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Inactivation of *Lactobacillus leichmannii* ribonucleotide reductase by F₂CTP: covalent modification[§]

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Abstract

Ribonucleotide reductase (RNR) from *Lactobacillus leichmannii*, a 76 kDa monomer using adenosylcobalamin (AdoCbl) as a cofactor, catalyzes the conversion of nucleoside triphosphates to deoxynucleotides and is rapidly (<30 sec) inactivated by one equivalent (eq.) of 2',2'-difluoro-2'-deoxycytidine 5'-triphosphate (F₂CTP). [1'-³H] and [5-³H]-F₂CTP were synthesized and used independently to inactivate RNR. Sephadex G-50 chromatography of the inactivation mixture revealed that 0.47 eq. of a sugar were covalently bound to RNR and that 0.71 eq. of cytosine were released. Alternatively, analysis of the inactivated RNR by SDS PAGE without boiling, resulted in 33% of RNR migrating as a 110 kDa protein. Inactivation of RNR with a mixture of [1'-³H]-F₂CTP/[1'-²H]-F₂CTP followed by reduction with NaBH₄, alkylation with iodoacetamide, trypsin digestion, and HPLC separation of the resulting peptides, allowed isolation and identification by MALDI-TOF mass spectrometry (MS) of a [³H/²H]-peptide containing C₇₃₁ and C₇₃₆ from the C-terminus of RNR accounting for 10% of the labeled protein. The MS analysis also revealed that the two cysteines were cross-linked to a furanone-species derived from the sugar of F₂CTP. Incubation of [1'-³H]-F₂CTP with C119S-RNR resulted in 0.3 eq. of sugar covalently bound to the protein and incubation with NaBH₄ subsequent to inactivation resulted in trapping of 2'-fluoro-2'-deoxycytidine. These studies and the ones in the accompanying manuscript allow proposal of a mechanism of inactivation of RNR by F₂CTP involving multiple reaction pathways. The proposed mechanisms share many common features with F₂CDP inactivation of the class I RNRs.

The nucleoside 2-deoxy-2',2'-difluorocytidine (F₂C, Gemzar[™]) is a clinically used anti-cancer drug, which targets ribonucleotide reductase (RNR) and DNA polymerase in addition to several other proteins involved in nucleotide metabolism.(1–3) Studies have shown that F₂C is therapeutically effective against a range of cancers(4–17) with tolerable levels of toxicity. The mechanism by which F₂C inactivates RNRs has been the subject of a number of investigations (18–21), modeled on our detailed understanding of the mechanism by which 2'-substituted 2'-deoxynucleotides inhibit these enzymes (Scheme 1).(22) These studies revealed that gemcitabine 5'-diphosphate (F₂CDP) in the case of the class I RNRs (*E. coli* and humans are both diphosphate reductases, RDPR) and gemcitabine-5-triphosphate (F₂CTP) in the case of the class II RNR from *Lactobacillus leichmannii* (a nucleoside triphosphate reductase, RTPR) are potent, mechanism-based inhibitors.(20,21,23–25)

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SUPPORTING INFORMATION AVAILABLE Outline of F₂C synthesis (Scheme S1). Depiction of PSD peptide fragments (Scheme S2). MS/MS analysis of RTPR peptide of 2020 Da (Figure S1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

Since the discovery of Thelander and Eckstein that 2'-chloro-2'-deoxynucleotides and 2'-azido-2'-deoxynucleotides are inhibitors of RNRs, a large number of studies have led to the general mechanism of inhibition shown in Scheme 1 ($X = \text{Cl}^-, \text{F}^-, \text{N}_3^-$). In this mechanism, a protein thiyl radical initiates the reduction process by 3'-hydrogen atom abstraction from the nucleotide, which is followed by rapid loss of the 2' substituent. The glutamate residue in the RNR active site likely facilitates this process by deprotonation of the 3' hydroxyl, and a cysteine facilitates C-X bond cleavage by protonation, depending on X ($\text{OH}, \text{Cl}^-, \text{F}^-, \text{N}_3^-$). The 3'-ketone with an adjacent 2'-radical (**3**, Scheme 1) is produced in all cases. This intermediate then undergoes different fates depending on the conformation on the nucleotide in the active site, the charge on the leaving group and the protonation state of the protein residues. In some cases, **3** is reduced from the top face (β face of the nucleotide) and in others, from the bottom face (α face). The 3'-ketodeoxynucleotide **4** is generated in both pathways. Recent computational studies have suggested that when X is anionic, the hydrogen bond network on the α face of the nucleotide is distinct from the hydrogen bonding when neutral water is released.(26) The binding affinity of **4** for the enzyme was calculated to be substantially reduced with X^- released relative to H_2O . Furthermore, when X^- was F^- , the calculations suggested that it was released prior to dissociation of **4**. When **4** dissociates from the active site, our previous studies showed that it decomposes in solution to generate free nucleic acid base, pyrophosphate (tripolyphosphate) and a reactive furanone (**5**). In the case of top face reduction (pathway A), alkylation is responsible for inactivation. In the case of β face reduction (pathway B), alkylation and destruction of the radical initiator is responsible for inactivation.

Studies with gemcitabine differ from other 2'-substituted mechanism based inhibitors in that there are two substituents at C-2'. In fact our initial studies on RNRs from *E. coli* and *L. leichmannii*, suggested that the mechanism differed from the paradigm in Scheme 1.(20,21) In the case of RTPR, its incubation with one equivalent (eq.) of F_2CTP resulted in 90% loss of the catalytic activity within 30 seconds. Analysis of products after 30 min revealed that the inactivation was accompanied by release of both fluorides, formation of 0.84 eq. of 5'-deoxyadenosine from the adenosylcobalamin (AdoCbl) cofactor and ultimately covalent attachment of cobalamin to C₄₁₉, one of the cysteines located on the α face of the nucleotide involved in supplying the reducing equivalents for the reduction. In addition, kinetic studies by stopped flow (SF) spectroscopy revealed that cob(II)alamin was generated with a k_{obs} of 50 s^{-1} when one equivalent of F_2CTP was reacted with RTPR, suggesting that the rate constant would be similar to cob(II)alamin formation with CTP (250 s^{-1}), if the nucleotide was present at saturating levels. Rapid freeze quench (RFQ) EPR experiments showed initial formation of the same exchange coupled thiyl radical-cob(II)alamin species (22 ms) observed with CTP, followed by stoichiometric conversion to a new radical-cob(II)alamin species over 255 ms, suggesting thiyl radical mediated nucleotide radical formation. Efforts to further study the mechanism of this inactivation were hampered by the unavailability of the isotopically labeled gemcitabine analogs.

