dTTP/dGTP gave a ~ 7 -fold less decrease in FA. Second, when these experiments were replicated on the wild type protein, CIATP gave rise to a significantly higher drop in FA (Figure 4b). These two data sets are further consistent with CIATP binding at the A site instead of the S site. Although the allosteric regulation of RNR is complex, the FA data (Figure 4a,b and S6a–e) and inhibition analysis of the D57N point mutant above (Figure S5a) are strongly consistent with the triphosphates binding to the A site.

Determination of Binding Site Specificities of CIADP and FIUDP: By Inhibition, Fluorescence Anisotropy, and Gel Filtration Analyses

Interestingly, the diphosphates of CIA and FIU also did not inhibit the D57N-RNR- α at concentrations at least 10-fold above wt K_i (Figure S5b), even though the C site in the mutant is fully functional.^{6,13} This result is striking for two reasons. (1) It confirms that neither CIADP nor FIUDP are mechanism-based inactivators, since the canonical mechanism-based inactivator, gemcitabine diphosphate (F2CDP), inhibits D57N- α as effectively as the wild type. (2) Since D57N- α is unable to adopt (a) deactivated hexameric state(s), the result is consistent with our proposal that oligomerization is a prerequisite for RNR inhibition. Conversely, the recently identified α_6 -inducer clofarabine diphosphate (CIFDP) can inhibit D57N- α -specific RNR activity.⁶ These differences likely reflect nuanced binding modes for the different ligands, which warrant further analyses that are beyond the scope of this present study. Nonetheless, we further established the binding-site specificities of the diphosphates of CIA and FIU by designing assays that combine FA and gel filtration analyses.

To demonstrate that diphosphate inhibitors do indeed associate with the C site, we performed two independent sets of experiments as follows. CIADP was used as a representative example in both. In experiment 1, wild type RNR- α was incubated with saturating dATP (at both S and A sites) such that only the C site was available for any newly introduced ligand. Under these conditions, RNR- α adopts hexameric states. The sample was

split into two equal portions, and to one, CIADP was added (sample A), and to the other, buffer was added in place of CIADP (sample B). The resultant samples were separately injected onto a gel filtration column pre-equilibrated with running buffer having no nucleotides, and the oligomeric state of eluted α was evaluated. A hexameric peak was observed only in sample A (Figure 4c). Our gel filtration results in the present study with CIADP (*vide supra*; Figure 3c) and in the previous studies with dATP from us⁶ and others²³ have shown that in the absence of excess dATP in the running buffer, RNR- α pretreated with excess dATP only elutes as lower-order species, whereas in the absence of CIADP in the running buffer, RNR- α pretreated with CIADP maintained its hexameric state—a remarkable feature of kinetic stability unique to the drug-induced hexamers (Figure 4c). Thus, in the present experiment, the observation of any hexameric peak in the gel filtration profile from sample A (and not in control sample B) could only arise from the interaction of CIADP to the C site of RNR- α , giving rise to the observed kinetically stable hexamers.

In experiment 2, the gel filtration analysis was coupled with FA measurements. Wild type RNR- α (6 μ M) was incubated with T*-dATP (1.2 μ M), and the S-site was subsequently preblocked by S-site-only binding ligand dTTP in saturating amounts (Figure S6c), we first showed the anisotropy decrease measured (~50%; Figure 4e) was similar to the change obtained in Figure 4a. The sample mixture was subsequently either treated with buffer alone or with saturating amounts of CIADP (75 μ M). Unlike treatment with CIATP as above, which resulted in ~20% further drop in anisotropy due to T*-dATP displacement, CIADP caused no statistically significant changes in anisotropy (Figure 4e), further consistent with CIADP interacting with neither S nor A site. However, when this sample was analyzed by gel filtration, the sample treated with CIADP resulted in an appreciable fraction of hexamers, whereas the control sample treated with buffer alone eluted exclusively as a monomer (Figure 4e). Thus, T*-dATP and CIADP must occupy RNR- α simultaneously under conditions in which CIADP site occupancy is appreciable, thereby leading to a significant proportion of hexameric state in the elution profile. Experiments 1 and 2 (Figure 4c and d,e) together provide convincing evidence that CIADP induces hexamerization through interaction with the C site only.

