SUPPORTING INFORMATION

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

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General Materials and Methods

Cladribine (ClA), fludarabine phosphate (FlUMP), and clofarabine (ClF) were purchased from AK Scientific. All other chemicals were from either Fisher or Sigma Aldrich in highest available purity and used without further purification. NH_4^+ salt of [5-^{3}H]-CDP (19.4 Ci/mmol) was from ViTrax. AG® 1-X8 resin acetate form was purchased from Bio-Rad. IAF (5-iodoacetamidofluorescein) and TMRIA (tetramethylrhodamine-5-iodoacetamide dihydroiodide) were from Invitrogen (catalog numbers: I-30451, and T-6006, respectively). PD-10 desalting columns and Sephadex G-25 resin and molecular weight standards were from GE Healthcare; DEAE-Sephadex A-25 chloride form was from Sigma life science; 7-amino-4-methylcoumarin (AMC) and Dowex® 50WX4 200–400 (H) were from Alfa Aesar, and TALON® metal affinity resin was from Clontech. *N*-alpha-CBZ-arginine 7-amino-4-methylcoumarin hydrochloride (z-Arg-AMC) was purchased from Santa Cruz Biotechnology. Alkaline Phosphatase Calf Intestinal (CIP) (10,000 U/mL) was from New England Biolabs. Sequencing grade modified trypsin was from Promega. Analytical TLC was performed on Merck TLC silica gel 60 F_{254} glass plates. Compounds were visualized by exposure to UV light. Streptomycin sulfate and isopropyl β-D-thiogalactopyranoside (IPTG) were from Gold Biotechnology. DL-dithiothreitol (DTT) and sodium phosphate monobasic monohydrate were from Amresco. Texas Red®-5-dATP (T*-dATP) was purchased from PerkinElmer®. Antibodies to mammalian ribonucleotide reductase α subunit (Ab81085), mammalian ribonucleotide reductase p53β (Ab8105), mammalian ribonucleotide reductase β subunit (Ab57653), mouse anti-rabbit IgG (HRP) (Ab99702) and goat anti-mouse IgG H&L (HRP) (Ab6789) were from Abcam. Fetal Bovine Serum (FBS) for cell experiments was from Sigma Aldrich. Other cell culture media, Bovine Calf Serum (BCS) and Trypan Blue Stain (0.4%) were purchased from Gibco® Life Technologies. AlamarBlue® dye for cell viability assay was from Thermo Fisher Scientific. Cloning cylinders (glass, 150 μL), 96 well tissue culture plates with 0.33 cm^2 cell growth area and 60 mm x 15 mm cell culture dishes were purchased from Sigma Aldrich, CELLTREAT® and Corning, respectively. pOG44 Flp-Recombinase Expression Vector was purchased from Thermo Scientific. Flp-In T-REx HEK293 cells were from Invitrogen. Ultrafiltration membranes (YM-30 and -

10), Centricons and Minicons were from Millipore. Human deoxycytidine kinase (HdCK) expression plasmid was a gift of Professor S. Eriksson (Swedish University of Agricultural Sciences, Uppsala, Sweden).[1] *E. coli* thioredoxin A [$(His)_6$-Trx, specific activity (SA) of 95 U/mg],[2] *E. coli* thioredoxin reductase [$(His)_6$-TrxR SA of 13,700 U/mg],[3] (His_6)-HdCK,[4] human (h)RNR α-subunit ($(His)_6$-α), RNR β-subunit ($(His)_6$-β), and RNR D57N α-subunit (D57N-$(His)_6$-α)[4] were isolated (and for β, anaerobically reconstituted)[5] as previously described. Proteins concentrations were determined using $\varepsilon_{280\ nm}/M^{-1}cm^{-1}$: 119,160 and 15,900 for $(His)_6$-α/ D57N-$(His)_6$-α and $(His)_6$-β, respectively (all concentrations reported are for monomer). All β used in this work has been reconstituted *in vitro* and the amount of tyrosyl radical is quantitated by electron paramagnetic resonance spectroscopy as previously reported.[5] ClFD(T)P and F2CDP were previously synthesized and concentrations were measured using extinction coefficients previously exported.[4,6] The extinction coefficients used to determine the concentrations of ClA(M/D/T)P and FlU(M/D/T)P were ε_{265}: 15,700 $M^{-1}cm^{-1}$ and ε_{263}: 16,400 $M^{-1}cm^{-1}$, respectively. The ^{3}H-counting was performed using LS 6500 multi-purpose Scintillation Counter (Beckman Coulter). Gel images were analyzed using Image Lab Version 4.1. Curve fittings and data analyses were performed using Kaleida Graph Version 4.1.2 (Synergy Software) and Prism v6.0 (Graphpad). Number of cells was counted by using Countess® II FL automated cell counter from Thermo Fisher Scientific. Fluorescence anisotropy assays were performed using Varian Cary Eclipse Fluorescence Spectrophotometer. Fluorescence intensities in FRET experiments and cell viability assays were measured using Cytation™ 3 from BioTek®.

Syntheses of ClAMP, ClADP, ClATP, FlUDP and FlUTP (Figure 1e, and Appendix)

ClAMP. Enzymatic synthesis of cladribine monophosphate (ClAMP) was achieved via phosphorylation of ClA using $(His)_6$-HdCK and the product formation was confirmed by ^{1}H-NMR and analytical TLC. The 10 mL reaction mixture which contained 1 mM ClA, 10 mM ATP, 2 mM DTT, 100 mM KCl and 10 mM $MgCl_2$ in Tris (pH 7.6) was pre-incubated at 37 °C for 2 min under gentle stirring. The reaction was initiated with 0.25

mg/mL $(His)_6$-HdCK and was incubated at 37 °C for another 45 min. The completion of the reaction was evaluated by silica gel TLC analysis using 10% MeOH in ethyl acetate as mobile phase. Anion-exchange purification of ClAMP was carried out at 4 °C on a 40 mL bed volume of DEAE A-25 Sephadex column that had been pre-equilibrated with 200 mL 5 mM triethylammonium bicarbonate [TEAB (pH 7.0)]. The desired product was eluted with a 150 mL × 150 mL linear gradient from 5 mM to 400 mM TEAB (pH 7.0). Fractions of 6 mL were collected and assayed for $A_{265\,nm}$. The solvent was removed under vacuum using lyophilizer. The resultant lyophilized white powder was characterized by ^{1}H and ^{31}P NMR spectroscopic analyses (59% isolated yield): ^{1}H NMR (600 MHz, D_2O) δ 8.38 (ppm) (*s*, 1H), 6.38 (*t*, ^{3}J = 6.8 Hz, 1H), 4.65–4.71 (*m*, 1H), 4.22–4.28 (*m*, 1H), 4.02 (*m*, 2H); ^{31}P NMR (501 MHz, D_2O, H_3PO_4 external reference) δ 2.02. HRMS (ESI) exact mass calculated for $C_{10}H_{12}ClN_5O_6P$ $[M\text{-}H]^-$ requires *m/z*: 364.02192, found 364.02231.

ClADP. *Tri*-ethylammonium (Et_3NH^+) salt of ClAMP (5.9 μmol) was dissolved in 500 μL H_2O and loaded onto a 60-mL bed volume of Dowex® 50WX4 200–400 (H) column that had been pre-equilibrated with 600 mL 20% pyridinium (pyH^+) solution. The pyH^+ salt of ClAMP was obtained by elution with H_2O and lyophilization under vacuum. The resultant salt was redissolved in H_2O (1 mL) and treated with $(n\text{-}Bu)_3N$ (12 μmol). The mixture was azeotroped with benzene (5 × 1.5 mL) to yield anhydrous tributylammonium $[(n\text{-}Bu)_3NH^+]$ salt of ClAMP. To a portion of this material (3.4 μmol) was added anhydrous dimethylformamide (DMF) (100 μL) under argon (Ar) atmosphere, followed by carbonyl diimidazole (CDI) (16.8 μmol) in DMF (100 μL) at room temperature (RT). The resultant clear solution was stirred overnight at RT under Ar atmosphere at which point anhydrous MeOH (3 μmol) was added. The suspension was stirred at RT for another 30 min at RT prior to the addition of 32 μL of 0.27 M solution of tributylammonium phosphate (TBAP) (prepared as previously reported)[4] to the reaction suspension. Stirring was continued at RT for 10 min and the reaction mixture was subsequently placed in a desiccator under vacuum until dry residue was obtained. The resultant residue was redissolved in DMF (200 μL), centrifuged (20,850 × g, 1 min, RT),

and supernatant was collected. This process was repeated 6×, and the combined supernatants were treated with an equal volume of MeOH and concentrated in vacuo. The residue was redissolved in ddH_2O (1 mL), and the ClADP was purified at 4 °C on a 20 mL bed volume of DEAE A-25 Sephadex column that had been pre-equilibrated with ddH_2O. The desired product was eluted with a 150 mL × 150 mL linear gradient from 0 to 1 M TEAB (pH 7.0). Fractions (5 mL) were assayed for $A_{265\,nm}$. ClADP was eluted at 550 mM TEAB. The solvent was removed using lyophilizer, and the material in 56% yield was judged to be homogenous by ^{1}H, ^{31}P NMR spectroscopic, and mass spectrometric analyses: ^{1}H NMR (600 MHz, D_2O) δ (ppm) 8.45 (*s*, 1H), 6.43 (*t*, ^3J = 6.8 Hz, 1H), 4.74–4.78 (*m*, 1H), 4.30 (*m*, 1H), 4.10–4.20 (*m*, 2H); ^{31}P NMR (501 MHz, D_2O, H_3PO_4 external reference) δ -10.53, -11.11; HRMS (ESI) exact mass calculated for $C_{10}H_{13}ClN_5O_9P_2$ $[M-H]^-$ requires *m/z*: 443.98825, found 443.98973.

ClATP. Anhydrous tributylammonium $[(n\text{-}Bu)_3NH^+]$ salt of ClAMP (5 μmol) was dissolved in anhydrous DMF (330 μL). The resultant cloudy suspension was then subsequently added CDI (25 μmol) in anhydrous DMF (170 μL) at RT under Ar. After stirring the colorless solution for 19 h under Ar, anhydrous MeOH (192 μmol) was added. Stirring at RT was continued for 30 min, followed by addition of a freshly prepared 0.1 M solution of commercially available tributylammonium pyrophosphate (TBAPP, 25 μmol) in DMF. The resultant suspension was stirred for another 10 min at RT and subsequently placed under vacuum overnight. Anion-exchange purification of ClATP was performed at 4 °C on a 20 mL bed volume of DEAE A-25 Sephadex column that had been pre-equilibrated with ddH_2O. The desired product was eluted with a 200 mL × 200 mL linear gradient from 0 to 1 M TEAB (pH 7.0). Fractions (5 mL) were assayed for $A_{265\,nm}$. The triphosphate was eluted at 630 mM TEAB. The solvent was removed using lyophilizer. The resultant lyophilized material (69% yield) was judged homogenous by ^{1}H, ^{31}P NMR spectroscopic, and mass spectrometric analyses: ^{1}H NMR (600 MHz, D_2O) δ (ppm) 8.50 (*s*, 1H), 6.46 (*t*, ^3J = 6.8 Hz, 1H), 4.31–4.33 (*m*, 1H), 4.15–4.28 (*m*, 2H); ^{31}P NMR (400 MHz, D_2O, H_3PO_4 external reference) δ -10.54, -11.23, -

23.10; HRMS (ESI) exact mass calculated for $C_{10}H_{14}ClN_5O_{12}P_3$ $[M\text{-}H]^-$ requires *m/z*: 523.95458, found 523.95505.

FlUDP. The commercially available FlUMP (31.8 μmol) was dissolved in 1 mL of 5 mM TEAB (pH 7.0) and was loaded on a 40 mL bed volume of DEAE A-25 Sephadex column that had been pre-equilibrated with 200 mL 5 mM triethylammonium bicarbonate [TEAB (pH 7.0)]. Anion-exchange of FlUMP was carried out at 4 °C. The desired product was eluted with a 150 mL × 150 mL linear gradient from 5 mM to 400 mM TEAB (pH 7.0). Fractions of 6 mL were collected and assayed for $A_{263\ nm}$. The solvent was removed in vacuo. The resultant *tri*-ethylammonium (Et_3NH^+) salt of FlUMP was split into 3 batches evenly and each of which was dissolved in 500 μL ddH_2O and loaded onto a 60-mL Dowex® 50WX4 200–400 (H) column that had been pre-equilibrated with 600 mL 20% pyridinium (pyH^+) solution. The pyH^+ salt of FlUMP was obtained by elution with ddH_2O. The aliquots from three batches were combined and lyophilized under vacuum. The resultant salt (13.6 μmol) was redissolved in H_2O (1 mL) and treated with $(n\text{-}Bu)_3N$ (27 μmol). The mixture was azeotroped with benzene (5 × 1.5 mL) to yield anhydrous tributylammonium $[(n\text{-}Bu)_3NH^+]$ salt of FlUMP (12.1 μmol). The synthesis of FlUDP was subsequently accomplished by following the same protocol as outlined for ClADP synthesis. Anion-exchange purification of FlUDP was performed at 4 °C on a 20 mL bed volume of DEAE A-25 Sephadex column that had been pre-equilibrated with ddH_2O. The desired product was eluted with a sequence comprising a 150 mL × 150 mL linear gradient from 0 to 300 mM TEAB (pH 7.0), an isocratic flow of 100 mL of TEAB (pH 7.0); and a 200 mL × 200 mL linear gradient from 300 to 700 mM TEAB (pH 7.0). Fractions (5 mL) were assayed for $A_{263\ nm}$. The diphosphate was eluted at 300 mM TEAB. The solvent was removed using lyophilizer and the 13% yield material was judged homogenous by ^1H, ^{31}P NMR spectroscopic, and mass spectrometric analyses: ^1H NMR (600 MHz, D_2O) δ (ppm) 8.43 (*s*, 1H), 6.34 (*d*, ^3J = 5.9 Hz, 1H), 4.61 (*t*, ^3J = 6.2 Hz, 1H), 4.49 (*t*, ^3J = 6.7 Hz, 1H), 4.32 (*m*, 2H), 4.19 (*m*, 1H), ^{31}P NMR (501 MHz, D_2O, H_3PO_4 external reference) δ -10.24, -11.01; HRMS (ESI) exact mass calculated for $C_{10}H_{13}FN_5O_{10}P_2$ $[M\text{-}H]^-$ requires *m/z*: 444.01272, found 444.01374.