We now report synthesis of [$1'^2\text{H}$], [$1'^3\text{H}$] and [$5'^3\text{H}$]-labeled gemcitabine nucleotides by a combination of chemical and enzymatic methods. Using these probes, we now report, in this paper and the accompanying paper, on the mechanism by which F_2CTP inhibits the class II. *L. leichmannii* RTPR.(27) We demonstrate that 0.47 eq. of sugar from F_2CTP covalently label RTPR and that in contrast to our previous studies, cytosine (~ 0.7 eq) is released to solution. Use of a mixture of [$1'^2\text{H}$, ^3H] F_2CTP for the inactivation of RTPR was critical for identification by mass spectrometric methods, the peptides modified by a sugar moiety derived from F_2CTP . In the accompanying paper [$1'^2\text{H}$]- F_2CTP has been used to establish that the new radical generated by the thiyl radical-cob(II)alamin species is nucleotide derived.(27) We also describe the fate of the adenosylcobalamin and the structure of an unusual nucleotide trapped with NaBH_4 which we believe is derived from the observed nucleotide radical

intermediate. These studies together have allowed us to formulate mechanisms for RTPR inactivation, that while complex, do follow the paradigms shown in Scheme 1.

MATERIALS AND METHODS

2-Deoxy-3,5-di-*O*-benzoyl-2,2-difluororibonolactone was a gift of Eli Lilly & Co. All reagents were purchased from Sigma or Mallinckrodt and used without further purification unless otherwise indicated. Analytical TLC was performed on E. Merck silica gel 60 F254 plate (≤ 0.25 mm). Compounds were visualized by cerium sulfate-ammonium molybdate stain and heating or by exposure to UV light. Human deoxycytidine kinase (dCK) expression plasmids were a gift of Dr. Staffan Eriksson,(28) and the protein (specific activity (SA) of 150 nmol $\text{mg}^{-1}\text{min}^{-1}$) was expressed, purified and stored in 20 mM Tris pH 7.9, 0.5 M NaCl, 10 mM DTT, 20% glycerol. UMP/CMP kinase expression plasmids were a kind gift of Dr. Anna Karlsson.(29) The protein was expressed and purified (SA of 2.1–4.8 $\mu\text{mol mg}^{-1}\text{min}^{-1}$) as described previously(30) and stored in 50 mM Tris pH 8.0 and 10 mM reduced glutathione. Its concentration was determined by Bradford assay with a BSA standard. Inorganic pyrophosphatase (baker's yeast), SA 868 units/mg, was obtained from Sigma as a lyophilized powder and taken up in 165 mM Tris pH 7.2 immediately prior to use. Pyruvate kinase (rabbit muscle), SA 450 U/mg, was obtained from Sigma as a lyophilized powder and prepared as a 1200 units/mL stock in 50 mM Tris pH 7.5, 80 mM KCl, 20 mM MgCl_2 . Calf intestine alkaline phosphatase was purchased from Roche as a 20 units/ μL stock in 100 mM Tris pH 8.5. *E. coli* thioredoxin (TR, SA of 40 units/mg) and *E. coli* thioredoxin reductase (TRR, SA of 1320 units/mg) were isolated as previously described.(31,32) RTPR (SA of 0.38–0.42 $\mu\text{mol mg}^{-1}\text{min}^{-1}$) was isolated as previously described procedure,(33) omitting the second anion exchange column. AdoCbl was handled with minimal exposure to light. All reactions including AdoCbl were kept wrapped in foil at all times. Transfer of AdoCbl containing solutions were performed under dim or red light conditions. Extinction coefficients used were: dCK ($\lambda_{280} = 56755$ at pH 7.0); F_2C , ($\lambda_{268} = 9360$ at pH 7.0);(34) cytidine ($\lambda_{271} = 9100$ at pH 7.0), cytosine ($\lambda_{267} = 6100$ at pH 7.0), adenosine ($\lambda_{259} = 15400$ at pH 7.0).(35) UV-vis spectra were taken on a Cary-3 spectrometer. Anion exchange column fractions were assayed using an Ultramark Bio-RAD fixed wavelength plate reader. Scintillation counting was performed using Emulsifier-Safe liquid scintillation counting cocktail (Perkin Elmer) on a Beckman LS6500 multipurpose scintillation counter.

Synthesis of [$1'$ - ^3H] 2-Deoxy-3,5-di-*O*-benzoyl-2,2-difluoro-1-*O*-methanesulfonyl-D-ribofuranoside (8, Scheme S1) (36, 37) and [$1'$ - ^3H] F_2C

F_2C was synthesized from 2-deoxy-3,5-di-*O*-benzoyl-2,2-difluororibonolactone (6) or the unblocked lactone by modifications of published procedures and is summarized in Scheme S1. (34) Only the step to incorporate tritium label at C-1' is described in detail. All solid reagents were dried over P_2O_5 under vacuum overnight. All solvents and liquid reagents were distilled from CaH immediately before use, except mesyl chloride, which was distilled from P_2O_5 . NaB^3H_4 (500 mCi NaB^3H_4 , SA 14.22 Ci/mM, Perkin Elmer, combined with 152 mg, 4 mmol NaBH_4) and were dissolved in diglyme (4 mL) under N_2 to make a 1.0 M solution. A three-necked flask fitted with cold finger, gas inlet, and dropping funnel was assembled and connected to a 10 mL conical flask through an exit from the cold finger using tygon tubing and a needle. This apparatus allowed B_2H_6 generated in the 3 necked flask to be transferred to the 10 mL flask. The entire apparatus was flame dried and cooled under N_2 . The cold finger attached to the three-necked flask was filled with dry ice/acetone. The NaB^3H_4 solution was added to the dropping funnel and boron trifluoride diethyl etherate (1.25 mL, 10 mmol, 2.5 eq.) was added to the three-necked flask in an oil bath at room temperature. The conical flask was immersed in a dry ice/acetone bath and tetrahydrofuran (THF, 2 mL) added. The needle was immersed in the THF and the N_2 flow turned to a minimum. The borohydride solution

was added dropwise over 1 h. The evolution of gas was immediately evident and a white precipitate (NaBF_4)(38) formed over the course of the reaction. The oil bath was heated to 70°C for 30 min to distill off any remaining B_2H_6 . The apparatus was disassembled, leaving the conical flask under N_2 in the dry ice/acetone bath. The concentration of $\text{BH}_3\text{:THF}$ was established by titration.(36, 37) The solution of $\text{BH}_3\text{:THF}$ was transferred to a ice/salt bath (-15°C) and 2-methyl-2-butene (1.03 mL, 9.6 mmol) was added slowly, dropwise via syringe over 1 h. The reaction mixture was stirred an additional 1 h at -15°C . The solution of disiamyl borane was transferred to an ice bath (0°C) and a solution of **6** (188 mg, 0.5 mmol) in 1 mL THF was added dropwise via syringe over 30 min. The reaction was stirred 1 h at 0°C followed by 16 h at room temperature. Water (1 mL) was then added slowly. Foaming occurred as H_2 gas evolved. The flask was equipped with a reflux condenser and the mixture was refluxed 30 min, then cooled to 0°C and the reflux condenser removed. Hydrogen peroxide (1 mL, 30% solution) was added dropwise over 5 min, along with enough 2N NaOH to keep the pH at 7.5–8.0 (monitored by microelectrode, ~200 mL over the course of the quench). The mixture was poured into EtOAc (100 mL) and washed with water (2×50 mL). The organic layer was dried over MgSO_4 , filtered, and the solvent removed *in vacuo*.