Trypsin Digestion Assays Suggest Conformationally Distinct Hexamers

Because our gel filtration data implied that RNR- α hexamers formed upon treatment with CIF/CIA/FIU nucleotides had different kinetic stabilities, we proceeded to understand this phenomenon further. As an initial step, we examined the extent to which individual hexamers are sensitive to protease cleavage. RNR- α hexamers were formed by the addition of various nucleoside di/triphosphates, then the samples were treated with trypsin and the products were monitored by SDS-PAGE. We unexpectedly found that the rates of cleavage were different, whereas no appreciable cleavage of RNR- α was observed for the natural ligands (Figure 5 and S7a). Two points of note: (1) The differences in observed cleavage rates were not associated with affinity or K_i values of the drugs; in other words, this finding is *not* depending on structure stabilization caused by ligand binding. For instance, FIUTP and FIUDP had clear differences in cleavage rates but have comparable binding affinities. (2) Trypsin activity was unperturbed by these drugs under the conditions used as shown by

the hydrolysis assay of fluorescent AMC-functionalized substrate of trypsin protease, Z-Arg-AMC (Figure S7b–c).

RNR- α Oligomerization Plays a Role in Cytotoxic Activity of CIA and FIU

We next evaluated the extent to which the oligomeric mode of RNR inhibition elucidated above plays a role in drug cytotoxicity profiles. Although toxicity to normal cells versus cancer cells depends on manifold parameters, we here focused on gaining initial insights into the importance of oligomeric regulation in the ultimate cytotoxic activity of these nucleosides.

We used Flp-In 293 T-REx cells that can stably incorporate a single copy of a selectable transgene at a defined genomic locus upon transient transfection with a plasmid encoding FLIP recombinase, and a suitable donor plasmid containing FRT sites. The cells constitutively express the tetracycline (tet)-repressor to facilitate tet-inducible expression of the transgene of interest. Isogenic lines expressing either RNR- α -Flag or D57N- α -Flag under a tet-controlled CMV-promoter were established. This system provides temporal control of protein expression, allowing us to spotlight the affects from RNR- α (wt or mutant) alone against a native background. These clonogenic lines showed undetectable expression of ectopic RNR- α as evidenced by Flag blot but, upon tet exposure, upregulated total RNR- α levels approximately 2–3-fold relative to endogenous after an 11-h treatment with tet (Figure S8a, b). The levels of both isoforms of the RNR- β subunit were unaffected under these conditions.

We set up a proliferation inhibition assay in which cells were seeded at 10% confluence in 96 well plates, then treated with tet, and after 11 h, the drug was added for 48 h (approximately 2.5 doubling times). After this time, total viable cells were measured using AlamarBlue reagent. Based on our *in vitro* data, we would predict that overexpression of RNR- α or D57N- α would similarly protect 293T cells from gemcitabine (F2C). This is because (1) F2CDP (the only active form that inhibits RNR) targets the active site (C site) irrespective of wild type or the reduced-affinity-A-site-binding mutant D57N- α ; (2) RNR- α overexpression is similar in both lines (Figure S8); and (3) D57N- and wt-RNR- α have similar specific activity. Consistent with this hypothesis over three separate runs, each with quadruplicate data, modest protection against F2CDP cytotoxicity was observed upon tet induction of RNR- α or D57N- α relative to the noninduced samples. The magnitude of the protection (3–5 fold) was on the order of the RNR- α overexpression we observed. The difference in fold protection between RNR- α and D57N- α was not significant (Figure 6a, b).

For the inhibitors of RNR- α in which inhibition is coupled with α -hexamerization, we hypothesized that overexpression of oligomerization-defective mutant D57N- α should rescue toxicity whereas wt-RNR- α should elicit some protection in cases where the K_i of the inhibitor under study is below or not much greater than the wt-RNR- α cellular concentration. Similar to the results with F2C, RNR- α overexpression elicited a small effect on the EC₅₀ relative to noninduced cells. By contrast, D57N- α overexpression was able to afford almost complete protection (>20-fold in each case) against both CIA and CIF treatment. Since RNR- α - and -D57N- α -expressing cells were still susceptible to artificial nucleotide-induced toxicity, we can rule out an unlikely scenario that the global nucleotide

pool is being perturbed by D57N- α overexpression. Thus, CIF and CIA selectively target RNR- α through oligomeric downregulation, as predicted above.