FlUTP. To obtained FlUTP, protocol identical to that of ClATP synthesis was followed starting with anhydrous tributylammonium [$(n\text{-Bu})_3NH^+$] salt of FlUMP (7.2 μmol). The desired product was eluted from the column with a 200 mL × 200 mL linear gradient from 0 to 1 M TEAB (pH 7.0). Fractions (5 mL) were assayed for $A_{263\ nm}$. The triphosphate was eluted at 600 mM TEAB. The solvent was removed using lyophilizer. The resultant lyophilized material (13% yield) was judged homogenous by ^1H, ^{31}P NMR spectroscopic and mass spectrometric analyses: ^1H NMR (600 MHz, D_2O) δ (ppm) 8.63 (*s*, 1H), 6.35 (*d*, ^3J = 6.0 Hz, 1H), 4.62 (*t*, ^3J = 6.4 Hz, 1H), 4.49 (*t*, ^3J = 7.0 Hz, 1H), 4.34 (*m*, 2H), 4.19 (*m*, 1H), ^{31}P NMR (501 MHz, D_2O, H_3PO_4 external reference) δ -10.47, -11.07, -22.95; HRMS (ESI) exact mass calculated for $C_{10}H_{14}FN_5O_{13}P_3$ $[M\text{-}H]^-$ requires *m/z*: 523.97905, found 523.96014.

Radioactive assays for [5-^{3}H]-CDP reduction in isolated system (Figure 1c, 2a–d and Figure S2a, c, e–f, S3a, c, S4, and S5)

Typical assay mixture (AM) for CDP reduction contained in a final volume of 135 μL in final concentrations: assay buffer (AB) [50 mM Hepes (pH 7.6), 15 mM $MgCl_2$], 3 mM ATP, 2 mM NADPH, 100 μM *E. coli* Trx, 1 μM *E. coli* TrxR, 0.5 mM [5-^{3}H]-CDP (SA 6,000 to 25,000 cpm/nmol) and indicated concentrations of limiting RNR-α [or D57N-α] and excess reconstituted RNR-β to assay α, or limited RNR-β and excess RNR-α [or D57N-α] to assay β. The AM without the substrate was pre-warmed at 37 °C for 2 min and the nucleotide reduction was initiated with the addition of substrate. Four aliquots (30 μL each) were removed at 40, 80, 130 and 180 sec time points post initiation, and quenched with 2% $HClO_4$ (30 μL), followed by neutralization with 0.4 M NaOH (30 μL). Dephosphorylation of resultant [5-^{3}H]-dCDP was achieved by adding 410 μL of dephosphorylation mixture [10 units CIP, 1.2 mM deoxycytidine (dC), 100 mM Tris (pH 8.6)] and incubating the mixture at 37 °C for 2 h. Unreacted [5-^{3}H]-CDP was removed by loading the resultant mixture on a borate column (method of Steeper and Steuart)[7] and amount of [5-^{3}H]-dCDP formed at each time point was analyzed by liquid scintillation counting.

Generation of mammalian expression plasmids (Figure 1c–d, and Figure S2)

An expression plasmid encoding mouse *(m)RNR-α* was constructed in the pCaggs expression vector. In contrast to the previously reported *mRNR-α* transgene construct,[8] the new construct included only the *mRNR-α* open reading frame (ORF), without UTR sequences. The *mRNR-α* ORF was amplified from pCaggs-*mRNR-α* with primers F: 5'-CTCGTCGACATGCATGTGATCAAGCGAGATGGC-3' and R: 5'-TCAGGATCCACACATCAGGCACTC-3'. The PCR product was cloned into pCR2.1 with the Topo-TA cloning kit (Invitrogen). Following sequencing analysis of the inserted fragment ends to confirm the absence of PCR-induced mutations, the PCR-amplified *mRNR-α* sequence was digested out with StuI and replaced by the non-amplified StuI fragment from pCaggs-*mRNR-α*. Fragment orientation was confirmed by SphI and EcoRI digestion. The *mRNR-α* ORF was then excised from pCR2.1 with SalI/XhoI and ligated into XhoI-linearized pCaggs. Fragment orientation was confirmed by BamHI and SalI/XhoI digests. The pCaggs-*D57N-mRNR-α* mutant construct was generated by site-directed mutagenesis of the pCaggs-*mRNR-α* plasmid using a Quick Change mutagenesis kit (Stratagene, La Jolla, CA) and primers D57N (5'-CCACAGTGGAACTGAACACCCTGGCTGCT-3') and D57N-AS (5'-AGCAGCCAGGGTCAGTTCCACTGTGG-3'). Following DpnI digestion, products were transformed into DH5α and cultured on NZY^+ medium. The presence of the mutation was confirmed by XcmI digest, as the D57N mutation destroys an XcmI site. The pCaggs-*D57N-mRNR-α* clone was confirmed to be free of other mutations by sequencing.

Generation of NIH-3T3 cells stably expressing wt- and D57N-mRNR-α (Figure 1c–d, and Figure S2)

NIH-3T3 fibroblasts overexpressing wt- and D57N- mouse (m)RNR-α were generated by transfecting pCaggs expression plasmids together with PGK-puro plasmid into 3T3 cells using FuGENE6 transfection reagent (Roche Diagnostics Co., Mannheim, Germany).

Transfected 3T3 cells were cultured in standard media (Dulbeco's Modification of Eagles Medium supplemented with 10% bovine calf serum, 1.0 mM L-glutamine, 0.1 mM MEM non-essential amino acids, 100 μg/mL streptomycin sulfate, 100 U/mL penicillin). 48 h after transfection, standard media was replaced with selection media containing 2 μg/mL puromycin, which was changed every 2 days. After 10–14 days, puromycin-resistant colonies were pooled together and expanded for further analysis and experiments. Western blotting was performed to confirm mRNR-α overexpression. Puromycin-resistant cells were maintained in standard media with 1.25 μg/mL puromycin.

Generation of T-REx cell lines expressing tetracycline-inducible wt- and D57N-RNR-α (Figure 6 and Figure S8)

For generation of Flp-In T-REx HEK293 cell lines expressing RNR-α- or D57N-α-2XFlag, Flp-In T-REx HEK293 cells were cultured in MEM supplemented with 10% FBS, 1X pyruvate and non-essential amino acids, 100 μg/mL zeocin and 1X penicillin/streptomycin in a humidified atmosphere at 37 ºC with 5% CO_2. 400,000 of the cells were seeded in 6-well plates. After 24 h plates were co-transfected (A) 3:1 and (B) 6:1 plasmid mix containing the POG44 vector encoding the Flp recombinase and pcDNA5/FRT/TO-RNR-α- or D57N-α-2XFlag (2.5 μg total plasmid) using Mirus 2020 (7.5 μL) in 6-well plates as per manufacturer's instructions. Two days after transfection or when the cells reached confluence, the cells were trypsinized, and the two separate transfection conditions (A and B) were pooled and plated in 10 cm dishes. After 1–1.5 days the cells were changed to selective media containing 150 μg/mL Hygromycin B. After 1.5–2 weeks, single colonies had formed. Once the colonies were large enough to pick, individual clones were selected using cloning cylinders. Individual clones were grown in 12-well plates, and verified for expression using anti-flag western blot (+/– 200 ng/mL tetracycline, 24 h) and immunofluorescence (which showed cytosolic Flag-tagged wt- or D57N-α upon tetracycline induction). For passaging lines post selection 100 μg/mL Hygromycin B was used.

NIH-3T3 **Cell culture protocol** (Figure 1c–d and Figure S2a–e)

NIH 3T3 cells overexpressed with either wt- or D57N-mRNR-α were cultured at 37 °C under 5% CO_2 atmosphere. The media used was DMEM supplemented with 10% BCS and 100 μg/mL streptomycin sulfate, 100 U/mL penicillin. Both cell lines were maintained and passaged in the presence of 1.25 μg/mL puromycin and puromycin-containing media were changed every two days. Cell passages were performed when the cells reached about 80% confluence by rinsing with 1X DPBS, and trypsinization followed by centrifugation (800 × g for 8 min at RT).

Cell viability assays (Figure 6 and Figure S8)

T-REx cells overexpressed with either wt- or D57N-RNR-α were seeded in 60 mm x 15 mm cell culture dishes (Corning) at 37 °C under 5% CO_2 atmosphere. The media used was DMEM supplemented with 10% FBS and 100 μg/mL streptomycin sulfate, 100 U/mL penicillin. After cells attached to the dishes (1–2 days), hygromycin was added at 50 μg/mL final concentration. Cells were trypsinized after they reached confluence and the number of cells was counted by using Trypan Blue Stain (Gibco® Life Technologies) and Countess® II FL automated cell counter (Thermo Fisher Scientific). 2500 cells were seeded in 96 well plates (CELLTREAT®) in 100 μL media without hygromycin. 50 μL of 300 ng/mL tetracycline or media alone (without tetracycline) was added into each well. Cells were cultured at 37 °C under 5% CO_2 atmosphere for 11 h. 50 μL of indicated drugs at 4x of indicated concentrations or media alone (for control) were added into designated wells. Cells were left at 37 °C under 5% CO_2 atmosphere for 45 h before 90 μL of media was removed and 11 μL of AlamarBlue® dye was added into each well. After 48 h of drug treatment, cell viabilities were determined using Cytation™ 3 (BioTek®) (λ_{ex} = 560 nm, λ_{em} = 590 nm). Normalized fluorescence intensities (y-axis) were plotted against log of drug concentrations (x-axis) and the data were analyzed their EC_{50} values using the equation: $y = 1/(1+(M/EC_{50}))$; whereas M = drug concentrations in M. Data analysis was performed using Prism 6.0.

Drug treatment in cells (Figure 1c–d, 6 and Figure S2)

In a 150 mm tissue culture dish (CELLTREAT®), either wt-mRNR-α or D57N-mRNR-α overexpressing 3T3 monolayer cultures at 80–100% confluence were treated with either DMSO (vehicle), ClF, ClA, or FlUMP at the indicated concentrations and incubated at 37 °C under 5% CO_2 atmosphere for 3 h. For each set of radioactive lysate assays, cells originating from two 150 mm plates of 80–100% confluent cultures were used. After harvest, each pallet was subsequently lysed according to previously published protocols.[5,9] The clarified lysate was immediately used in radioactive lysate activity assays.

Lysate activity assay protocol (Figure 1c and Figure S2a, c, e)

The α-subunit activity for CDP reduction was determined in an analogous way to that described under *Radioactive assays for [5-^{3}H]-CDP reduction in isolated system*, but recombinant RNR-α was replaced with the lysate mentioned in *Drug-treatment in cells* above, and 10 mM DTT was used as a reducing agent instead of 2 mM NADPH, 100 μM *E. coli* Trx and 1 μM *E. coli* TrxR.

Western blot of cell lysates (Figure 1d and Figure S2b, d, S8a)

Western blot analysis was carried out as previously reported.[5,9] Antibodies to mammalian ribonucleotide reductase α subunit (Ab81085) and goat anti-mouse IgG H&L (HRP) (Ab6789) were used for primary and secondary antibodies, at 1:1000 and 1:5000 dilutions, respectively. For loading controls, membranes were re-probed with mouse monoclonal anti-GAPDH-peroxidase (1:30000 dilution) (monomer, 37 kDa) (Figure 1d and Figure S2b, d). For Figure S8a: Antibodies to mammalian ribonucleotide reductase α subunit (Ab81085) and mouse anti-rabbit IgG (HRP) (Ab99702) were used for primary and secondary antibodies, at 1:1000 and 1:4000 dilutions, respectively. In order to probe mammalian RNR-p53β, antibodies to mammalian RNR-p53β (Ab8105) at 1:2000

dilution and mouse anti-rabbit IgG (HRP) (Ab99702) at 1:4000 dilution were used for primary and secondary antibodies, respectively. Antibodies to mammalian ribonucleotide reductase β subunit (Ab57653) at 1:2000 dilution and goat anti-mouse IgG H&L (HRP) (Ab6789) 1:4000 dilution were used for primary and secondary antibodies to probe mammalian ribonucleotide reductase β subunit, respectively. Mouse monoclonal anti-GAPDH-peroxidase (1:20000 dilution) was used for loading controls.