The residue **7** (Scheme S1) was dried over P_2O_5 and dissolved in CH_2Cl_2 (5 mL). The reaction was cooled to 0°C and triethyl amine (TEA, 0.5 mL, distilled from KOH) and mesyl chloride (0.39 mL, 5 mmol) were added. The ice bath was removed and the reaction stirred 3 h at room temperature. The reaction was diluted to 100 mL with CH_2Cl_2 and the organic layer washed with 1N HCl, water, saturated NaHCO_3 , and water. The organic layer was dried over MgSO_4 , filtered, and the solvent removed *in vacuo*. Purification using silica gel chromatography (1 cm $\times$ 15 cm column, 4:1 hexanes:ethyl acetate elutant) yielded **8** (143 mg, 0.313 mmol, 63%) as a clear oil. The ^1H -NMR spectrum was consistent with previously reported data.(34)

Bis(trimethylsilyl)cytosine (**9**) was made from cytosine (39) and coupled to **8** using trimethylsilyl trifluoromethanesulfonate as previously described.(34) Compound **10** as a 6:4 α : β mixture was obtained and quantified by UV absorbance ($\epsilon_{268} = 9360$ at pH 7.0) to give 190.5 mmol, 76% from **8**). ^1H -NMR was consistent with that reported for **10** (34) and it was judged to be >95% pure.

2'-Deoxy-2',2'-difluorocytidine-5'-monophosphate (F_2CMP).(28,30)

F_2C was converted to its monophosphate directly from the α : β mixture. The reaction contained in a final volume of 1 mL: 5 mM β - F_2C , 10 mM ATP, 2 mM DTT, 0.5 mg/mL BSA, 1.4 mg/mL human dCK, 50 mM Tris pH 7.6, 100 mM KCl, and 10 mM MgCl_2 . The reaction was initiated by addition of dCK and incubated at 37°C for 45 min. The reaction mixture was loaded on a DEAE Sephadex A25 column (20 mL, 20 cm $\times$ 1 cm) equilibrated in 5 mM triethylammonium bicarbonate (TEAB) pH 6.8 and the column washed with 50 mL of 5 mM TEAB. The product was eluted using a 100 mL $\times$ 100 mL linear gradient from 5 mM $\rightarrow$ 400 mM TEAB. Fractions (5 mL) were assayed for $A_{260\text{ nm}}$ and the nucleotide containing fractions were combined and the solvent was removed *in vacuo*. ^1H -NMR analysis of the flow-through from the initial wash showed only the presence of the α anomer of **10**, (34). F_2CMP eluted at 250 mM TEAB (4.45 mmol, 90%). ^1H -NMR (500 MHz, D_2O), selected peaks, δ 7.83 (d, $J = 7.5$ Hz, 1H), 6.15 (t, $J = 6.4$ 1H), 6.05 (d, $J = 7.5$ Hz, 1H), 4.36 (dd, $J = 12.4, 21.1$ Hz, 1H), 4.02–4.09 (m, 2H), 3.91–3.96 (m, 1H). ^{31}P -NMR (121.5 MHz, D_2O , phosphoric acid external reference) δ 4.6.

2'-Deoxy-2',2'-difluorocytidine-5'-diphosphate (F_2CDP).(29,30,40)

The reaction mixture contained in a final volume of 5 mL: 2 mM F_2CMP , 8 mM ATP, 2 mM DTT, 50 mM Tris pH 8, and 25 mM MgCl_2 , 66.5 mg/mL UMP-CMP kinase. The reaction was

incubated 30 min at 37°C, diluted to 50 mL with cold water and loaded onto a DEAE-Sephadex A-25 column (30 mL, 18 cm × 1.5 cm). The column was washed with water (100 mL) and the product eluted with a 400 mL × 400 mL linear gradient from 0 → 600 mM TEAB. The fractions (7.5 mL) were assayed by A₂₆₀ nm, with F₂CDP eluting along with ADP at 450 mM TEAB. The fractions were combined and the solvent removed *in vacuo*. The product was co-evaporated with 50% ethanol in water (5×20 mL) to remove excess TEAB. To remove contaminating adenosine nucleotides, this material was dissolved in water (24 mL) and NaIO₄ (0.5 mmol, 1 mL of a 0.5 M solution) was added. The reaction was incubated 10 min at 37°C, then methylamine (2.5 mmol, 640 mL of 3.9 M solution in water, pH adjusted to 7.5 with phosphoric acid) added and the reaction incubated an additional 20 min. The reaction was quenched by addition of rhamnose (1 mmol, 1 mL of a 1 M solution). To this mixture was added 6.6 mL of 8 mM MgCl₂, 165 mM Tris pH 7.2 and yeast inorganic pyrophosphatase (165 mL of a 50 units/mL stock in 165 mM Tris pH 7.2) to give final concentrations of 2 mM MgCl₂, 55 mM Tris pH 7.2 and 0.25 units/mL inorganic pyrophosphatase. This mixture was incubated 45 min at 37°C, diluted to 300 mL with cold water, and loaded onto a DEAE Sephadex A25 column (30 mL, 18 cm × 1.5 cm). The column was washed with water (100 mL) and the product eluted with a 400 mL × 400 mL linear gradient from 0 → 600 mM TEAB. The fractions (7.5 mL) were assayed by A_{260nm} and the F₂CDP containing fractions eluting at 450 mM TEAB were combined and the solvent was removed *in vacuo*. The product was redissolved in water (1 mL) giving 5.6 mmol, 56% yield. ¹H-NMR (500 MHz, D₂O) δ 7.71 (d, *J* = 7.7 Hz, 1H), 6.09 (t, *J* = 7.2 Hz, 1H), 5.96 (d, *J* = 7.7 Hz, 1H), 4.40 (dd, *J* = 13.3, 22.2 Hz, 1H), 4.05–4.21 (m, 2H), 3.99–4.03 (m, 1H). ³¹P-NMR (121.5 MHz, D₂O) δ (phosphoric acid external reference) –9.5 (d, *J* = 22.1 Hz), –10.6 (d, *J* = 22.1 Hz). The ³¹P NMR showed no contamination by inorganic pyrophosphate.