One subtle point of note is that both ClADP and ClATP failed to inhibit recombinant D57N- α (Figure S5), but for CIF, the mutant is only sensitized to ClFTP and not ClFDP.⁶ Our in-cell data showed cells overexpressing D57N- α resisted both CIA and CIF (Figure 6b). On the basis of the significant rescue observed with D57N- α overexpression, we infer that the triphosphate of CIF predominates over the diphosphate in these lines. Existing data on cellular concentrations of the nucleotides of CIF agree with this conclusion.^{24,25} In the case of FIUMP, no protection was observed in either case. For RNR- α overexpression, given the K_i 's calculated for the FIUDP and FIUTP (Table 1), and the relatively low amount of protein overexpression we observe (Figure S8), any rescue would be unlikely. These observations imply that RNR- α is the principal target of CIF and CIA nucleotides whereas RNR- α is a low-priority target of FIU nucleotides.

Conclusions

We initiated this study with three major objectives: (1) to establish α -hexamerization as a unified model for RNR downregulation using a set of antileukemic agents, (2) to interrogate the extent to which α -hexamerization is coupled to enzyme inhibition, and (3) to gain initial insights into the role of oligomeric regulation in cytotoxic action conferred by these anticancer nucleotides in clinical use. The study satisfied all of these aims. During these studies, we also gained initial evidence of structural plasticity of drug-induced RNR- α -hexamers; such a result is not expected *a priori* and has profound implications for rational design of small-molecule allosteric drugs that stabilize reduced-activity oligomers of RNR. Unmasking the mystery shrouding the complex allosteric regulation that is the hallmark of mammalian RNRs may also be the ultimate missing piece of the jigsaw that elucidates the enigmatic tumor suppressor role specific to α .⁴

Notably, the hexamerization model was applicable across a diverse landscape of small-molecule inhibitors: CIA and FIU nucleotides differ in their affinity for RNR- α 10–20 fold (Table 1). Their affinities are reasonably well dispersed across this range. These nucleotides also showed comparable off-rates under assay conditions that are on the order of minutes—a time scale 5–10-fold shorter than that of gel filtration (Figure 3a–f and Figure S4). FIUDP-treated RNR- α eluted as a monomer under the gel filtration time scale, unless FIUDP itself was present in the running buffer (Figure 3e). All other α_6 -inducing drugs did not require inhibitor supplementation in the running buffer to maintain a hexameric state (Figure 3b–d,f and Figure 4c,d). This result is surprising given that FIUDP and FIUTP have similar affinities (Table 1). The simplest explanation for this result is that hexameric states formed by different nucleotide drugs are *structurally* different, and these differences are retained post ligand dissociation. The different rates of trypsin protease cleavage among these hexamers induced by different unnatural nucleotide inhibitors provides strong evidence for this proposal (Figure 5 and Figure S7).

Interestingly, despite the fact that diphosphates of CIA and FIU interact with the C site, they are unable to inhibit D57N- α appreciably. This observation supports the allosteric communication between CB and NTD (Figure S1c) that is presumably required for the

diphosphates to elicit hexamerization.¹⁶ The fact that hexamerization can be achieved across manifold ligands binding through different sites further attests to the versatility of the oligomeric mode of inhibition. Interestingly, ClFDP—a previously characterized high-affinity C-site-binder and the only known hexamer-inducer inhibitor prior to this work⁴—with 10–100-fold higher affinity than ClADP and FIUDP⁶ is able to inhibit RNR- α . Differences in binding-site affinity and unique chemical/structural properties of different nucleotides likely account for the fact that ClFDP can inhibit D57N whereas ClA/FIUDP cannot.

The popularity of suicide inactivation of RNR enzymes stems from the relative ease of handling of *E. coli* RNRs, and the fact that the catalytic chemistry of class Ia RNRs to which *E. coli*, yeast, and mammals belong is conserved. Our recent finding with antileukemic agent ClF implied that non-natural nucleotides might be able to mimic the endogenous feedback regulation by dATP that confers dNTP pool balance.⁴ Nonetheless, the sole example with ClF, among the wealth of suicide nucleotide inactivators,^{2,5} was potentially an anomaly. We chose to investigate ClA and FIU, adenine analogs used to treat acute myeloid and lymphoblastic leukemias.⁹ RNR inhibition by ClA and FIU was presumed to be a direct consequence of ClATP and FIUTP mimicking dATP.⁹ The precise mechanisms have remained poorly defined. The diphosphates were not considered as active drug metabolites, although the intracellular presence of both the di- and triphosphates is established. Our data thus provide valuable mechanistic information on under-characterized inhibitors in clinical use.