Dose-dependent titration: apparent K_i measurement protocol (Figure 2a–d)

Typical inhibition mixture (IM) contained in a final volume of 50 μL in final concentrations 3 mM ATP, 15 mM $MgCl_2$, 2 mM NADPH, 100 μM *E. coli* Trx, 1 μM *E. coli* TrxR, 0.5 mM [5-^{3}H]-CDP, 1 μM wt- (or D57N-) RNR-α and 3 μM RNR-β in 50 mM Hepes (pH 7.6). The IM was pre-incubated at 37 °C for 2 min prior to the addition of either ClADP, ClATP, FlUDP or FlUTP at various concentrations as indicated. Subsequent to 3-min incubation period, the reaction was quenched with 2% $HClO_4$ (30 μL), followed by neutralization with 0.4 M NaOH (30 μL). Dephosphorylation was achieved by adding 390 μL of dephosphorylation mixture [10 units CIP, 1.2 mM deoxycytidine (dC), 100 mM Tris (pH 8.6)] and the amount of [5-^{3}H]-dCDP production was analyzed as described under *Radioactive assays for [5-^{3}H]-CDP reduction in isolated system*.

Time-dependent inhibition assays for RNR-β subunit (Figure S3a)

Typical inhibition mixture (IM) contained in a final volume of 70 μL (for control, 3-AP, FlUDP and FlUTP) or 35 μL (for ClADP and ClATP) in final concentrations 2 μM RNR-α and 2 μM RNR-β, indicated inhibitor or buffer-alone for control, 3 mM ATP, 15 mM $MgCl_2$, and 10 mM DTT in 50 mM Hepes (pH 7.6). The IM was pre-incubated at 37 °C for 2 min prior to the addition of either 3-AP (6 μM), ClADP (6 μM), ClATP (6 μM), FlUDP (6 μM), or FlUTP (6 μM). At the designated time points, 9 μL (for control, 3-AP, FlUDP and FlUTP) or 3 μL (for ClADP and ClATP) aliquots were removed from IM and

diluted into the assay mixture (AM) (that had been pre-incubated for 2 min at 37 °C) containing in a final volume of 21 μL (for control, 3-AP, FlUDP and FlUTP) or 57 μL (for ClADP and ClATP) final concentrations 3 μM RNR-α (for control, 3-AP, FlUDP and FlUTP) or 2 μM RNR-α (for ClADP and ClATP), 2 mM NADPH, 100 μM *E. coli* Trx, 1 μM *E. coli* TrxR, 0.5 mM [5-^{3}H]-CDP, 3 mM ATP, 15 mM $MgCl_2$ in 50 mM Hepes (pH 7.6). Subsequent to 3-min incubation period, the reaction was quenched with 2% $HClO_4$ (30 μL), followed by neutralization with 0.4 M NaOH (30 μL). Dephosphorylation was achieved by adding 410 μL (for control, 3-AP, FlUDP and FlUTP) or 380 μL (for ClADP and ClATP) of dephosphorylation mixture [containing in final concentrations 10 units CIP, 1.2 mM deoxycytidine (dC), 100 mM Tris (pH 8.6)] and the amount of [5-^{3}H]-dCDP production was analyzed as described under *Radioactive assays for [5-^{3}H]-CDP reduction in isolated system*.

Recovery of ClADP subsequent to enzyme inhibition (Figure 3g)

The IM contained in a final volume of 200 μL in final concentrations: 25 μM RNR-α, 25 μM RNR-β, 50 mM DTT, 2.5 mM ATP, 15 mM $MgCl_2$, 0.5 mM CDP, and 25 μM ClADP in 50 mM Hepes (pH 7.6). The IM was incubated at 37 °C for 10 min. Subsequent to inhibition, the enzymes were denatured by boiling the sample (100 °C) for 2 min. (Control experiments contained ddH_2O in place of α and β). The mixture was centrifuged at 10,000 × g, for 30 sec at RT. The supernatant was collected and the residue was rinsed with 50 μL H_2O, followed by spinning at 10,000 × g, 30 sec at RT. After collection of the supernatant, the rinsing process was repeated one more time. The combined supernatant was filtered by using an ultracentrifugation device (YM-30 minicon). Ultracentrifugation was performed at 6,000 × g for 10 min at 4 °C for five cycles. After each cycle of filtration, the flow-through was collected and the YM-30 minicon was topped up with additional 400 μL of ddH_2O and ultracentrifugation was continued. The solvent of the combined supernatant was removed using lyophilizer. The resultant residue was redissolved in 130 μL H_2O, filtered with 0.22 μm Millex-GV syringe filter unit (Merck Millipore), and the 100 μL of the final solution was injected

onto the Ultra IBD (150 × 4.6 mm) column (Restek) that had been pre-equilibrated with 1% MeOH in buffer A [20 mM NH_4OAc (pH 5.8)] at 1 mL/min flow rate. The nucleotides were eluted isocratically with 1% MeOH in buffer A for 5 min, followed by linear gradients to 3% and 30% MeOH in buffer A over 25 min and 15 min, respectively. The percentage of MeOH in buffer A was brought up to 60% in the next 5 min and the last linear gradient to 100% MeOH was applied over 15 min. The retention time of ClADP was in 21 min and the amount of ClADP averaged over two runs was quantified using the known extinction coefficient (ε_{265}: 15,700 $M^{-1}cm^{-1}$) against control experiments that were run under identical conditions.

Gel filtration analysis [Size Exclusion Chromatography (SEC)] (Figure 3a–f and 4c, e)

The typical reaction mixture contained in a final volume of 150 μL final concentrations: 3 mM DTT, 10 μM RNR-α and 250 μM inhibitor [ClADP or ClATP or FlUDP or FlUTP] or 500 μM FlUTP in 50 mM Hepes (pH 7.6). The sample mixture was incubated for 3 min at 37 °C, followed by a short centrifugation at 10,000 × g, 30 sec at RT. The resultant supernatant was filtered using 0.22 μm Millex-GV syringe filter unit (Merck Millipore). 130 μL of the resulting filtrate was injected into a Superdex™ 200 10/300 GL column (24 mL, 10 × 300 mm, GE Healthcare), attached to a Prominence HPLC with a photodiode array (PDA) detector (SPD-M20A) (Shimadzu Corporation), that had been pre-equilibrated at RT at 0.5 mL/min flow-rate with elution buffer [50 mM Hepes (pH 7.6), 15 mM $MgCl_2$, 150 mM NaCl]. The protein was eluted at 0.5 mL/min flow-rate at RT. GE Healthcare MW standards (M.W., retention time): thyroglobulin (669 kDa, 18.7 min); ferritin (440 kDa, 21.7 min); aldolase (158 kDa, 25.7 min); conalbumin (75 kDa, 28.8 min); and ovalbumin (43 kDa, 30.7 min) were run under identical conditions at the end of each set of experiments and $A_{280\ nm}$ and $A_{260\ nm}$ were monitored. Additional standards: β-amylase (200 kDa, 23.8 min); alcohol dehydrogenase (150 kDa, 25.7 min); and BSA (66 kDa, 28.0 min) were also independently run. An additional experiment performed for FlUDP included 20 μM FlUDP in the elution buffer under otherwise

identical conditions (Figure 3a–f). For Figure 4c, 10 μM RNR-α was incubated with 4 mM dATP before splitting into two fractions. 300 μM ClADP and assay buffer were added to the first and second fraction, respectively. For Figure 4e, samples were directly taken from fluorescence anisotropy assays. These samples were prepared and analyzed using same conditions as mentioned above (130 μL of samples in Figure 4c and 110 μL of samples from Figure 4e were injected into HPLC column). Detailed protocols for fluorescence anisotropy assays are described elsewhere in this supporting information.

Fluorescence Resonance Energy Transfer (FRET) assays (Figure 2e–f and Figure S3b)

Fluorescent Dye Labelling of RNR-α. The protocol was modeled upon the recently reported procedure.[2] The labelling reaction in a final volume of 835 μL in final concentrations contained 10 μM RNR-α, 30 μM 5-IAF or 5-TMRIA, 15 mM $MgCl_2$, 1 mM DTT in 50 mM Hepes (pH = 7.6). The presence of DTT was necessary for α stability. The mixture was incubated in the dark for 20 min at RT by end-to-end rotation on a rotary mixer. The reaction mixture was desalted using a PD-10 Sephadex G-25M column that had been pre-equilibrated with 50 mM Hepes (pH = 7.6), 15 mM $MgCl_2$ and 5 mM DTT. Fractions containing labeled proteins were pooled and the final concentration was adjusted to 1 μM using the same buffer containing DTT and $MgCl_2$. The labeling stoichiometry and catalytic activity of labeled proteins were evaluated as previously reported.[2] The 5-IAF labeled RNR (F-α) and 5-TMRIA labeled RNR (T-α) were then mixed in 1:1 ratio (in equal concentrions and in equal volumes). Labeled proteins were immediately used in the subsequent FRET assay.

Biotek Cytation™ 3 platereader-based FRET assay. The pre-mixed protein solution containing a 1:1 mixture of F-α : T-α was diluted into the assay mixture to yield final concentrations of either 0.8 or 0.4 μM total proteins, respectively for the experiments with ClADP, FlUD(T)P, and ClATP. The assay mixture contained in final concentrations 50 mM Hepes buffer (pH 7.6), 1 mM DTT, and 15 mM $MgCl_2$. This solution (100

μL) was pipetted into individual wells of a Costar 96 half-area black opaque plate, and the indicated concentrations of respective nucleotide inhibitors [prepared as stock solutions in 50 mM Hepes (pH 7.6), 15 mM $MgCl_2$, 5 mM DTT] were added using a P2 pipette to the final volume of 102 μL across all wells. The plate was placed in the Biotek Cytation™ 3 plate-reader that was programmed with the following sequence: gently shaking the plate at 410 rpm for 20 min [ClADP, FlUD(T)P], and 35 min (ClATP) to allow for complete equilibration of the quenched state; delaying for 5 sec post gentle shaking; and recording the drop in quenching of donor fluorophore intensity using green filters: excitation 485 ± 10 nm, emission 528 ± 10 nm, top 510 nm (optics position), and gain 35. All FRET experiments were performed as independent triplicates. The normalized fluorescence intensities against concentrations were plotted and the data were fitted to the equation: normalized fluorescent intensity = 1/{1+10^[X-Log(IC_{50})]} in which X represents the log of inhibitor concentration was performed using Prism 6.0.

Fluorescence anisotropy assays of T*-dATP with either wt- or D57N-α (Figure 4a–b, d–e and Figure S6)

The assay mixture (final volume = 150 μL) contained in final concentrations of either wt- or D57N-α (6 μM), T*-dATP (1.2 μM), and nucleotides of interest (dTTP, dATP, dGTP, ClADP, or ClATP at 94, 700, 250, 75 and 31 μM, respectively) or buffer alone as control (Figure 4a–b, 4d–e and Figure S6a), KCl (100 mM), $MgCl_2$ (15 mM), DTT (5 mM) and 5% glycerol in Tris base (50 mM) (pH=7.6) at RT. The assay mixture was prepared by incubating T*-dATP with either wt- or D57N-α for one min before adding nucleotides of interest (dTTP, dATP, dGTP, or ClATP at indicated final concentrations) or buffer alone (control). The mixture was thoroughly mixed and incubated for another minute. Fluorescence anisotropy was then measured using a fluorescence spectrophotometer (Varian Cary Eclipse) (λ_{ex} = 580 nm and λ_{em} = 620 nm) (Figure 4a–b and Figure S6a). For Figure 4d–e, the mixture without T*-dATP was pre-incubated for one minute with saturating amounts of S-site binding nucleotide dTTP (or buffer alone as control) to pre-

block the S-site. T*-dATP was then added into the mixture and the mixture was mixed thoroughly at which point the nucleotide inhibitor (ClADP or ClATP at indicated final concentrations or buffer alone as control) was added. The mixture was incubated for another minute and fluorescence anisotropy was measured as mentioned above. Thereafter, 110 μL of each of the two samples (wt-RNR-α and T*-dATP in the presence of dTTP alone, or in the presence of both dTTP and ClADP) was directly injected into HPLC following the same protocol as described in *Gel filtration analysis [Size Exclusion Chromatography (SEC)]* section. This procedure further validated the resultant oligomeric state of RNR-α subsequent to the fluorescence anisotropy measurements (Figure 4e).

For titration studies (Figure S6b–e), the mixture without any nucleotides (dTTP, dATP, dGTP and ClATP) was pre-incubated with T*-dATP for one minute. Then, nucleotides of interest at indicated final concentrations [namely, dTTP (3.5, 10.4, 31.3, 94 and 282 μM), dATP (87.5, 175, 350, 700 μM and 1.4 mM), dGTP (9.3, 27.8, 83.3, 250 and 750 μM) or ClATP (2, 4, 8, 16 and 31 μM)] were added into the assay mixture and incubated for another minute before the measurements were made as above. For the analysis of potential non-specific binding of T*-dATP, please refer to Figure S6b: the corresponding plots at the top right and bottom right of the sub-figure. The order of addition of T*-dATP or 700 μM dATP to the assay mixture is designated on the x-axis of each plot.

Regain of activity post dilution (estimation of off-rates) (Figure S4)

To approximate the off-rates, inhibited enzyme was diluted to promote inhibitor dissociation and regain of RNR-α activity was subsequently measured as a function of time. The experimental details pertinent to each inhibitor are listed below.