2'-Deoxy-2',2'-difluorocytidine -5'-triphosphate (F₂CTP)

The reaction (20 mL) contained 2 mM F₂CDP, 4 mM phosphoenolpyruvate (PEP), 50 mM Tris pH 7.5, 80 mM KCl, 20 mM MgCl₂, and 120 units/mL pyruvate kinase. The reaction was incubated at 37°C for 1 h. The reaction was then diluted with cold water (50 mL) and loaded on a DEAE-Sephadex A-25 column (60 mL, 20 cm × 2 cm), the column was washed with water (100 mL), and the product eluted with a 400 mL × 400 mL linear gradient from 0→750 mM TEAB. The fractions (10 mL) were assayed for A_{260nm}. The triphosphate containing fractions eluting at 550 mM TEAB were combined and the solvent removed *in vacuo*. The product was dissolved in 5 mL ddH₂O to give 35 mmol, 88% yield. ¹H-NMR (500 MHz, D₂O), selected peaks, δ 7.75 (d, *J* = 7.7 Hz, 1H), 6.09 (t, *J* = 7.2 Hz, 1H), 6.02 (d, *J* = 7.3 Hz, 1H), 4.33–4.40 (m, 1H), 4.21–4.26 (m, 1H), 4.10–4.14 (m, 1H), 4.03–4.06 (m, 1H). ³¹P-NMR (121.5 MHz, D₂O) δ (phosphoric acid external reference) –9.3, –10.5, –21.4.

[1'-²H]-F₂C

The synthesis of **8** was repeated with the substitution of NaB²H₄ (98% [²H], Sigma) for NaBH₄. The yield of [1'-²H]-F₂C, 274 mmol, was 64%. [2H] incorporation was 93% by ¹H-NMR and ESI-MS. The corresponding mono, di and triphosphates were prepared enzymatically in 86%, 60% and 76% yield, respectively.

[1'-³H]-F₂CTP

Enzymatic phosphorylations to the mono, di, and tri phosphates were carried out on as described above to give: 85 %, SA 8700 cpm/nmol, 60%, SA 8620 cpm/nmol, and 88% SA 8620 cpm/nmol, respectively.

[5-³H]-F₂CTP

[5-³H]-F₂CDP was synthesized from [5-³H]-F₂C provided by Eli Lilly.(30) [5-³H]-F₂CDP (2.2 μmol) was converted to the triphosphate by the PEP/pyruvate kinase procedure described above. Yield 1.85 mmol, 85%, SA 10060 cpm/nmol.

RTPR assay (spectrophotometric.)

The assay mixture contained in a final volume of 500 μL: RTPR (typically 0.25, 0.5 or 1 μM), CTP (1 mM), dATP (100 μM), NADPH (0.2 mM), TR (20 μM), TRR (0.2 μM), HEPES (25 mM, pH 7.5), EDTA (4 mM), and MgCl₂ (1 mM). The reaction was initiated with AdoCbl (20 μM) at 37°C and monitored by the change in A_{340nm} (ε = 6220 M⁻¹cm⁻¹). The SA of RTPR was typically 0.28–0.44 mmol mg⁻¹ min⁻¹.

RTPR assay monitoring [¹⁴C-] dCTP

The assay mixture contained in a final volume of 300 μL: RTPR (1 μM), [¹⁴C-CTP] (SA of 1500–2500 cpm/nmol, 1 mM), dATP (100 μM), NADPH (1 mM), TR (20 μM), TRR (0.2 μM), HEPES (25 mM, pH 7.5), EDTA (4 mM), and MgCl₂ (1 mM). The reaction was initiated with AdoCbl (20 μM) and incubated at 37°C; a control aliquot was removed before addition of AdoCbl. Four aliquots (50 μL) were removed at time points over 5 min, and the reaction quenched by addition of 2% perchloric acid (25 μL). The aliquots were neutralized with 0.4 M KOH (25 μL) after all time points were collected. Cytosine (50 nmol) and dC (50 nmol) were added to each aliquot, then 20 units alkaline phosphatase in 500 mM Tris pH 8.6, 1 mM EDTA (26.5 μL) was added and the aliquots were incubated 2h at 37°C. The dCDP was quantitated by the method of Steeper and Steuart.(41)

Time dependent inactivation of RTPR with F₂CTP.(19,21)

The inactivation mixture contained in final volume of 100 μL: pre-reduced RTPR (5 μM), dATP (100 μM), NADPH (1 mM), TR (20 μM), TRR (0.2 μM), AdoCbl (20 μM), HEPES (25 mM, pH 7.5), EDTA (4 mM), and MgCl₂ (1 mM). To study inactivation in the absence of reductant, NADPH, TR, and TRR were omitted. The inactivation was initiated by addition of F₂CTP (or isotopically labeled derivative, 5 μM) and the reaction carried out at 37°C. Assay for activity was carried out by measuring [¹⁴C] dCTP production.

Size Exclusion Chromatography (SEC) on RTPR inactivated with [1'-³H]- or [5-³H]-F₂CTP

Inactivation mixtures were prepared as described above in a final volume of 500 μL. Experiments were run both in the presence and absence of reductants. Inactivations were initiated by addition of F₂CTP ([1'-³H], SA 7200 cpm/nmol or [5-³H], SA 10800 cpm/nmol) and incubated for 2 min at 37°C. An aliquot was assayed for activity using the spectrophotometric assay before initiation and at the inactivation end point. After 2 min, an aliquot (200 μL) was loaded on Sephadex G-50 columns (1 cm × 20 cm, 20 mL), equilibrated with and eluting with 25 mM HEPES pH 7.5, 1 mM MgCl₂, 4 mM EDTA and 1 mL fractions were collected. A second aliquot (210 μL) was mixed with 630 μL 8 M guanidine, then loaded on a Sephadex G-50 column (1 cm × 20 cm, 20 mL) equilibrated in and eluted with 2M guanidine in assay buffer and 800 μL fractions were collected. In all cases, fractions were assayed for A_{260nm} and A_{280nm} and for radioactivity by scintillation counting (500 μL aliquots). Recovery of radioactivity was typically >95%. Recovery of protein determined from A_{280nm} was typically >90%.

Elimination of cytosine deaminase activity from pre-reduced RTPR

RTPR stock solution (~1 mM, 250 μL) was mixed with 5 mM *o*-phenanthroline (750 μL) and DTT was added to a final concentration of 30 mM. The solution was incubated 20 min at 37°

C, and RTPR was isolated by Sephadex G-25 chromatography and quantified by standard procedures. Alternatively, the cytosine deaminase was removed by SEC on a Sephacryl S-300 column.