A key conclusion of this study is that hexamerization is coupled with RNR enzyme inhibition. This finding helps explain why the diphosphates of ClF, FIU, and ClA are not mechanism-based inhibitors, because under this model, binding *necessitates* rapid modulation of RNR- α to a conformation that is incapable of PCET. Of note, the ligand cannot simply trap a pre-existing hexameric state present in low amounts in the ground state because of the kinetic stability of the hexameric state induced by most of these drugs. This coupling was hinted at by the fact that K_i 's for all of the inhibitors studied (Figure 2a–d) and EC_{50} 's of oligomerization (Figure 2e–f) have similar values (Table 1). However, more compelling evidence was obtained by studying the oligomerization-defective mutant, D57N- α . As expected, ClATP and FIUTP both failed to inhibit D57N- α under the conditions that these nucleotides inhibited the wt (Figures 2b,d and S5a). This observation and the fluorescence anisotropy data (Figures 4a,b,d and S6) both suggest the triphosphates exclusively interact with the A site.

Since D57N- α is similarly active to the wt enzyme⁶ (and hence interacts with substrate similarly), one would expect that both ClADP and FIUDP would inhibit D57N- α similarly to wt because they bind to the fully functional NDP-substrate-binding C site (Figure S1a). Remarkably, neither of the diphosphates inhibited D57N- α (Figure S5b). We also confirmed by multiple orthogonal experiments involving gel filtration and fluorescence anisotropy (Figures 4c,e and S6) that the diphosphates bind exclusively to the C site. One interpretation is that for there to be inhibition, ClADP and FIUDP must not only bind to the substrate-binding C site but also induce a subsequent step. Since D57N- α cannot hexamerize, ClADP and FIUDP precluded inhibition of D57N- α and accompanying hexamer formation. The

mechanism-based inactivator F2CDP that binds to the C site and irreversibly targets the postulated $\alpha_2\beta_2$ holocomplex (Figure S1a)² promoted inactivation of D57N- α as well as wt- α —both *in vitro* and in cells (Figures S1a and S2e,f). These data reinforce that D57N- α behaves similarly to the wt in terms of catalytic chemistry/substrate binding. We interpret these findings as a corroboration of the tight linkage between hexamerization and inhibition, presumably through intraprotein allosteric signaling between the C site and A site. This proposition is consistent with our recent studies suggesting the existence of structural coupling and contiguous interactions between the RNR- α catalytic body that houses the catalytic C site and N-terminal domain that bears the allosteric A site (Figure S1c).¹⁶ The interdomain coordination is an essential prerequisite for inhibitory regulation through inhibitor-induced α -hexamerization. However, CIFDP is still able to inhibit both wt- and D57N- α with similar potency. The discrepancy between CIFDP and CIADP/FIUDP may indicate that subtle differences in chemical structures of the nucleotide inhibitors alter allosteric and oligomeric regulatory outcomes in a complex pattern. The significantly higher affinity of CIFD(T)P relative to CIA and FIU nucleotides (Table 1) may also contribute to these differences.

Many elegant studies have revealed complex mechanisms by which nucleoside analogs such as CIA and FIU induce cytotoxicity in both dividing and quiescent cells.^{9,27,28} Although RNR inhibition is a key route to depleting cellular dNTPs and indirectly suppressing DNA replication in proliferating cells, the molecular mechanisms leading to this state are mostly unclear.⁴ Surprisingly, the role of RNR- α oligomeric regulation has never been considered for any artificial nucleoside except CIF.⁴ Of equal importance to the detailed mode of inhibition, drug resistance has proven to be one of the latest pressing issues in advancing cancer treatment.²⁹ Conventional views largely attribute mechanisms of nucleotide drug resistance to a reduction of intracellular concentration of active species and/or suppression of apoptosis induction.^{27,29} We evaluated the functional impacts of defective oligomeric regulation of RNR- α under conditions where drug uptake and metabolism, interaction with other targets, and apoptotic pathway induction processes are unaltered. Our data show that hexamerization is an additional pathway to cytotoxic resistance to hexamer-inducer nucleotides such as those of CIF and CIA and set them apart from the more well-characterized conventional suicide inactivators such as F2C.