ClADP. The inhibition mixture (IM) contained in a final volume of 20 μL final concentrations: 15 μM RNR-α, 37.5 μM RNR-β, 3 mM ATP, 15 mM $MgCl_2$, 5 mM DTT and 30 μM ClADP (or ddH_2O for control) in 50 mM Hepes (pH 7.6). The IM without the

inhibitor was pre-warmed at 37 °C for 2 min at which point ClADP (or ddH_2O) was added. After 2-min incubation at 37 °C, the IM was diluted by the addition of 2,380 μL pre-warmed buffer 50 mM Hepes (pH 7.6) containing 15 mM $MgCl_2$ and 5 mM DTT (120-fold dilution at this step). At the indicated time points, 50 μL aliquots were removed and mixed with 5 μL of 11x stock of assay mixture (AM) (a further 1.1-fold dilution of original IM at this step). The 11x stock of AM contained in 65 μL final concentrations: 33 μM RNR-β, 33 mM ATP, 15 mM $MgCl_2$, 11 mM NADPH, 1.1 mM Trx, 11 μM TrxR, and 5.5 mM [5-^{3}H]-CDP in 50 mM Hepes (pH 7.6) and individual aliquots of the 11x AM stock was pre-warmed for 2 min at 37 °C prior to mixing with the respective aliquots of the diluted IM at each indicated time point. dCDP production was assayed over 9 min for each time point, prior to quenching with 2% $HClO_4$ (30 μL). Standard neutralization, dephosphorylation, and subsequent purification and scintillation counting procedures ensued. Under the experimental conditions employed for inhibition and dilution, and based on the K_i values derived for ClADP (Figure 2a, Table 1), 92% of α was anticipated to be in the inhibitor-bound form, and 86% of the initial formed E•I complex was expected to be dissociated upon 132-fold dilution. These technical limitations in part attributed to incomplete recovery of full enzyme activity (Figure S4a).

ClATP. The inhibition mixture (IM) contained in a final volume of 15 μL final concentrations: 16.5 μM RNR-α, 20 μM RNR-β, 3 mM ATP, 15 mM $MgCl_2$, 5 mM DTT and 16.5 μM ClATP (or ddH_2O for control) in 50 mM Hepes (pH 7.6). The IM without the inhibitor was pre-warmed at 37 °C for 2 min at which point ClATP (or ddH_2O) was added. After 2-min incubation at 37 °C, the IM was diluted by the addition of 2,235 μL pre-warmed buffer 50 mM Hepes (pH 7.6) containing 15 mM $MgCl_2$ and 5 mM DTT (150-fold dilution at this step). At the indicated time points, 150 μL aliquots were removed and mixed with 15 μL of 11x stock of assay mixture (AM) (a further 1.1-fold dilution of original IM at this step). The 11x stock of AM contained in 195 μL final concentrations: 33 μM RNR-β, 33 mM ATP, 15 mM $MgCl_2$, 11 mM NADPH, 1.1 mM Trx, 11 μM TrxR, and 5.5 mM [5-^{3}H]-CDP in 50 mM Hepes (pH 7.6). The individual aliquots of the 11x AM stock was pre-warmed for 2 min at 37 °C prior to mixing with the

respective aliquots of the diluted IM at each indicated time point. dCDP production was assayed over 9 min for each time point, prior to quenching with 2% $HClO_4$ (30 μL). Standard neutralization, dephosphorylation, and subsequent purification and scintillation counting procedures ensued. Under the experimental conditions employed for inhibition and dilution, and based on the K_i values derived for ClATP (Figure 2b, Table 1), ~85% of α was anticipated to be in the inhibitor-bound form, and ~83% of the initial formed E•I complex was expected to be dissociated upon 165-fold dilution. These suppositions in part accounted for incomplete recovery of full enzyme activity (Figure S4a).

FlUDP and FlUTP. The inhibition mixture (IM) contained in a final volume of 30 μL final concentrations: 2 μM RNR-α, 5 μM RNR-β, 3 mM ATP, 15 mM $MgCl_2$, 5 mM DTT and 90 μM FlUDP or FlUTP (or ddH_2O for control) in 50 mM Hepes (pH 7.6). The IM without the inhibitor was pre-warmed at 37 °C for 2 min at which point inhibitor (FlUDP or FlUTP) or ddH_2O was added. After 2-min incubation at 37 °C, the IM was diluted by the addition of 870 μL pre-warmed buffer 50 mM Hepes (pH 7.6) containing 15 mM $MgCl_2$ and 5 mM DTT (30-fold dilution at this step). At the indicated time points, 50 μL aliquots were removed and mixed with 5 μL of 11x stock of assay mixture (AM) (a further ~1.1-fold dilution of original IM at this step). The 11x stock of AM contained in 65 μL final concentrations: 33 μM RNR-β, 33 mM ATP, 15 mM $MgCl_2$, 11 mM NADPH, 1.1 mM Trx, 11 μM TrxR, and 5.5 mM [5-^{3}H]-CDP in 50 mM Hepes (pH 7.6) and individual aliquots of the 11x AM stock was pre-warmed for 2 min at 37 °C prior to mixing with the respective aliquots of the diluted IM at each indicated time point. dCDP production was assayed over 9 min for each time point, prior to quenching with 2% $HClO_4$ (30 μL). Standard neutralization, dephosphorylation, and subsequent purification and scintillation counting procedures ensued. Under the experimental conditions employed for inhibition and dilution, and based on the K_i values derived for FlUDP and FlUTP (Figure 2c–d, Table 1), 90% and 93% of α was anticipated to be in the inhibitor-bound form in each case, respectively, and 75% and 70% of the initial formed E•I complex was expected to be dissociated upon 33-fold dilution, respectively.

Incomplete recovery of full enzyme activity was expected under these conditions (Figure S4b).

Trypsin digest analysis of hexamers induced by different nucleotide inhibitors and trypsin sensitivity of RNR-α in the presence of natural nucleotide ligands (Figure 5 and Figure S7a)

The assay mixture contained in a final volume of 103 μL in final concentrations RNR-α (3 μM), $MgCl_2$ (15 mM), DTT (0.5 mM), trypsin (1 μM), and appropriate inhibitors at the concentrations at least 10-fold above K_i (Table 1) [ClFDP, ClADP, ClATP, FlUDP, or FlUTP in 3, 14, 5, 94, and 68 μM, respectively] (Figure 5) or natural nucleotides ligands at the concentrations at least 10-fold above reported K_d's[3,10] [dTTP, dGTP, dATP or ATP in 18, 44, 700 μM and 3 mM, respectively] (Figure S7a) or ddH_2O as control, in 50 mM Hepes (pH 7.6). The mixture without trypsin was pre-warmed at 37 ºC for 3 min and cleavage reaction was initiated by the addition of trypsin. Aliquots (18 μL) were removed at the designated time points and quenched with 10 μL of 4x Laemmli dye containing 8.4% BME. The mixture was immediately boiled (100 ºC) for 10 min. The relative rates of trypsin digestion of hexamers induced by different nucleotide inhibitors were evaluated by standard SDS-PAGE analysis.

Trypsin activity assay in the presence and absence of nucleotide inhibitors (Figure S7b–e)**.** To ensure that differences in the protease cleavage rates observed were not due to some of the nucleotide inhibitors unexpectedly inhibiting the protease activity of trypsin, the following sets of experiments were performed.

(1) Standard curve of AMC (i.e., increase in fluorescence intensity as a function of AMC concentration) (Figure S7b). The assay mixture contained in a final volume of 100 μL in final concentrations: 15 mM $MgCl_2$ and AMC at the designated concentrations in 50 mM Hepes (pH 7.6). The fluorescence signal intensities were recorded using a Biotek Cytation™ 3 plate-reader (λ_{ex} = 380 nm and λ_{em} = 460 nm). The data were fit to a linear regression equation (Figure S7b).

(2) K_m measurement of z-Arg-AMC for trypsin (Figure S7c). The assay mixture contained in a final volume of 100 μL in final concentrations: 10 ηM trypsin (or assay buffer for background), 15 mM $MgCl_2$ and z-Arg-AMC at the indicated concentrations in 50 mM Hepes (pH 7.6). Fluorescence signal intensities were recorded over 17 min using a Biotek Cytation™ 3 plate-reader (λ_{ex} = 380 nm and λ_{em} = 460 nm). The rates of AMC production from the hydrolysis of z-Arg-AMC at the indicated concentrations were calculated using the linear regression equation from the AMC standard curve (Figure S7b). The hydrolysis of z-Arg-AMC by trypsin was fit to the Michaelis Menten equation to yield K_m ~ 84 ± 15 μM (Figure S7c).

(3) Trypsin activity in the presence of nucleotide inhibitors (Figure S7d). The assay mixture contained in a final volume of 100 μL in final concentrations: 10 ηM trypsin, 15 mM $MgCl_2$, 110 μM z-Arg-AMC (~K_m value derived from Figure S7c) and 3 μM ClFDP (or 14 μM ClADP, 5 μM ClATP, 94 μM FlUDP, 68 μM FlUTP, or assay buffer for background) in 50 mM Hepes (pH 7.6). Fluorescence signal intensities were recorded over 10 min using a Biotek Cytation™ 3 plate-reader (λ_{ex} = 380 nm and λ_{em} = 460 nm). The rates of AMC production from the hydrolysis of z-Arg-AMC in the absence or presence of each nucleotide inhibitor were calculated using the linear regression equation from the AMC standard curve (Figure S7b).

(4) Percentage of fluorescence quenching of AMC by nucleotide inhibitors (i.e., confirming the lack of non-specific AMC-fluorophore quenching by nucleotides) (Figure S7e). The assay mixture contained in a final volume of 100 μL in final concentrations: 0.25 μM AMC, 15 mM $MgCl_2$ and 3 μM ClFDP (or 14 μM ClADP, 5 μM ClATP, 94 μM FlUDP, 68 μM FlUTP, or assay buffer for background) in 50 mM Hepes (pH 7.6). The fluorescence signal intensities were measured over 17 min using a Biotek Cytation™ 3 plate-reader (λ_{ex} = 380 nm and λ_{em} = 460 nm) (Figure S7e).

SUPPORTING FIGURES

Figure S1.

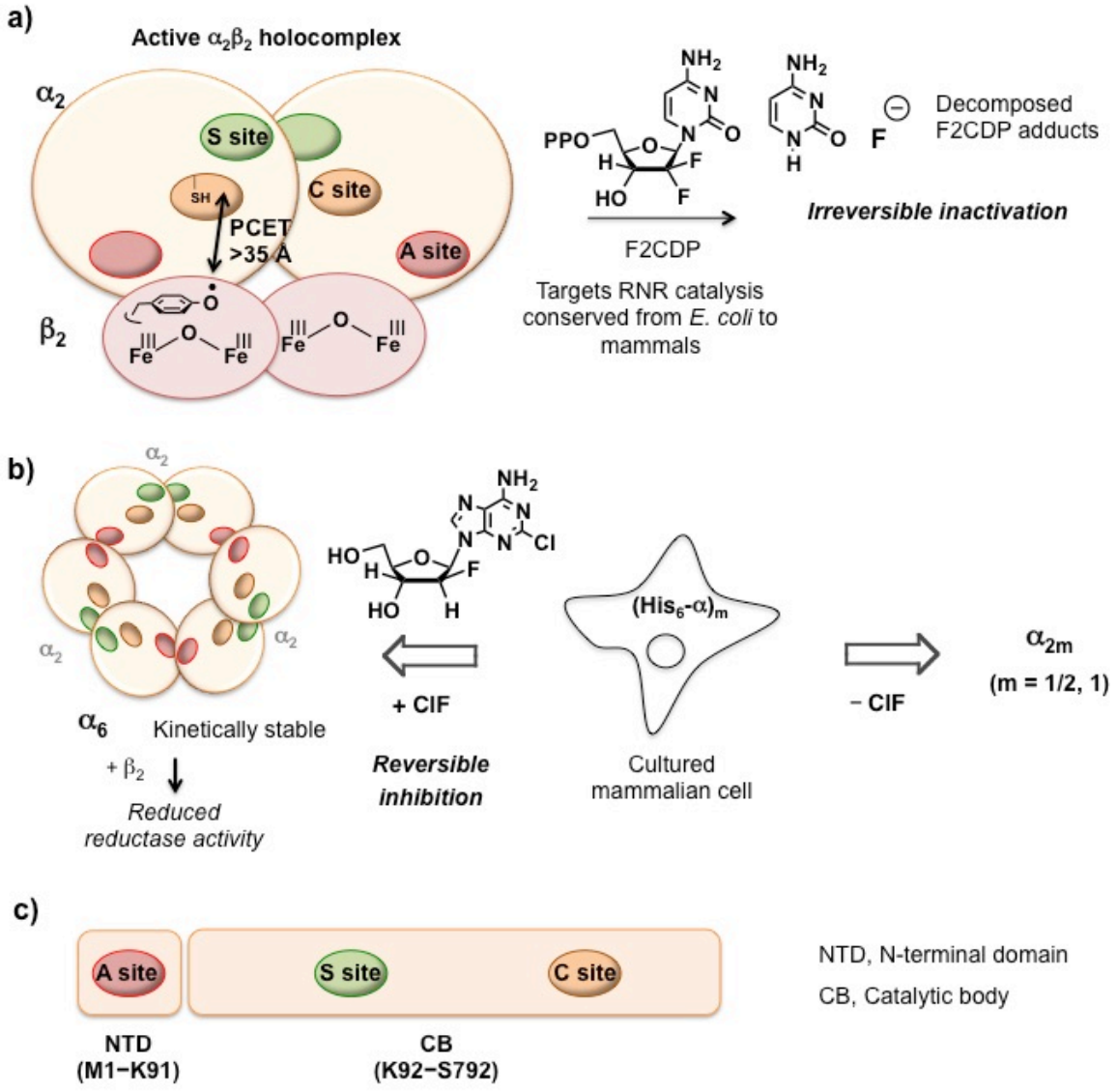


Figure S1. Mechanistically distinct RNR inhibition pathways. (**a**) Irreversible inactivation of $\alpha_2\beta_2$ holocomplex has been the paradigm of RNR inactivation by the first-line cancer drug gemcitabine diphosphate (F2CDP), and other nucleotide analogs.[11] RNR catalysis is conserved from *E. coli* to humans. F2CDP hijacks this catalytic chemistry for enzyme inactivation. It binds to the C site of the activated RNR holocomplex [in which tyrosyl radical within β has been transferred via the forward proton-coupled electron

transfer (PCET) pathway to the thiyl radical on the α C site], and irreversibly crosslinks with α, leading to suicide enzyme inactivation. (**b**) By contrast, α-specific targeting by antileukemic nucleoside clofarabine (ClF) in cells involves formation of kinetically stable α hexamers (α_6) with reduced activity.[4,9,11] This new mechanism is biochemically and structurally validated *in vitro*, and is exclusive to ClF among all RNR-targeting drugs at the outset of the present work.[12] (c) Schematic representation of the two domains of RNR-α. Amino acids shown correspond to human RNR sequence.