Spectrophotometric Detection of cytosine deaminase activity in the RTPR preparation

The conversion of cytosine ($\lambda_{\text{max}} = 269 \text{ nm}$) to uracil ($\lambda_{\text{max}} = 258 \text{ nm}$) was monitored spectrophotometrically. The reaction mixture contained in 500 μL : 50 μM cytosine, 5 μM RTPR, 20 μM AdoCbl, 5 mM DTT, 25 mM HEPES pH 7.5, 1 mM MgCl_2 , and 4 mM EDTA. Three different preparations of RTPR were assayed: as isolated, purified by S-300 SEC, or pre-treated with 3 mM *o*-phenanthroline. The UV spectra were collected at $t = 0, 30 \text{ min}, 1 \text{ h}, 6 \text{ h},$ and 1 day.

Analysis for cytosine generated during the inactivation of RTPR with F_2CTP

The reaction mixture in a final volume of 220 μL (or 1020 μL), contained pre-reduced RTPR (50 μM), dATP (500 μM), AdoCbl (50 μM), HEPES (25 mM, pH 7.5), EDTA (4 mM), and MgCl_2 (1 mM). $[5\text{-}^3\text{H}]\text{-F}_2\text{CTP}$ (50 μM , 7200 cpm/nmol). $[5\text{-}^3\text{H}]\text{-F}_2\text{CTP}$ (50 μM , 10,800 cpm/nmol) was added to initiate the reaction which was then incubated 2 min at 37°C. Before initiation with F_2CTP and after the inactivation was complete, 10 μL aliquots were removed and assayed for RTPR activity by the spectrophotometric assay. At 2 min, the inactivation mixture was filtered through a YM-30 membrane (30,000 MW cutoff minicon), 10 min at $14,000 \times g$ at 4°C. F_2C (50 nmol) and cytosine (50 nmol) were added as carrier before filtration. Twenty units of alkaline phosphatase were added to the filtrate and the reaction was incubated for 3h at 37°C and filtered again using a YM-30 centricon.

The sample was then purified by HPLC using a Rainin SD-200 HPLC and an Altech Adsorbosphere Nucleotide Nucleoside C-18 column (250 mm $\times$ 4.6 mm) with elution at a flow rate of 1 mL/min. The solvent system was: Buffer A, 10 mM NH_4OAc , pH 6.8; Buffer B: 100% methanol. The flow program was 100% A for 10 min followed by a linear gradient to 40% B over 25 min, then to 100% B over 5 min. Under these conditions, standards eluted as follows: cytosine, 5.7 min; uracil, 7.9 min; C, 12.6 min; *ara*-C-17.4 min; dC, 19.0 min; F_2C , 23.2 min. Fractions (1 mL) were collected; for samples with radiolabeled F_2CTP , 200 μL of each fraction was analyzed by scintillation counting. The cytosine peak was isolated and recovery calculated based on carrier added and scintillation.

Stability of $[^3\text{H}]\text{-RTPR}$ generated by inactivation with $[1'\text{-}^3\text{H}]\text{-F}_2\text{CTP}$ to trypsin digestion conditions

The mixture in final volume of 300 μL contained: pre-reduced RTPR (15 μM), dATP (100 μM), AdoCbl (22.5 μM), HEPES (25 mM, pH 7.5), EDTA (4 mM), and MgCl_2 (1 mM). The reaction was initiated by addition of $[1'\text{-}^3\text{H}]\text{-F}_2\text{CTP}$ (SA 7200 cpm/nmol) to a final concentration of 15 μM . The reaction was incubated 2 min at 37°C. The mixture, 200 μL aliquot, was passed through Sephadex G-50 columns (1 cm $\times$ 20 cm, 20 mL) equilibrated and eluted in 0.1 M NH_4HCO_3 , pH 8, 2M urea (trypsin digestion buffer) and the protein containing fractions assayed for $A_{280\text{nm}}$ and radioactivity. The sample (1.5 mL) was loaded into a Slidalizer dialysis cassette (10,000 MW cutoff) and dialyzed against the same buffer in which the sample was eluted. Aliquots (250 μL) were removed at 1h, 2h, 4h, and 6h. The dialysis buffers were changed and the samples were dialyzed an additional 14h. A final aliquot was removed, and all aliquots were assayed for radioactivity by scintillation counting.

Trypsin Digestion of RTPR inactivated with $[1'\text{-}^3\text{H}]\text{-F}_2\text{CTP}$

The reaction in a final volume of 220 μL contained: pre-reduced RTPR (50 μM), dATP (500 μM), AdoCbl (75 μM), HEPES (25 mM, pH 7.5), EDTA (4 mM), and MgCl_2 (1 mM).

[1'-³H]-F₂CTP (50 μM, SA 7200 cpm/nmol) was added to initiate the reaction, and the reaction incubated for 2 min at 37°C. Before initiation with F₂CTP and after the inactivation was complete, 10 μL aliquots were removed and assayed for RTPR activity. At 2 min the inactivation mixture was quenched with 50 μL of 250 mM NaBH₄, 400 mM Tris pH 8.3 and incubated for an additional 5 min at 37°C. The NaBH₄ solution was prepared by combining solid NaBH₄ with the buffer solution immediately before use; vigorous foaming occurred upon addition to the inactivation mixture. To this mixture was added 750 μL 8M guanidine, 40 mM DTT, 5.33 mM EDTA, 400 mM Tris pH 8.3 and the reaction incubated 30 min at 37°C. Iodoacetamide was added to a final concentration of 250 mM (25000 eq. per RTPR) and the reaction incubated an additional 1 h at 37°C. The protein was then separated from small molecules on a Sephadex G-50 column (1cm × 20 cm, 20 mL) equilibrated into 0.1 M NH₄HCO₃ pH 8.2 (or 0.1 M NH₄HCO₃ pH 8.2, 2M urea). The protein containing fractions were combined (typically 2 mL total volume, 90% recovery of protein, >95% recovery of loaded radioactivity, 0.28 eq. of radioactivity coeluted with protein). Trypsin (Worthington) was added from a freshly prepared stock solution to a final concentration of 4:1 RTPR:trypsin w/w, and incubated 2 h at 37°C. The reaction was quenched by addition of 25 μL trifluoroacetic acid (TFA, to pH ~2), and the peptides were immediately separated using a Waters 2487 HPLC with a Phenomenex Jupiter C18 peptide column (150 × 4.6mm, 5 micron, 300 Å pore size) with a flow rate of 1 mL/min. The peptides were eluted with Buffer A, 0.1% TFA in ddH₂O and Buffer B, 0.1% TFA in acetonitrile by a linear gradient of 0–45% B over 90 min. Fractions were collected (1 mL) and aliquots (100 μL) of each fraction were assayed for radioactivity by scintillation counting. The recovery of injected radioactivity was typically 80%. Four regions of radioactivity were observed (Figure 1). Fractions were pooled based on the observed peaks of radioactivity, concentrated to < 1 mL on a lyophilizer, and repurified using the same column and flow rate with Buffer A, 10 mM NH₄OAc, pH 6.8 and buffer B, 100% acetonitrile using a gradient of 0–35% B over 90 min. Fractions (1 mL) were collected and aliquots of each fraction (250 μL) were assayed for radioactivity. The major peaks of radioactivity were pooled and submitted for mass spectrometry analysis without concentration. Reactions were also carried out as described above substituting NaB²H₄ for NaBH₄ or F₂CTP that was a mixture of [1'-²H] and [1'-³H], SA 7200 cpm/nmol, to give a stock with 60% [1'-²H] with SA 2600 cpm/nmol.