In summary, the present study unambiguously showed that the emerging evidence of clinically relevant oligomeric regulation is a generalizable avenue to modulate RNR activity. This understanding is critical for the development of new RNR inhibitors because this inhibitory phenotype enables rapid inhibitor screening. Directing the focus of RNR drug design to more target-specific allosteric modulators that also side-step complete activity shut-off offers potential development of small-molecule antagonists that would circumvent toxicity issues commonly associated with nucleotide chemotherapy.⁴ The existence of distinct conformational states among the hexamers renders a versatile manifold for added pharmacological benefits. From a broader context, deeper knowledge of how this important class of antimetabolites inhibits one of their key pharmacological targets is valuable at both the basic and translational levels.

METHODS

The Supporting Information includes detailed methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Ananda Mahidol Fellowship (to S.W.), a Hill Undergraduate Research Fellowship (to W.A.B.), and a seed grant from the Meyer Cancer Center (to R.S.W. and Y.A.). Instrumentation used in this research was funded in part by a National Institutes of Health Director's New Innovator Award (1DP2GM114850), the Arnold and Mabel Beckman Foundation through a Beckman Young Investigator Award, a Sloan Foundation Fellowship, and an NSF CAREER Award (CHE-1351400; to Y.A.). We thank J. Stubbe and her laboratory for plasmids encoding human RNR-wt- and D57N- α and - β genes; P. Chen for the use of a fluorimeter; Y. Fu and S. Parvez for helpful discussions and scientific collaborations; X. Xu and J. Levy for constructing pCaggs wt- and D57N-mRNR- α expression plasmids; and I. Keresztes for assistance with high-resolution mass spectrometric characterizations. ESR data for the reconstituted RNR- β subunit were collected at the National Biomedical Research Center for AdvanCed ESR Technology (ACERT) at Cornell University, supported by NIH/ NIGMS grant P41M103721 [J. Freed (PI); we thank B. Dzikovski for his assistance]. NSF MRI [CHE-1531632, Y.A. (PI)] was acknowledged for NMR instrumentation support.

References

1. Terstappen GC, Schlupen C, Raggiaschi R, Gaviraghi G. Target deconvolution strategies in drug discovery. *Nat Rev Drug Discovery*. 2007; 6:891–903. [PubMed: 17917669]
2. Stubbe J, van der Donk WA. Ribonucleotide reductases: radical enzymes with suicidal tendencies. *Chem Biol*. 1995; 2:793–801. [PubMed: 8807812]
3. Nordlund P, Reichard P. Ribonucleotide reductases. *Annu Rev Biochem*. 2006; 75:681–706. [PubMed: 16756507]
4. Aye Y, Li M, Long MJ, Weiss RS. Ribonucleotide reductase and cancer: biological mechanisms and targeted therapies. *Oncogene*. 2015; 34:2011–2021. [PubMed: 24909171]
5. Licht, S.; Stubbe, J. Mechanistic Investigations of Ribonucleotide Reductases. Poulter, CD., editor. Elsevier; Amsterdam: 1999. p. 163p. 203
6. Aye Y, Stubbe J. Clofarabine 5'-di and -triphosphates inhibit human ribonucleotide reductase by altering the quaternary structure of its large subunit. *Proc Natl Acad Sci U S A*. 2011; 108:9815–9820. [PubMed: 21628579]
7. Fu Y, Lin HY, Wisitpitthaya S, Blessing WA, Aye Y. A fluorimetric readout reporting the kinetics of nucleotide-induced human ribonucleotide reductase oligomerization. *ChemBio-Chem*. 2014; 15:2598–2604.
8. Aye Y, Brignole EJ, Long MJ, Chittuluru J, Drennan CL, Asturias FJ, Stubbe J. Clofarabine targets the large subunit (α) of human ribonucleotide reductase in live cells by assembly into persistent hexamers. *Chem Biol*. 2012; 19:799–805. [PubMed: 22840768]
9. Bonate PL, Arthaud L, Cantrell WR Jr, Stephenson K, Secrist JA 3rd, Weitman S. Discovery and development of clofarabine: a nucleoside analogue for treating cancer. *Nat Rev Drug Discovery*. 2006; 5:855–863. [PubMed: 17016426]
10. Foran JM, Oscier D, Orchard J, Johnson SA, Tighe M, Cullen MH, de Takats PG, Kraus C, Klein M, Lister TA. Pharmacokinetic study of single doses of oral fludarabine phosphate in patients with "low-grade" non-Hodgkin's lymphoma and B-cell chronic lymphocytic leukemia. *J Clin Oncol*. 1999; 17:1574–1579. [PubMed: 10334546]
11. Robertson LE, Denny AW, Huh YO, Plunkett W, Keating MJ, Nelson JA. Natural killer cell activity in chronic lymphocytic leukemia patients treated with fludarabine. *Cancer Chemother Pharmacol*. 1996; 37:445–450. [PubMed: 8599867]