Figure S2.

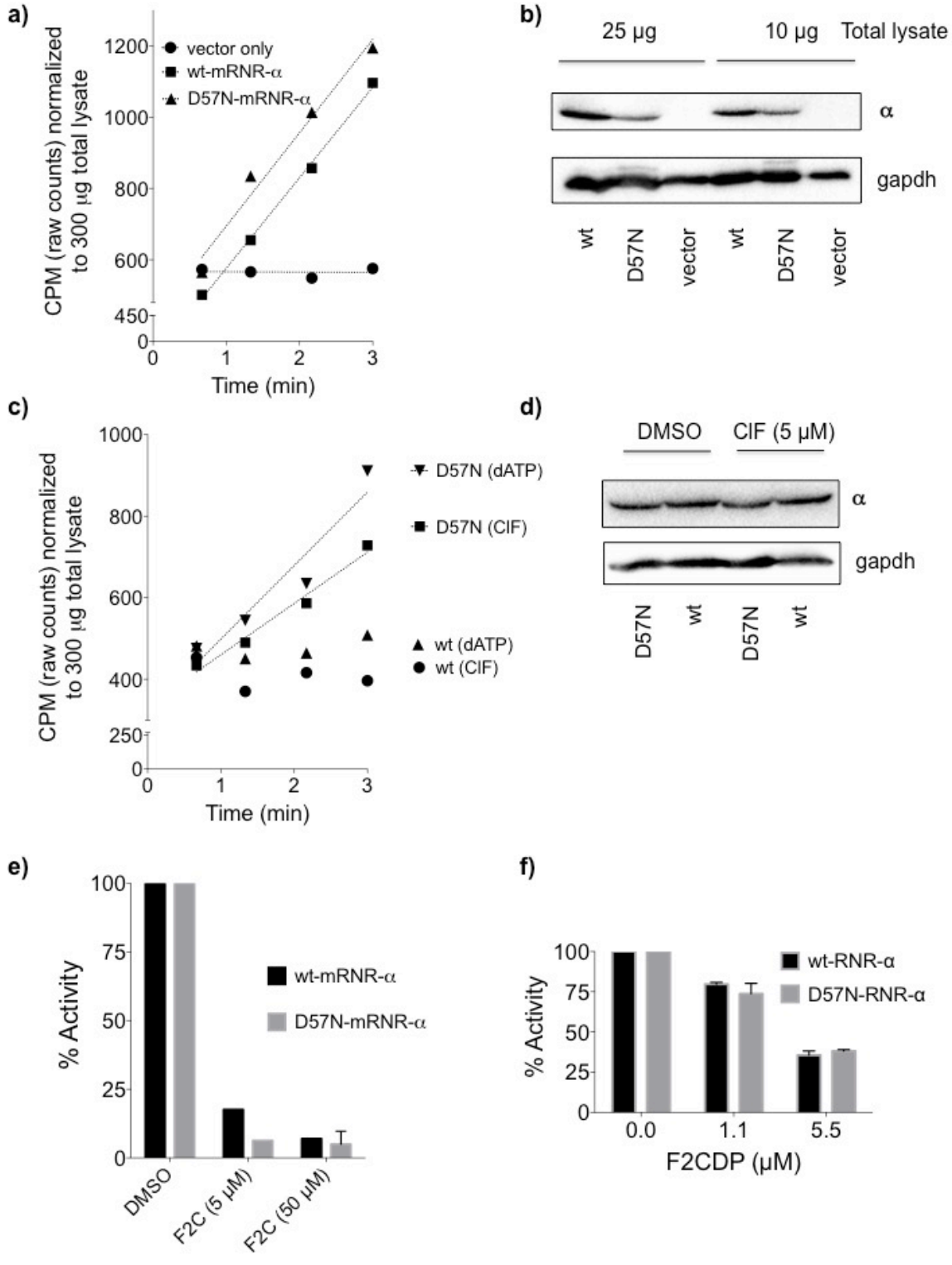

a)
CPM (raw counts) normalized to 300 µg total lysate
vector only
wt-mRNR-α
D57N-mRNR-α
Time (min)
b)
25 µg
10 µg
Total lysate
α
gapdh
wt
D57N
vector
wt
D57N
vector
c)
CPM (raw counts) normalized to 300 µg total lysate
D57N (dATP)
D57N (CIF)
wt (dATP)
wt (CIF)
Time (min)
d)
DMSO
CIF (5 µM)
α
gapdh
D57N
wt
D57N
wt
e)
% Activity
wt-mRNR-α
D57N-mRNR-α
DMSO
F2C (5 µM)
F2C (50 µM)
f)
% Activity
wt-RNR-α
D57N-RNR-α
F2CDP (µM)

Figure S2. (a–d) NIH-3T3 cell lines stably expressing catalytically functional wild type and mutant (D57N) mouse (m)RNR-α, and (e) the effects of gemcitabine (F2C) in cells and (f) its active form (F2CDP) *in vitro*. (**a**) Time-dependent radioactive lysate assays reporting the rate of CDP reduction. 300 μg total lysate from freshly lysed cells stably transfected with either wt- (■) or D57N- (▲) mRNR-α, or empty vector (●), was incubated with assay mixture including reconstituted recombinant RNR-β^5 (6 μM), Mg•ATP, Trx, TrxR, NADPH, and [5-^{3}H]-CDP in 50 mM Hepes (pH 7.6). (**b**) Western blot analysis of the same cell lysate used in the assays. **(c–d)** Mutant mRNR-α expressing cells, as opposed to wt-mRNR-α, selectively resist to ClF treatment in cells and dATP treatment in lysate. (**c**) Time-dependent radioactive lysate assays reporting the rate of CDP reduction. 300 μg total lysate derived from cells expressing either wt- (●,▲) or D57N- (■,▼) mRNR-α treated with ClF (5 μM, 3 h) (●,■) was incubated with assay mixture including reconstituted recombinant RNR-β^5 (6 μM), Mg•ATP, Trx, TrxR, NADPH, and [5-^{3}H]-CDP in 50 mM Hepes (pH 7.6). See SI Methods for details. In another set of samples, fresh lysates from the DMSO-alone-treated cells were incubated with the mentioned assay mixture containing dATP (200 μM) (▲,▼). (**d**) Western blot analysis of the same cell lysate used in the assays showing similar protein levels between treated and untreated wt or mutant cells. GAPDH is used as a loading control in western blots. See SI Methods for details. (**e**) Mutant (D57N) mRNR-α- and wt-mRNR-α-expressing NIH-3T3 cells were both sensitive to F2C treatment. Percentage activity was derived from time-dependent radioactive lysate assays reporting the CDP reductase activities in DMSO-, or F2C (5 or 50 μM)-treated either wt- and D57N-mRNR-α-expressing cells, normalized over equal amounts of total lysate proteins in each sample. See SI Methods for details. (**f**) Both the recombinant wt- and mutant-RNR-α were inhibited by F2CDP to similar efficiencies. Percentage activity was derived from the radioactive assays reporting the amount of [5-^{3}H]-dCDP formed during 3-min incubation of either recombinant wt- or D57N-RNR-α (1 μM) either in the presence or absence of F2CDP in the reaction mixture that contains reconstituted recombinant RNR-β^5 (3 μM), Mg•ATP, Trx, TrxR, NADPH and [5-^{3}H]-CDP in 50 mM Hepes (pH 7.6). Error bars indicate SD (n = 3).

Figure S3.

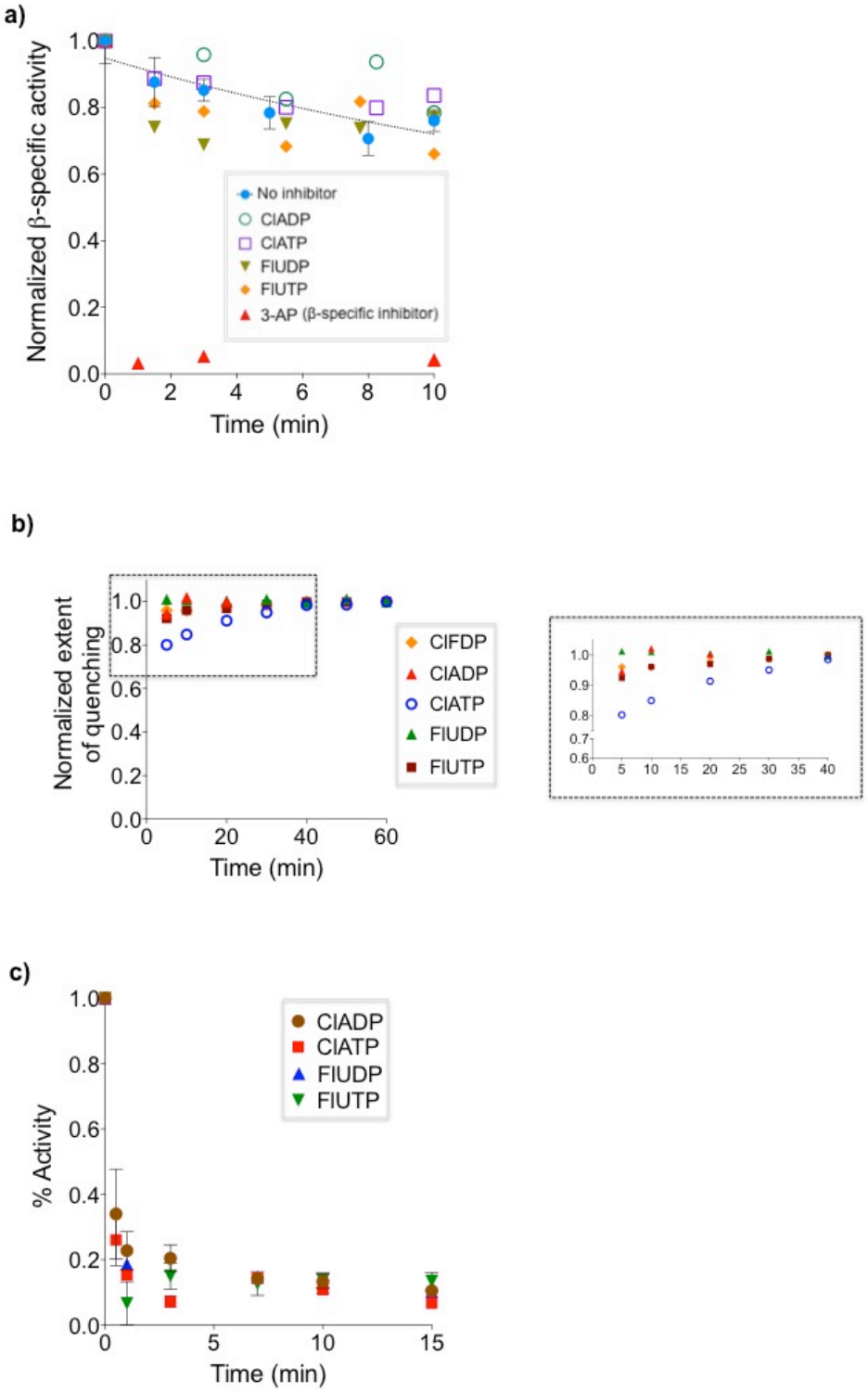

a)
Normalized β-specific activity
Time (min)
No inhibitor
ClADP
ClATP
FlUDP
FlUTP
3-AP (β-specific inhibitor)
b)
Normalized extent of quenching
Time (min)
ClFDP
ClADP
ClATP
FlUDP
FlUTP
c)
% Activity
Time (min)
ClADP
ClATP
FlUDP
FlUTP

Figure S3. (a) RNR-β-subunit-specific activity is unaffected by ClADP, ClATP, FlUDP, and FlUTP but inhibited by 3-AP.[5] The time-dependent assessments of RNR-β-specific-activity in the inhibition mixture containing 1:1 RNR-α : RNR-β (2 μM) in the presence of the designated inhibitor, Mg•ATP, and DTT in 50 mM Hepes (pH 7.6), were performed by removing the aliquots of the inhibition mixture at the indicated time points and diluting it into the assay mixture containing excess and saturating amounts of RNR-α, Mg•ATP, Trx, TrxR, NADPH and [5-^{3}H]-CDP in 50 mM Hepes (pH 7.6). For each time point, the dCDP production was measured over 3 min prior to quenching. Normalized β-specific activity of 1.0 corresponds to the counts generated at time zero. The time-dependent intrinsic decay of diferric-tyrosyl radical cofactor-bearing mammalian RNR-β subunit (●) is known.[5] See SI Methods for details. **(b) Time-dependent analysis of the extent of fluorescence quenching in the FRET assay.** The assay mixture contained in final concentrations 1:1 F-α : T-α and saturating amounts of respective inhibitors. The extent of quenching was derived based on a control experiment in which buffer replaced the inhibitors. Monomer concentrations of α, 0.2–0.4 μM. Inset shows an expanded view of the early assay period. See SI Methods for details. Also see Figure 2e–f. **(c) Time-dependent inhibition analysis of RNR-α-subunit-specific activity in the presence of ClADP, ClATP, FlUDP, and FlUTP** (5, 5, 50, and 12.5 μM, respectively). Assay procedure used was identical to that reported previously except the indicated inhibitors of shown respective final concentrations replaced ClFDP and ClFTP.[4]

Figure S4.