Peptide mass spectrometry.(42)

MALDI-TOF and tandem MS/MS mass spectrometry were performed by Dr. John Leszyk at UMass Medical School on a Kratos Axima CFR (Shimadzu Instruments) matrix-assisted-laser desorption/ionization (MALDI) mass spectrometer. Samples (0.5 mL, containing 50–100 fmol of F₂CTP labeled peptide, determined by specific activity) were applied to the target and mixed with 0.5 μL of matrix which was 2,5-dihydroxybenzoic acid at 15mg/mL in (CH₃CN:0.1%TFA 50:50). Samples were allowed to air dry prior to insertion into the mass spectrometer. Peptides were analyzed in positive ion mode in mid mass range (100–3000 Da) with an accuracy to within 100 ppm. The instrument was externally calibrated with bradykinin (757.40), P14R (MS mass standard, Sigma-Aldrich, 1533.86 Da), and adrenocorticotrophic hormone fragment 18–39 (Sigma-Aldrich, 2465.20 Da). Post-source decay (PSD) analysis were performed on the same instrument using a timed ion gate for precursor selection with a laser power about 20% higher than for MS acquisition. PSD fragments were separated in a curved field reflectron that allowed for a seamless full mass range acquisition of the spectrum, with an accuracy of within 1000 ppm. All spectra were processed with Mascot Distiller (Matrix Sciences, Ltd.) prior to database searching.(43) Database searches were performed with Mascot (Matrix Sciences, Ltd.). For MS searches the Peptide Mass Fingerprint program was used with a peptide mass tolerance of 150 ppm. For MS/MS searching, the MS/MS Ion Search program was used with a Precursor tolerance of 150 ppm and a fragment tolerance of 1 Da.

SDS PAGE of RTPR inactivations with F₂CTP

Inactivations were performed as described above. Each sample in final volume of 300 μ L contained: pre-reduced RTPR (25 μ M), dATP (500 μ M), AdoCbl (37.5 μ M), HEPES (25 mM, pH 7.5), EDTA (4 mM), and MgCl₂ (1 mM). The reaction was initiated by addition of F₂CTP to a final concentration of 25 μ M. Reactions containing reductant used 5 mM DTT. Aliquots were quenched by mixing with an equal volume of 2x Laemmli buffer. An aliquot was removed immediately after F₂CTP addition (0 min) and two aliquots were removed at 5 min: one was not boiled and the other was boiled for 3 min. Samples were analyzed on a 10% SDS-PAGE gel and band intensities measured. The band intensities were quantified using Bio-Rad Quantity One software.

SEC on C₁₁₉S RTPR inactivated with [1'-³H]-F₂CTP

SEC experiments were performed with C₁₁₉S-RTPR *-analyzed as described above for wt RTPR in the absence of reductants.

Characterization of major nucleoside product(s) isolated from a NaBH₄ quenched C₁₁₉S RTPR/F₂CTP inactivation mixture

The reaction mixture contained in a final volume of 200 μ L: C₁₁₉S RTPR (50 μ M), dATP (250 μ M), AdoCbl (75 μ M), F₂CTP (50 μ M), HEPES (25 mM, pH 7.5), EDTA (4 mM), and MgCl₂ (1 mM). The inactivation mixture was quenched at 2 min with 50 μ L of 250 mM NaBH₄ in 500 mM Tris pH 8.5 in a 4.0 mL falcon tube and incubated 5 min at 37°C. The NaBH₄ solution was prepared by using solid NaBH₄ as described above. The solution was then filtered through a YM-30 membrane for 15 min at 14,000 $\times$ g at 4°C, and the flow through was treated with 200 units alkaline phosphatase for 3 h at 37°C, followed by filtration through a second YM-30 membrane. The sample was acidified by addition of glacial acetic acid (5% of the final volume) and the resulting mixture was lyophilized to dryness to hydrolyze borate esters. The residue was then taken up in 1 mL 10 mM NH₄OAc and purified on an Altech Adsorbosphere Nucleotide Nucleoside C-18 column (250 mm $\times$ 4.6 mm) using a 2 mL injection loop with elution at a flow rate of 1 mL/min. The solvent system for elution and analysis for nucleosides was described above for cytosine identification. Fractions were collected (1 mL) and aliquots (100 μ L) of each fraction were assayed for radioactivity by scintillation counting. The major peak of radioactivity (43% of total counts) was found to co elute with a standard of 2'-fluoro-2'-deoxycytidine. The presence of this compound was confirmed by ESI-MS: (C₉H₁₂FN₃O₄) *m/z* (M + Na⁺) calcd 268.0710, obsd 268.0718; (M + H⁺) calcd 246.0890, obsd 246.0901.

RESULTS

Synthesis of isotopically labeled F₂CTP

The synthesis of [1'-³H] F₂C (10) was carried out using modifications of the previously published procedure and is summarized in Scheme S1. (34) The reduction of the difluorinated lactone **6** used disiamylborane made from NaB³H₄ and 2 methyl-2-butene. The presence of the two fluorines required an increased ratio of disiamylborane to **6** relative to ribonolactone to obtain high yields.(36) The reduced **7** was then converted directly to the mesylate **8** with mesyl chloride and TEA without purification in 85% yield from the lactone **6**. The mesylate was coupled to *bis*-trimethylsilylcytosine **9** prepared by refluxing cytosine in 10:1 HMDS:TMSCl. Efforts to make **9** from cytosine in HMDS using ammonium sulfate as a catalyst were unsuccessful on the small scale used to make [1'-³H] F₂C. **9** added to **8** in xylenes and refluxed in the presence of trimethylsilyl trifluoromethanesulfonate resulted in production of protected nucleoside as a 6:4 α : β mixture that was directly deprotected with sodium methoxide to give α : β **10** in 74% yield from **8**. The published protocols separate the F₂C

anomers by crystallization at either the benzoate protected or fully deprotected stage. This approach is incompatible with the scale used for radiolabeled syntheses. After investigating a number of options, we determined that it is most convenient to resolve the α : β mixture enzymatically during the conversion of F_2C to F_2CMP .