12. Månsson E, Spasokoukotskaja T, Sällström J, Eriksson S, Albertioni F. Molecular and Biochemical Mechanisms of Fludarabine and Cladribine Resistance in a Human Promyelocytic Cell Line. *Cancer Res.* 1999; 59:5956–5963. [PubMed: 10606241]
13. Kashlan OB, Cooperman BS. Comprehensive model for allosteric regulation of mammalian ribonucleotide reductase: refinements and consequences. *Biochemistry.* 2003; 42:1696–1706. [PubMed: 12578384]
14. Rofougaran R, Vodnala M, Hofer A. Enzymatically active mammalian ribonucleotide reductase exists primarily as an $\alpha 6\beta 2$ octamer. *J Biol Chem.* 2006; 281:27705–27711. [PubMed: 16861739]
15. Fairman JW, Wijerathna SR, Ahmad MF, Xu H, Nakano R, Jha S, Prendergast J, Welin RM, Flodin S, Roos A, Nordlund P, Li Z, Walz T, Dealwis CG. Structural basis for allosteric regulation of human ribonucleotide reductase by nucleotide-induced oligomerization. *Nat Struct Mol Biol.* 2011; 18:316–322. [PubMed: 21336276]
16. Fu Y, Long MJ, Rigney M, Parvez S, Blessing WA, Aye Y. Uncoupling of allosteric and oligomeric regulation in a functional hybrid enzyme constructed from *Escherichia coli* and human ribonucleotide reductase. *Biochemistry.* 2013; 52:7050–7059. [PubMed: 24024562]
17. Ando N, Li H, Brignole EJ, Thompson S, McLaughlin MI, Page JE, Asturias FJ, Stubbe J, Drennan CL. Allosteric Inhibition of Human Ribonucleotide Reductase by dATP Entails the Stabilization of a Hexamer. *Biochemistry.* 2016; 55:373–381. [PubMed: 26727048]
18. Weinberg G, Ullman B, Martin DW Jr. Mutator phenotypes in mammalian cell mutants with distinct biochemical defects and abnormal deoxyribonucleoside triphosphate pools. *Proc Natl Acad Sci U S A.* 1981; 78:2447–2451. [PubMed: 7017732]
19. Sasvari-Szekely M, Spasokoukotskaja T, Szoke M, Csapo Z, Turi A, Szanto I, Eriksson S, Staub M. Activation of deoxycytidine kinase during inhibition of DNA synthesis by 2-chloro-2'-deoxyadenosine (Cladribine) in human lymphocytes. *Biochem Pharmacol.* 1998; 56:1175–1179. [PubMed: 9802328]
20. Copeland, RA. *Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medicinal Chemists and Pharmacologists.* 2nd. John Wiley & Sons, Inc; NJ: 2009.
21. Aye Y, Long MJ, Stubbe J. Mechanistic studies of semicarbazone triapine targeting human ribonucleotide reductase *in vitro* and in mammalian cells: tyrosyl radical quenching not involving reactive oxygen species. *J Biol Chem.* 2012; 287:35768–35778. [PubMed: 22915594]
22. Reichard P, Eliasson R, Ingemarson R, Thelander L. Cross-talk between the allosteric effector-binding sites in mouse ribonucleotide reductase. *J Biol Chem.* 2000; 275:33021–33026. [PubMed: 10884394]
23. Hofer A, Crona M, Logan DT, Sjöberg BM. DNA building blocks: keeping control of manufacture. *Crit Rev Biochem Mol Biol.* 2012; 47:50–63. [PubMed: 22050358]
24. Parker WB, Shaddix SC, Chang CH, White EL, Rose LM, Brockman RW, Shortnacy AT, Montgomery JA, Secrist JA 3rd, Bennett LL Jr. Effects of 2-chloro-9-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)adenine on K562 cellular metabolism and the inhibition of human ribonucleotide reductase and DNA polymerases by its 5'-triphosphate. *Cancer Res.* 1991; 51:2386–2394. [PubMed: 1707752]
25. Xie KC, Plunkett W. Deoxynucleotide pool depletion and sustained inhibition of ribonucleotide reductase and DNA synthesis after treatment of human lymphoblastoid cells with 2-chloro-9-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl) adenine. *Cancer Res.* 1996; 56:3030–3037. [PubMed: 8674058]
26. Sauvageot CM, Weatherbee JL, Kesari S, Winters SE, Barnes J, Dellagatta J, Ramakrishna NR, Stiles CD, Kung AL, Kieran MW, Wen PY. Efficacy of the HSP90 inhibitor 17-AAG in human glioma cell lines and tumorigenic glioma stem cells. *Neuro Oncol.* 2009; 11:109–121. [PubMed: 18682579]
27. Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues: mechanisms of drug resistance and reversal strategies. *Leukemia.* 2001; 15:875–890. [PubMed: 11417472]
28. Jordheim LP, Durantel D, Zoulim F, Dumontet C. Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. *Nat Rev Drug Discovery.* 2013; 12:447–464. [PubMed: 23722347]

29. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer*. 2013; 13:714–726. [PubMed: 24060863]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

(a) RNR catalyzes NDP reduction, central to DNA metabolism. Nucleoside anticancer agents F2C and ClF inhibit RNR *via* vastly different mechanisms.⁴ (b) RNR-inhibiting nucleosides for which mechanisms are known *in vitro* (F2C)^{2,5} as well as in cells (ClF)^{6–8} and unknown (ClA and FIU).⁴ (c) RNR- α subunit-specific CDP/ATP-reductase-activity (500 μ g of total lysate, measured over four time points) post 3-h treatment of 3T3 cells stably expressing either wt- or mutant (D57N)-mouse(m)RNR- α by 5, 50, and 300 μ M ClF, ClA, and FIUMP, respectively. Error bars, SD ($n = 3$). (d) mRNR- α protein levels in samples from c. GAPDH, loading control. Also see Figure S2. (e) Chemoenzymatic syntheses of mono-, di-, and triphosphates of ClA and FIU. See SI for characterizations.

Figure 2. (a–d) Dose-dependent inhibition of RNR- α -specific CDP/ATP-reductase-activity (assay period, 3 min). Fitting these data to tight-binding equation²⁰ gives K_i 's as 1.4 ± 0.7 , 0.5 ± 0.1 , 9.4 ± 1.7 , and $6.8 \pm 1.3 \mu\text{M}$ for (a) CIADP, (b) CIATP, (c) FIUDP, and (d) FIUTP, respectively. Error bars designate SD., $n = 3$. (e,f) FRET-quenching assay reporting the ligand-driven RNR- α oligomerization.⁷ The ribbon structure represents the known 9.0 Å dATP-bound human α_6 crystal structure (5D1Y).¹⁷ (f) CIADP (●), CIATP (▲), FIUDP (■), and FIUTP (◆). Error bars designate SD ($n = 3$). See also Table 1 and Figures S3–S5.

Figure 3.

(a–f) Representative gel filtration profiles. In b–f, — and ··· indicate A280 and A260 traces, respectively. (a) MW standards: thyroglobulin (669 kDa, 17.6 min); ferritin (440 kDa, 20.6 min); aldolase (158 kDa, 24.6 min); conalbumin (75 kDa, 27.8 min); ovalbumin (44 kDa, 29.8 min). Additional standards: β -amylase (200 kDa, 23.8 min); alcohol dehydrogenase (150 kDa, 25.7 min); and BSA (66 kDa, 28.0 min). (b) RNR- α alone. (c and d), RNR- α treated with CIADP or CIATP (250 μM , 2 min, 37 $^{\circ}\text{C}$) was analyzed. Running buffer contained no nucleotides. (e) As in (c) and (d), except 250 M FIUDP replaced CIAD(T)P

(Trace labeled **T**). Running buffer contained 20 M FIUDP (Trace labeled **R**). (f) Trace labeled “**1x**”: as in c and d, except 250 μ M FIUTP replaced CIAD(T)P. Trace labeled “**2x**”: 500 μ M FIUTP was used. (g) CIADP was recovered quantitatively post enzyme inhibition. Left panel: HPLC diode array traces of nucleotides and small molecules extracted from inhibition mixture. Inset shows expansion at the retention time expected for CIADP (21 min; by comparison to an authentic standard under otherwise identical conditions). Red and blue traces designate the reaction and control (identical conditions except no *a*), respectively. Right panel: UV–vis absorbance spectra corresponding to the peak maxima at the 21 min within red (···) and blue (—) traces.