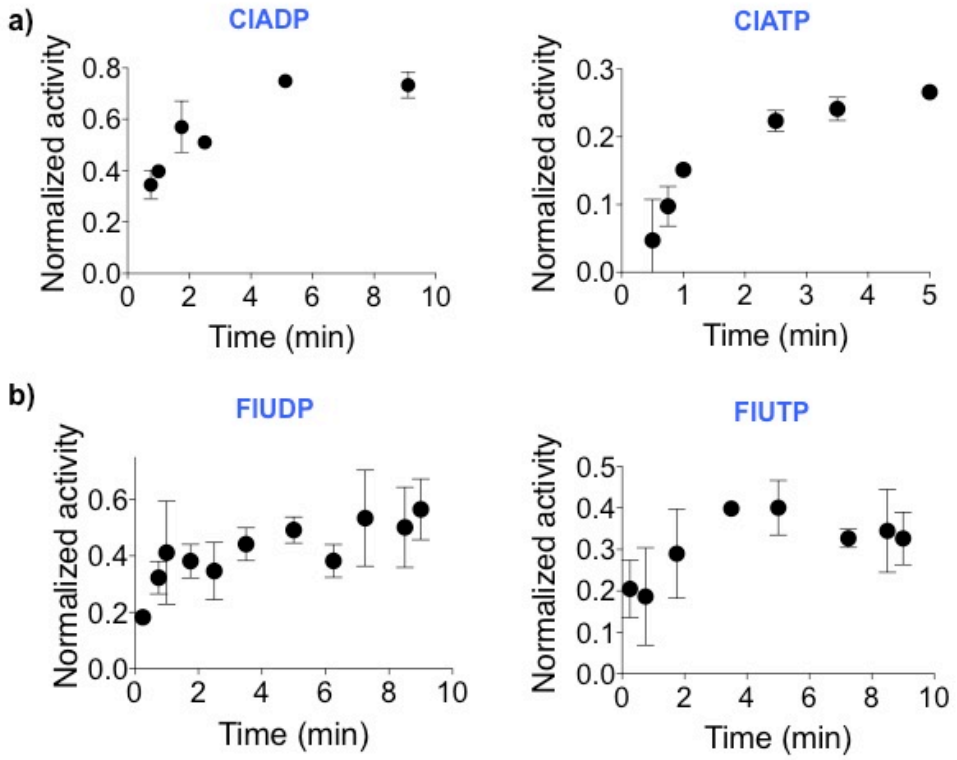


Figure S4. Evaluation of the time-dependent regain of RNR-α-subunit-specific activity subsequent to dilution-assisted dissociations of (a) ClA and (b) FlU nucleotides. The inhibition mixture containing RNR-α : RNR-β in the presence of the designated inhibitor, Mg•ATP, and DTT in 50 mM Hepes (pH 7.6) (see SI Methods for details) were rapidly diluted to promote dissociation of α•I (enzyme•inhibitor) complex. At indicated time points, aliquots were removed and measured for α-specific activity in the assay mixture containing excess RNR-β, Mg•ATP, Trx, TrxR, NADPH and [5-^{3}H]-CDP in 50 mM Hepes (pH 7.6). Percentage activities were derived based on the results from control experiment in which buffer replaced the inhibitor. Incomplete recovery was in part due to technical limitations associated with incomplete dissociation as judged by the estimated apparent K_i's of the inhibitors (Figure 2a–d, Table 1) and final concentrations of α and respective inhibitors within the diluted samples. See SI Methods for details.

Figure S5.

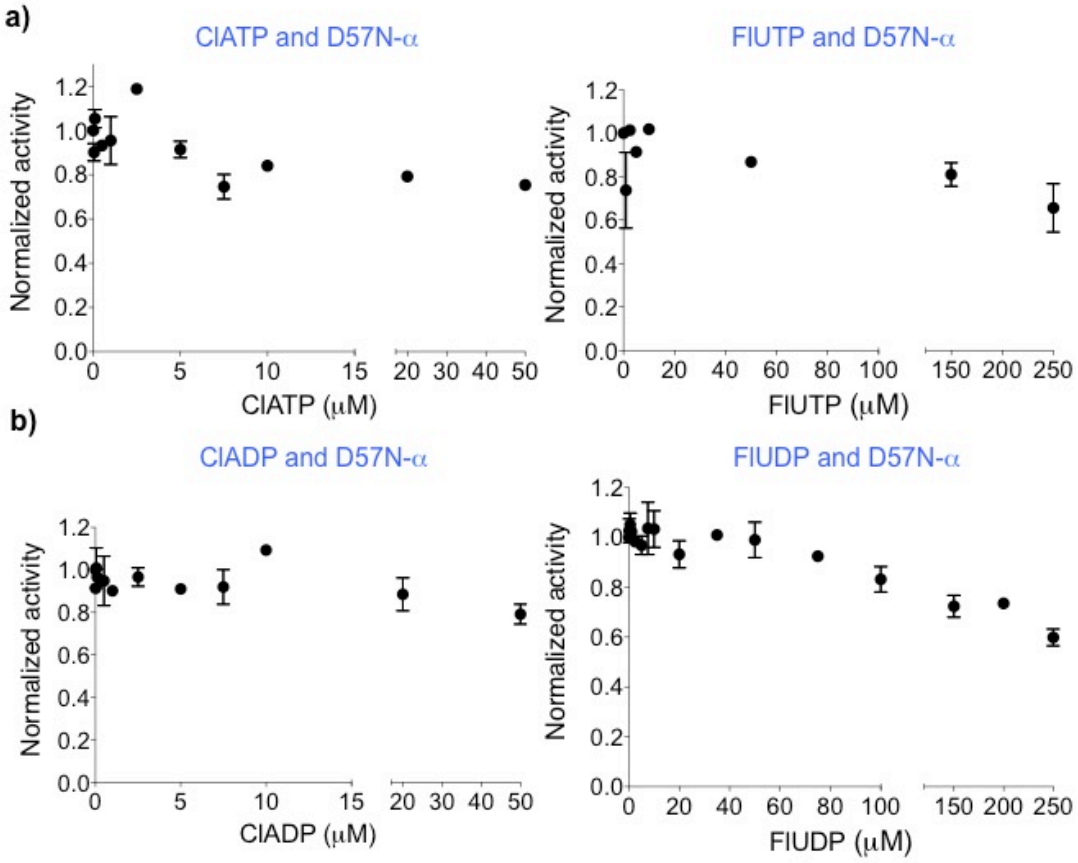


Figure S5. The recombinant mutant α remained largely uninhibited by (a) triphosphates and (b) diphosphates of ClA and FlU under the conditions in which the wt-α was inhibited (Figure 2a–d). The inhibition mixture contained 1 μM RNR-α, 3 μM RNR-β in the presence of the designated inhibitor, Mg•ATP, NADPH, Trx, TrxR, and [5-^{3}H]-CDP in 50 mM Hepes (pH 7.6) (see SI Methods for details). Percentage activities were calculated based on the results from control experiment in which buffer replaced the inhibitor. The ranges of concentrations were guided by the concentrations of respective inhibitors that were sufficient to inhibit the wt-α (Figure 2a–d, Table 1).

Figure S6.

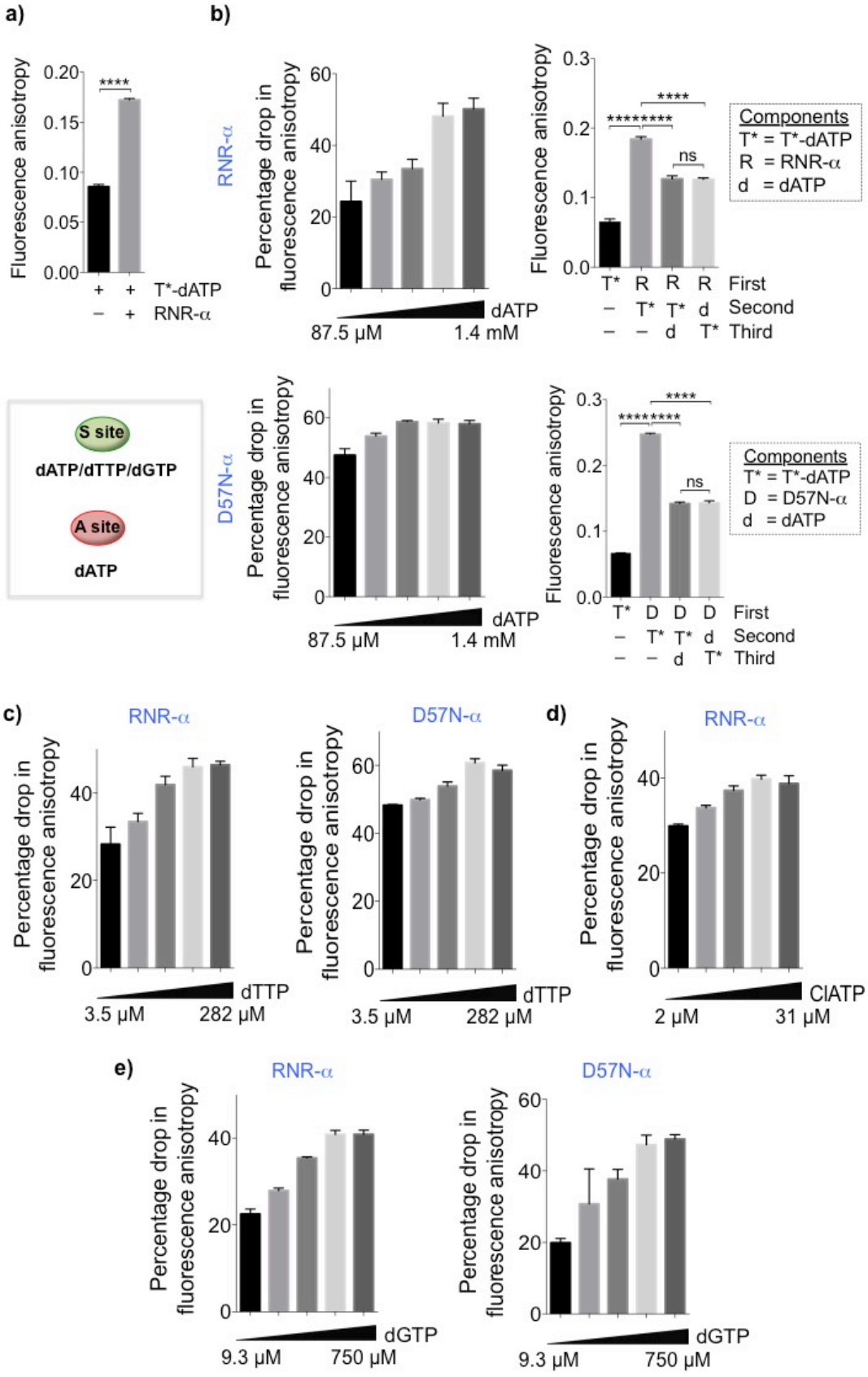

a)
b)
Fluorescence anisotropy
0.20
0.15
0.10
0.05
0.00
****
+ + T*-dATP
– + RNR-α
RNR-α
Percentage drop in fluorescence anisotropy
60
40
20
0
dATP
87.5 μM
1.4 mM
Fluorescence anisotropy
0.3
0.2
0.1
0.0
****
****
ns
Components
T* = T*-dATP
R = RNR-α
d = dATP
T* R R R First
– T* T* d Second
– – d T* Third
S site
dATP/dTTP/dGTP
A site
dATP
D57N-α
Percentage drop in fluorescence anisotropy
60
40
20
0
dATP
87.5 μM
1.4 mM
Fluorescence anisotropy
0.3
0.2
0.1
0.0
****
****
ns
Components
T* = T*-dATP
D = D57N-α
d = dATP
T* D D D First
– T* T* d Second
– – d T* Third
c)
RNR-α
Percentage drop in fluorescence anisotropy
40
20
0
dTTP
3.5 μM
282 μM
D57N-α
Percentage drop in fluorescence anisotropy
60
40
20
0
dTTP
3.5 μM
282 μM
d)
RNR-α
Percentage drop in fluorescence anisotropy
40
20
0
ClATP
2 μM
31 μM
e)
RNR-α
Percentage drop in fluorescence anisotropy
40
20
0
dGTP
9.3 μM
750 μM
D57N-α
Percentage drop in fluorescence anisotropy
60
40
20
0
dGTP
9.3 μM
750 μM

Figure S6. Fluorescence anisotropy (FA) studies with T*-dATP. The FA measurements were made using the conditions described elsewhere in the SI. Briefly, the final concentrations of T*-dATP and protein (RNR-α or D57N-α) used were kept consistent at 1.2 μM and 6 μM, respectively. **(a)** Representative increase in FA upon addition of the wt protein to T*-dATP. See also Figure 4a. (Note: this measurement needs to be performed each time the FA assays are set up since absolute values of FA fluctuate between different experiments whereas relative drop is consistent for a given protein and ligand(s). **(b) FA of T*-dATP and RNR-α** [wt (top) or D57N (bottom)] **in the presence of increasing concentrations of dATP.** The lowest concentration of dATP used was 87.5 μM and the concentrations were doubled up to 1.4 mM as the highest concentration. *The existence of non-specific binding of T*-dATP to RNR-α wt (top) or mutant (bottom) was confirmed by comparing FA of the system in which the protein was sequentially incubated with T*-dATP and saturating (700 μM) dATP in a different order of addition*. In both cases, FA failed to return to the value achieved in "dye-alone" samples. Also see discussion in the main text. *However, because these FA experiments measure specific release of the fluorophore from specific sites through ligand competition, any non-specific binding does not bias our data interpretation and conclusion*. **Inset** shows the binding site specificities of the three natural nucleotide ligands employed in these FA studies. **(c) FA of T*-dATP and RNR-α (left panel) or D57N-α (right panel) in the presence of increasing concentrations of dTTP**. 3.5 μM dTTP was used as the lowest concentration and the concentrations were tripled up to 282 μM. **(d) FA measurements in the presence of ClATP, T*-dATP and saturating dTTP that blocks S-site [see inset in (b)].** T*-dATP was added to RNR-α in which the S-site was pre-blocked with 94 μM dTTP. ClATP was then added to the mixture and FA was measured. The concentrations of ClATP was doubled from 2 to 31 μM. **(e) FA of T*-dATP and RNR-α (left) or D57N-α (right) in the presence of increasing concentrations of dGTP**. The titration started with 9.3 μM dGTP and the concentrations were tripled up to 750 μM. Error bars designate SD (n=3).