Phosphorylation of F_2C

F_2C is very difficult to phosphorylate chemically using activated phosphates due the enhanced acidity of its 3'-OH which results in rapid formation of the 3',5' cyclic phosphate. Previous studies had reported the ability to generate F_2CMP using human dCK and to generate F_2CDP using F_2CMP and UMP/CMP kinase. (6,42,43,47–49). Treatment of α : β - F_2C with human dCK and ATP allowed selective conversion of the β anomer to the 5'-monophosphate (F_2CMP) in 85% yield. The desired product readily separated from the remaining a nucleoside by anion exchange chromatography.

To make the F_2CTP , we first made the F_2CDP , which also has also been used to investigate the class I RNRRs which use NDP substrates. F_2CDP was generated enzymatically from F_2CMP and ATP with human UMP/CMP kinase. However, this procedure yields an equilibrium mixture of starting material and products: F_2CDP and ADP. Since these products co-elute on anion exchange chromatography, a periodate cleavage step was introduced to destroy ADP. (30,40) $NaIO_4$ (aq) selectively reacts with the *syn*-diol of the ADP to cleave the 2'C-3'C bond generating a dialdehyde. The F_2CDP is unreactive. After removal of excess periodate, $MeNH_2$ (pH 7.5) is added to catalyze elimination of pyrophosphate from the dialdehyde. Addition of inorganic pyrophosphatase converts the inorganic pyrophosphate to inorganic phosphate. Anion exchange chromatography of this reaction mixture allows recovery of homogenous F_2CDP in 56% yield.

In the development of the UMP-CMP kinase procedure for production of F_2CDP , experiments were initially carried out in the presence of pyruvate kinase and PEP to recycle the ADP to ATP and avoid the problem of its separation from F_2CDP .(30) However, HPLC analysis indicated that the F_2CDP was phosphorylated under the conditions investigated. This observation suggested that pyruvate kinase could be used to make F_2CTP . Two eq. of PEP and 120 units/mL pyruvate kinase resulted in 88% conversion to F_2CTP in 1 h. The enzymatic phosphorylation methods have been used to prepare all of the isotopically labeled gemcitabine nucleotides ($[5\text{-}^3H]\text{-}F_2CD(T)P$, and $[1'\text{-}^2H]\text{-}F_2CD(T)P$ in 10–100 μmol quantities.

Covalent labeling of RTPR by $[1'\text{-}^3H]\text{-}$ and $[5\text{-}^3H]\text{-}F_2CTP$

Our previous studies suggested that one eq. of F_2CTP was sufficient for 90% inactivation of RTPR within 30 s in the presence and absence of reductants (TR/TRR/NADPH), and 95% by 2 min (21) At that time we suggested that inactivation might in part result from covalent labeling of RTPR and in part by covalent labeling of C_{419} by a cobalamin species.(21) The availability of $[1'\text{-}^3H]\text{-}$ or $[5\text{-}^3H]\text{-}F_2CTP$ has now allowed us to test these proposals by tracking the fate of the ribose ring in the case of $[1'\text{-}^3H]\text{-}F_2CTP$ and the cytosine base in the case of $[5\text{-}^3H]\text{-}F_2CTP$. RTPR was incubated with these compounds for two min, and the small molecules separated from the protein by SEC in the presence or absence of denaturant. The results are summarized in Table 1. With $[1'\text{-}^3H]\text{-}F_2CTP$, 0.47 ± 0.02 eq. co-elute with RTPR in both cases. With $[5\text{-}^3H]\text{-}F_2CTP$, only 0.16 eq. co-elute with RTPR in the presence and absence of reductants, with a slight reduction in recovered label under denaturing conditions. The substoichiometric labeling, the greater than 90% inactivation and the observation that C_{419} can be modified with the corrin of AdoCbl, suggest a branching mechanism for inactivation of RTPR.(21)

Release of cytosine accompanies inactivation

It was surprising that there was significantly less cytosine bound to RTPR than ribose (Table 1), as our earlier studies failed to detect any cytosine subsequent to the inactivation.(21) Given our results with [5-³H] F₂CTP and the observation that cytosine is released when the class I RDPRs from *E. coli* or human are inactivated with F₂CDP,(20,23–25) our efforts focused on determining if cytosine was being converted to uracil. The inactivation was carried out with [5-³H]- F₂CTP and the small molecules analyzed by RP-HPLC subsequent to dephosphorylation of the nucleotides with alkaline phosphatase. The retention time of the new [³H]-labeled material was identical to a uracil standard. Isolation of this material and examination by UV and NMR spectroscopy confirmed its identity. These results suggested that RTPR preparations were contaminated with *E. coli* cytosine deaminase.(44) Several different types of experiments confirmed this to be the case and allowed its removal (see Methods). Inactivation studies with [5-³H]-F₂CTP were then repeated and the small molecules analyzed by HPLC subsequent to dephosphorylation. Starting with a 1:1 ratio of [5-³H]-F₂CTP:RTPR, 0.71 eq. of cytosine and 0.19 eq. of unreacted F₂C were recovered. In addition, 0.15 eq. of cytosine co-eluted with RTPR, accounting for all of the radiolabel.

Identification of peptide(s) of RTPR labeled during its inactivation with F₂CTP and insight into the structure of the sugar moiety(ies) responsible for labeling

As summarized in Table 1, 0.47 [³H]-labels from [1'-³H] F₂CTP were found covalently attached to RTPR and are likely associated with loss of 47% of the RTPR activity. Subsequent to inactivation of RTPR by [1'-³H]-F₂CTP, the protein was denatured in 8M guanidine and the cysteines alkylated with iodoacetamide. The alkylated RTPR was exchanged into the trypsin digest buffer (0.1 M NH₄HCO₃, pH 8.2) by Sephadex G-50 chromatography and the protein containing fractions were pooled and analyzed for protein and radioactivity. While 80% of the protein was recovered, only 0.14 eq. of radioactivity co-eluted with the protein indicating substantial loss of the label during the denaturation and iodoacetamide treatment. Furthermore, when the [³H]-labeled RTPR was digested with trypsin and the peptides were separated by reverse-phase HPLC, 10% of the radioactivity eluted in the void volume of the column and the remaining radioactivity eluted in a very broad peak between 45 min and 65 min (data not shown). These results suggested that inactivation results in multiple sites of labeling or more likely that the label is redistributed to multiple residues during the workup.