Figure 4.

Determination of binding site specificity by fluorescence anisotropy (FA). (a,b) CIATP A-site-binding specificity. Also see Figure S5 for independent validations by activity assays and Figure S6 for additional FA data and titration studies. (a) Addition of CIATP to a sample containing RNR- α , T*-dATP (S- and A-site binder) and dTTP (S-site-exclusive binder in saturating amounts) led to a further decrease in FA, suggesting CIATP interacts at the A site. Also see Figure S6. (b) Using the reduced-affinity-A-site-binding mutant D57N- α , the addition of CIATP to a sample containing this mutant and T*-dATP (binds S-site of the mutant) resulted in a minute decrease in FA compared to the values obtained when S-site-exclusive binders (dTTP or dGTP) were added (left panel). When wt- α was used in place of a mutant in which T*-dATP can interact with both A and S sites, higher levels of anisotropy drop were measured upon the addition of CIATP (right panel) compared to that obtained in the case with mutant (left panel). (c) CIADP C-site-binding specificity determined by size exclusion chromatography (SEC). Gel filtration analysis of RNR- α treated with buffer alone

(... dark), saturating amounts of dATP alone (— green), CIADP alone (... blue), or both (— magenta). α_6 peak observed only when CIADP treatment was additionally included, but not in dATP-saturated sample (A and S sites occupied), suggesting CIADP binds at the C site. (d,e) CIATP and CIADP A-site- and C-site-binding specificities, respectively. FA was first measured for a sample containing RNR- α , T*-dATP, and dTTP (saturating amounts at S site). Unlike treatment with CIATP, which resulted in a ~20% further drop in FA due to T*-dATP displacement, CIADP (e) caused no statistically significant changes in FA consistent with CIADP interacting with neither the S nor A site. By gel filtration, the sample treated with CIADP (right-hand bar in the plot) resulted in an appreciable fraction of hexamers, whereas the control sample (first bar in plot) eluted exclusively as a monomer. Error bars designate SD ($n = 3$). See text for discussion. The experimental data in this figure and Figure S6 are interpreted on the assumption that T*-dATP binds to the same site as dATP.

Figure 5.

(a) Different nucleotides gave rise to conformationally distinct hexamers. SDS-PAGE analysis of the samples in which RNR- α pretreated with buffer (“no drug” control) or respective drugs was incubated with buffer (“no trypsin” control) or trypsin protease (1 mg mL⁻¹) over the indicated time. M, MW marker. See also Figure S7. (b) Quantification of the designated band intensities relative to the 92 kDa band in the sample without drug or trypsin (left, 92 kDa monomer; right, ~60 kDa band).

Figure 6.

(a,b) Proliferation inhibition assays show protection from drug-induced cytotoxicity is selectively achieved in cells expressing oligomerization-defective D57N-α provided inhibitors cause α-hexamerization (CIF, CIA, and not F2C). See text for discussion with FIUMP. See also Figure S8. (a) Quantification of the data in b. Inset shows the experimental hypothesis. (b) Dose-dependent viability assays. 3-AP is an RNR-β-specific inhibitor²¹ (also see Figure S3a). 17-AAG is an HSP90 inhibitor.²⁶

Table 1Inhibition Constants and EC₅₀ Values of Inhibitor-Induced Oligomerization

	$K_i/\mu\text{M}$ (inhibition)	$\text{EC}_{50}/\mu\text{M}$ oligomerization
CIADP	1.4 ± 0.7	1.7 ± 0.5
CIATP	0.5 ± 0.1	0.5 ± 0.1
FIUDP	9.4 ± 1.7	5.2 ± 1.2
FIUTP	6.8 ± 1.3	4.6 ± 0.8
CIFDP	0.017	0.19
CIFTP	0.04	0.07