Figure S7.

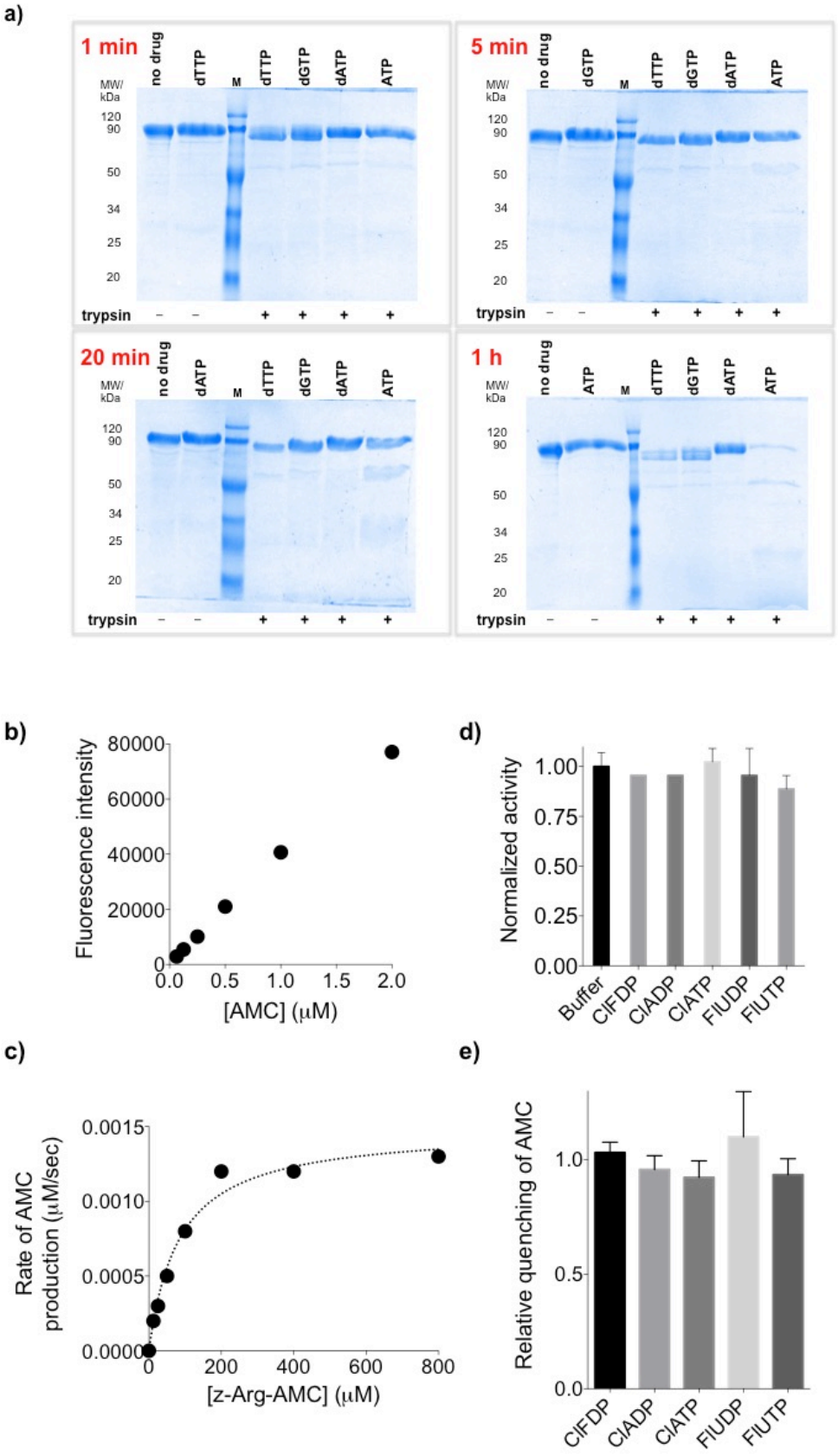

a)
1 min
5 min
20 min
1 h
no drug
dTTP
dGTP
dATP
ATP
M
MW/ kDa
120
90
50
34
25
20
trypsin
b)
Fluorescence intensity
[AMC] (μM)
c)
Rate of AMC production (μM/sec)
[z-Arg-AMC] (μM)
d)
Normalized activity
Buffer
ClFDP
ClADP
ClATP
FlUDP
FlUTP
e)
Relative quenching of AMC

Figure S7. RNR-α in the presence of natural nucleotide ligands was resistant to trypsin and trypsin activity is unaffected by the non-natural nucleotide inhibitors. (**a**) In the presence of natural nucleotide ligands, RNR-α showed no appreciable sensitivity to trypsin protease—a result markedly different from the data in Figure 5 with non-natural *persistent* hexamer-inducing nucleotide inhibitors. Interestingly, treatment with dATP, under the conditions known to induce hexamers, also does not result in protease sensitivity. This observation is taken as a further support for intrinsically different hexameric states induced by dATP versus the persistent hexamers induced by many of the non-natural nucleotide inhibitors.[11] (**b**) Concentration-dependent fluorescence intensity change (standard curve) for AMC (product of the trypsin catalyzed hydrolysis of z-Arg-AMC). (**c**) Determination of K_m of trypsin for substrate z-Arg-AMC by using product AMC formation rate. $K_m \sim 84 \pm 15$ μM. (**d**) Relative protease activity of trypsin (10 nM) in hydrolyzing z-Arg-AMC substrate (110 μM) in the presence of the indicated inhibitors 3, 14, 5, 94, and 68 μM, respectively, in the order shown. The absolute intensities measured over 10 min were normalized over that measured for a control experiment in which buffer replaced the inhibitor. (**e**) Under identical conditions, the nucleotide inhibitors showed no significant perturbation of fluorescence intensity of AMC. Error bars designate standard error from independent duplicates.

Figure S8.

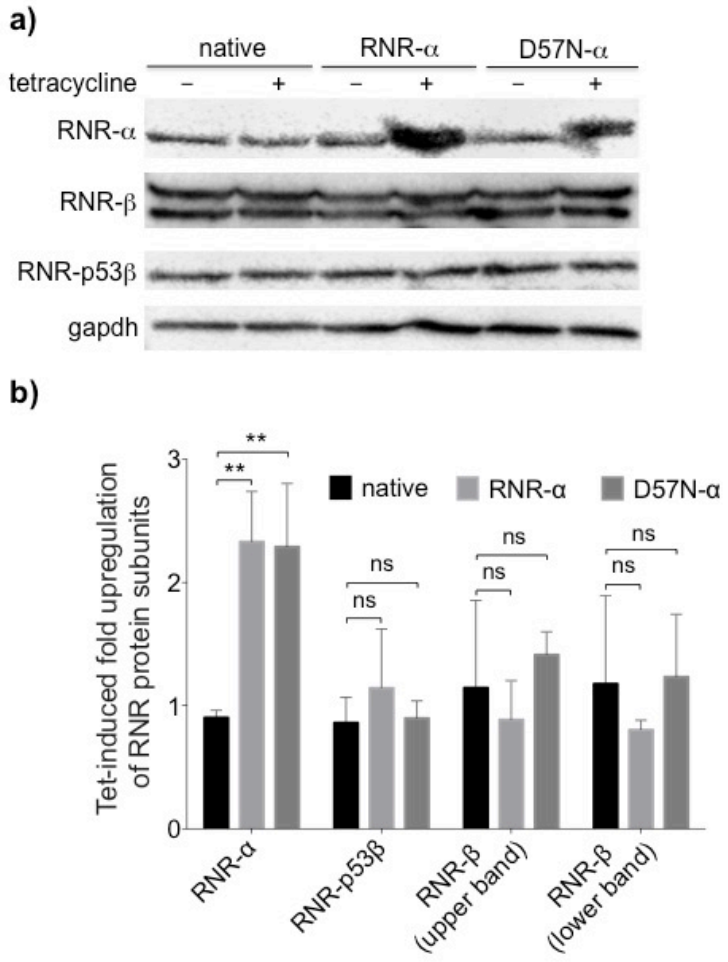
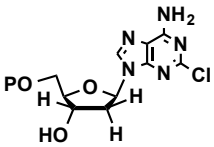


Figure S8. Validation of tetracycline-induced RNR-α or D57N-α overexpression in Flp-In T-REx HEK293 cell lines. Protein fold upregulation was analyzed using indicated antibodies to RNR-α subunit and the two isoforms of RNR-β subunit. GAPDH was used as a loading control. Dilutions and sources of the antibodies are described elsewhere in the supporting information. (**a**) A representative western blot. (**b**) Quantification of fold upregulation normalized over GAPDH. Mammalian RNR-β is known to be labile for cleavage at the N-terminal PEST domain.[13]

Appendix: ^{1}H and ^{31}P NMR spectra

ClAMP

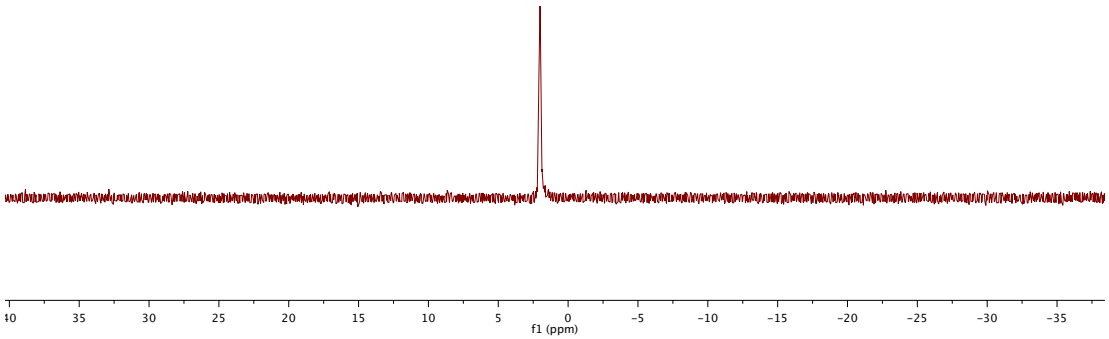


20131209-4
STANDARD PROTON PARAMETERS

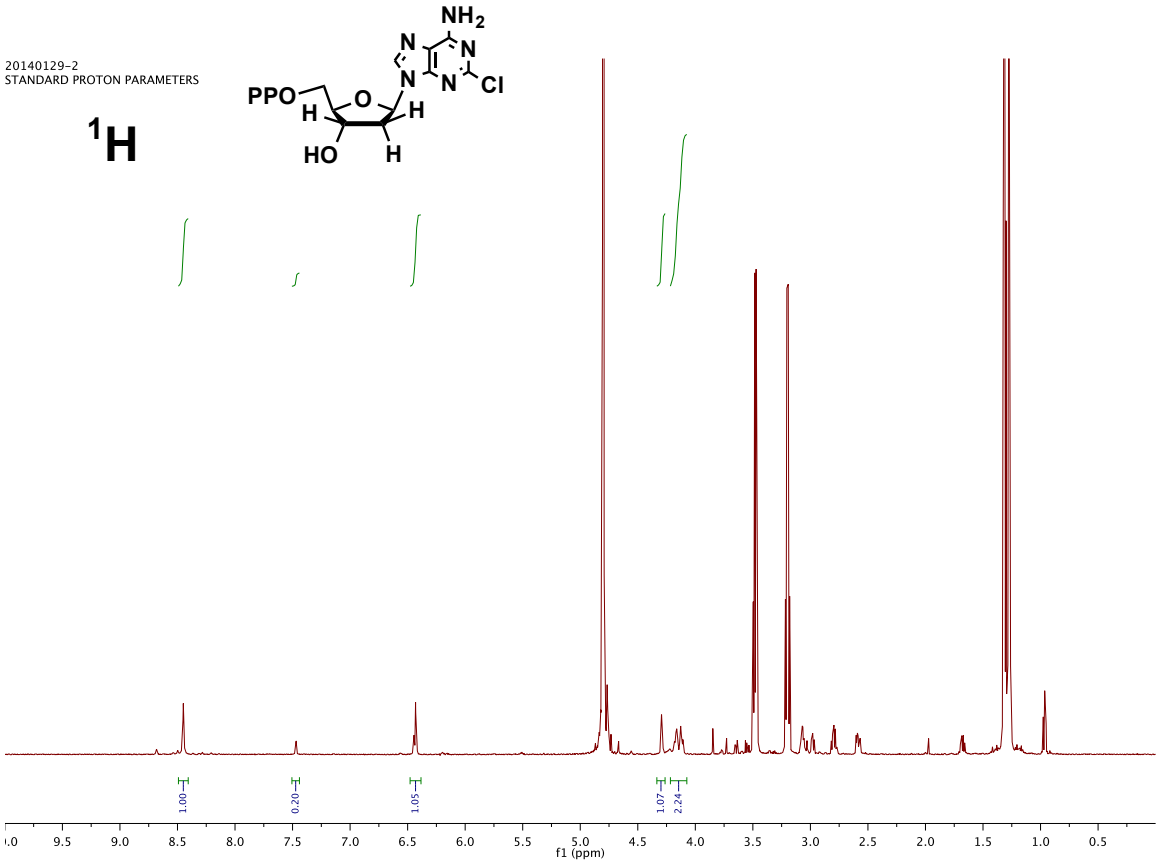
^{1}H

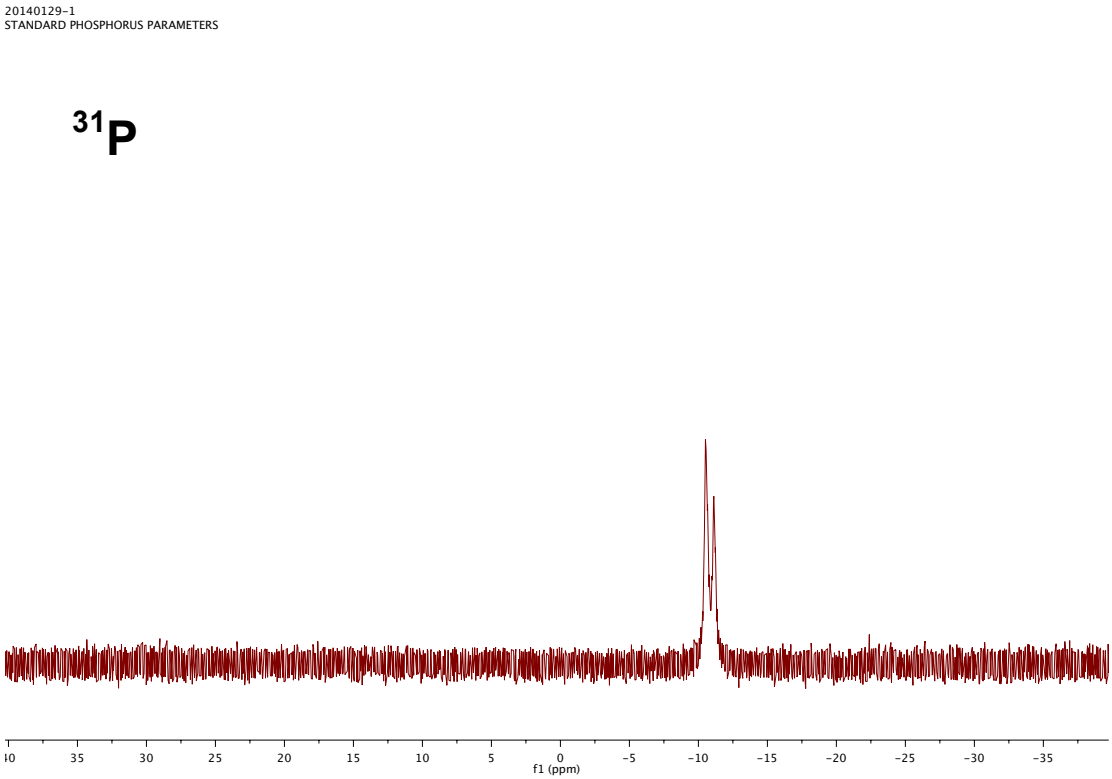
20131209-2
STANDARD PHOSPHORUS PARAMETERS

^{31}P

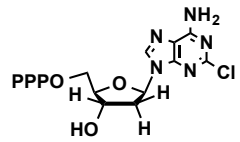


ClADP


20140129-2
STANDARD PROTON PARAMETERS
1H
NH2
N
N
N
N
Cl
PPO
H
O
H
HO
H
1.00
0.20
1.05
1.07
2.24
f1 (ppm)


20140129-1
STANDARD PHOSPHORUS PARAMETERS
31P
f1 (ppm)

ClATP

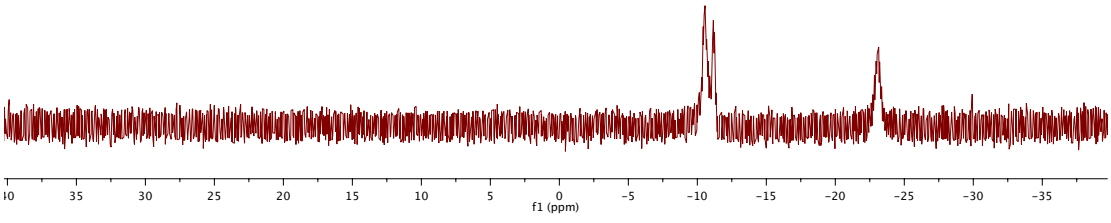


20140121-2
STANDARD PROTON PARAMETERS

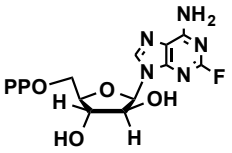
^{1}H

20140120-1
STANDARD CARBON PARAMETERS

^{31}P

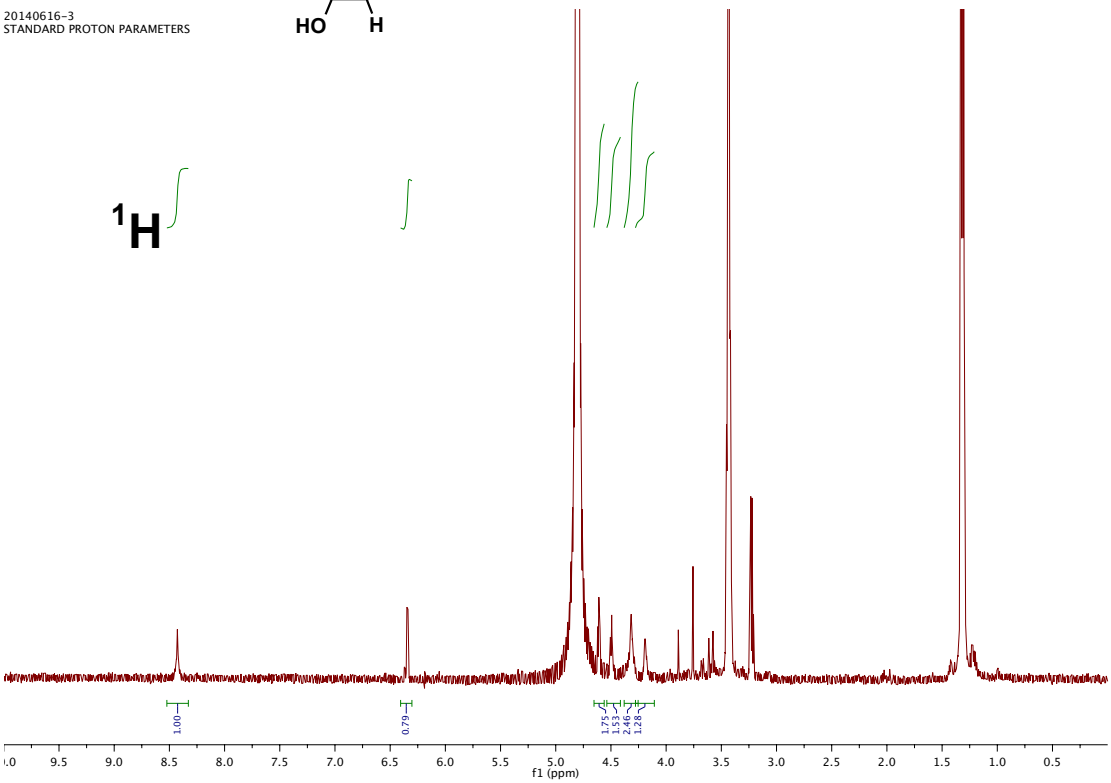


FlUDP



20140616-3
STANDARD PROTON PARAMETERS

^{1}H



20140902-2
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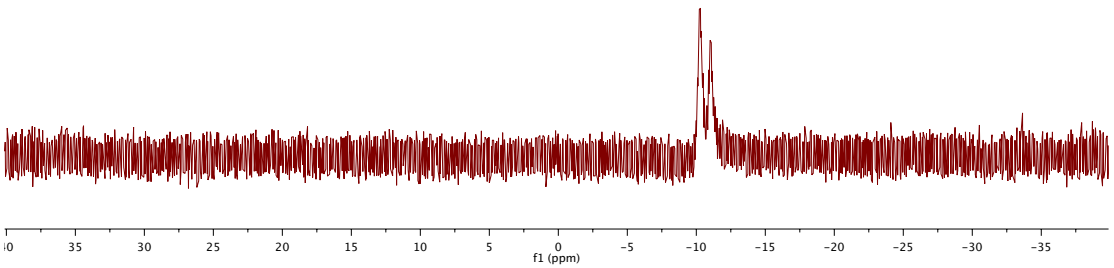
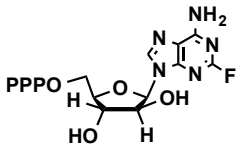
^{31}P

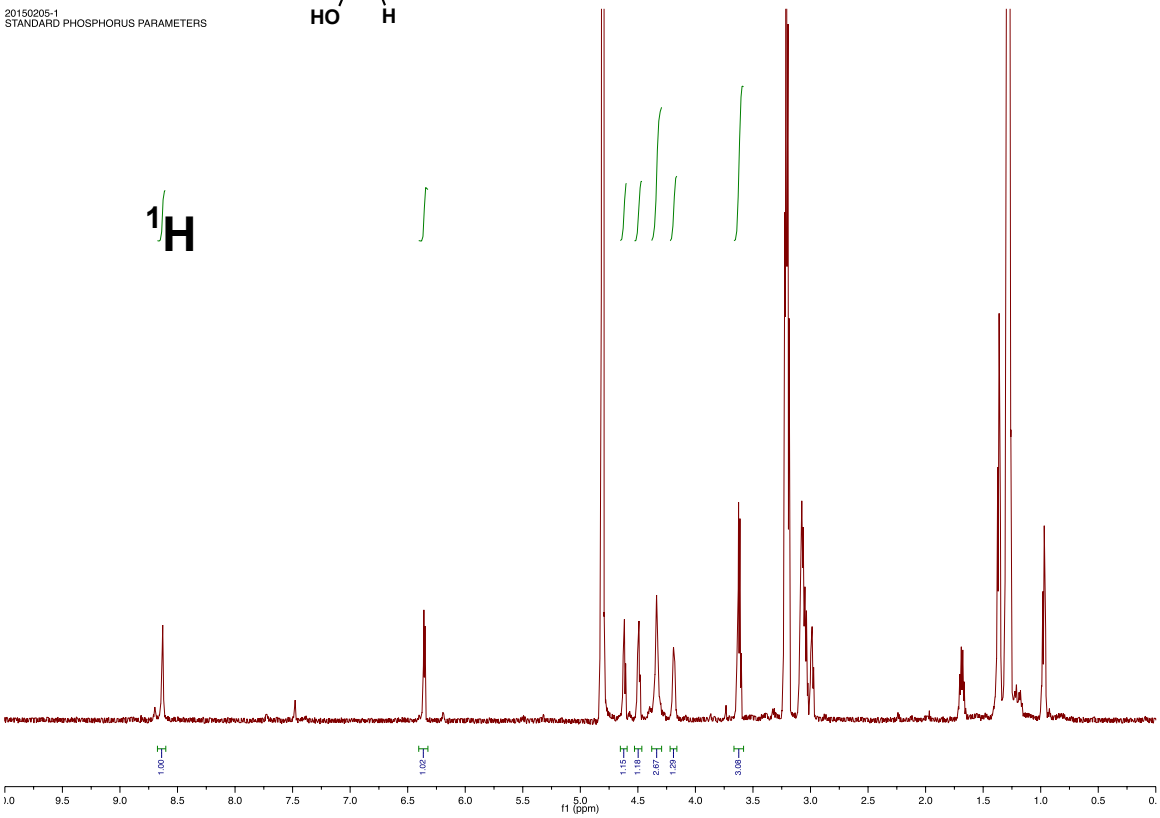


FlUTP



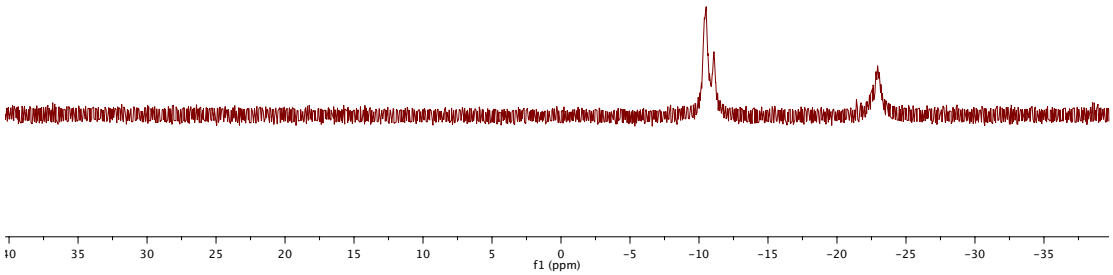
20150205-1
STANDARD PHOSPHORUS PARAMETERS

^{1}H



20140425-4
STANDARD PHOSPHORUS PARAMETERS

^{31}P



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