Our previous experience with identifying the site of label attachment to RTPR inactivated by the mechanism based inhibitor, [2'-³H]-2'-chloro-2'-deoxynucleotide (Scheme 2), suggested that the loss of label might be minimized by reduction with NaBH₄. Thus, subsequent to inactivation, the reaction mixture was treated with 50 mM NaBH₄. The labeled-RTPR recovered (Table 1) was reduced from 0.45 to 0.33. Under the conditions of alkylation, trypsin digestion, and HPLC purification of the resulting peptides, 0.22 eq. of radiolabel were recovered. The results of a typical chromatography are shown in Figure 1 where four main regions of radioactivity are identified. The average total recovery of radiolabel from column chromatography from 6 experiments was 74 ± 5 % with 20 ± 2% in Region I, 26 ± 4% in Region II, 20 ± 5% in Region III and 21 ± 4 % in Region IV.

Fractions from each region of the chromatogram were pooled and rechromatographed at pH 6.4 in potassium phosphate buffer. The results from Region I are shown in Figure 1B. Regions II, III and IV were also rechromatographed (data not shown). All label was lost from region IV.(45)

Analysis of labeled peptide isolated from region I by MALDI-TOF mass spectrometry

MALDI analysis was performed on the peptides isolated from regions I (Figure 2A, B and C and Figure 3 A and B), II and III (data not shown). To facilitate identification of peptides

containing a sugar fragment derived from F₂CTP, two additional experiments were carried out. In one set of experiments [1'-²H and ³H]-F₂CTP with ~60% ²H at the 1' position and a specific activity of 2600 cpm/nmol was used for the inactivation and in the second set of experiments NaB²H₄ replaced NaBH₄. Inactivation reactions involving these changes will result in the same, labeled peptides, but with additional mass and predictable mass distribution. When using partially [²H]-labeled F₂CTP, any peptides incorporating the sugar ring of F₂CTP show an isotopic distribution shifted such that the +1 Da peak increases in intensity (Figures 2B, 3A II and 3B II), allowing confirmation of the source of the label. Alternatively, the experiments quenched with NaB²H₄ will show a shift of x Da over the peptides quenched with NaBH₄ (Figures 2C, 3A III and 3B III), where x indicates the number of hydrogens in the label originating from the borohydride quench.

The MALDI spectra of the peptides isolated from Region I under the three sets of conditions are shown in Figure 2 A–C and Figure 3A and B. Two peptides, one with a mass of 2004 Da and one with 2063 Da show shifts in isotopic distribution from the non-deuterated material (Compare Figure 3A I with 3A II and III, and Figure 3B I with 3B II and III). No peptides from Regions II and III (Figure 1) showing the shift in isotopic distribution were detected in the MALDI analysis, despite these regions representing a substantial percentage of the radioactivity recovered from the trypsin digests. We propose the lack of identifiable labeled peptides is due to a combination of a number of potential cross-linked species with low incidence of each specific sequence (see discussion of mechanism below) and that some of these cross-linked peptides might be too large to be seen in the high resolution MALDI experiments.

Our previous studies with RTPR inactivated by [2'-³H]-2'-chloro-2'-deoxy-UTP followed by NaBH₄ trapping, identified alkylation of cysteines within the C-terminus of RTPR.(46) The C-terminal tail contains a pair of cysteines (C731 and C736) essential to the function of the enzyme, re-reducing the active site disulfide formed after every nucleotide reduction.(47–50) The expected mass of this C-terminal peptide with both cysteines (C731 and C736 within peptide 722–739: D₇₂₂LELVDTDCEGGACPIK₇₃₉) labeled with acetamide is 2019.90, and should be present in our digests if this region is alkylated. This peptide is detected at 2020.04 Da (Figure 2 inset) and serves as an internal control for the identification of C-terminal peptides labeled by F₂CTP derivatives.

Since one or both of these cysteines could be the site of alkylation, the labeled peptide could be missing one or both acetamides (loss of one or two 58 Da adducts gives rise to 2020 – 2 (58) = 1904 Da or 2020 – 58 = 1962 Da species). If *both* acetamides are missing, a sugar moiety from F₂CTP would need to have a mass of 100 Da (1904 + 100 = 2004) to give the observed mass of 2004 Da. Alternatively if the peptide at 2063 Da corresponds to the C-terminal peptide with *one* acetamide, then the group derived from F₂CTP would have a mass of 101 Da (1904 + 58 + 101 = 2063).

To learn more about the structure of the labeled species and the number of hydrogen atoms derived from NaBH₄, the inactivation mixture with [1'-³H]-F₂CTP and NaB²H₄ (98% D) was examined. Trapping with NaB²H₄ can occur by 1,2 reduction of a ketone or the 1,4 reduction of an unsaturated ketone (Scheme 2). In either case the observed mass of the adduct would result in the addition of 1 Da. In addition, the ketone subsequent to 1,4 reduction, could then itself be reduced, resulting in the incorporation of a second deuterium (+2 Da). The analysis of peptides isolated subsequent to this treatment are shown in Figure 2C and Figure 3AIII and 3BIII.

In the expansion on the 2004 Da peak (Figure 3A), the mass of the peptide shifts from 2004 Da to 2005 Da after NaB[²H]₄ trapping (Figure 3A I and III). In the expansion on the 2063 Da

peak (Figure 3B), the mass is shifted by 2 Da to 2065 Da (Figure 3B I and 3B III). These results establish that one hydrogen in the species with mass 2004 Da and two hydrogens in the species with a mass of 2063 Da come from NaBH_4 .

Analysis by MS/MS

To gain more information about the structure of the modification(s), the sequence of the peptide and the residues alkylated, MS/MS analysis was performed. Initial focus was on the peptide of mass 2020 Da (Figure 2A inset) where the mass itself is consistent with two cysteines modified by acetamide. This assignment was confirmed by post-source decay (PSD) fragmentation (Figure 1S). In this method, additional energy is applied to the peptide of interest, initiating predictable fragmentation patterns. The major site of fragmentation is at peptide bonds and gives rise to two series of ions: the y series in which the charge is retained on the N-terminus, and the b series with the charge retained on the C-terminus (Scheme S2). (51, 52) Each mass peak within a given series is thus associated with a truncated peptide, and the difference in mass between each peak within a given series is the mass of one amino acid in the sequence. The relative abundance of each ion series detected is highly peptide dependent and, in the case of 2020 Da peptide (Figure S1), the y series dominates the spectrum. A number of additional peaks can be seen that are -18 and -17 Da from the main y series. These are the y^0 and y^* series, representing loss of water or ammonia from the y series ions. The peaks from the major "y" series are marked (vertical line), and the difference in mass indicates the amino acid lost, allowing sequencing of the peptide. Most remaining peaks visible in the spectrum are b series peptides. This spectrum allows N-terminal sequencing by viewing the y series. Fourteen y ions are observable, showing mass differences consistent with the C-terminal RTPR peptide, with both cysteines modified by acetamide.

The MS/MS of the 2063 Da peptide shows the same series of ions as in the doubly acetamide labeled C-terminal peptide (Figure S1), but shifted by $+43$ Da (Figure 4). The same series is visible in the 2065 Da peptide from the NaB^2H_4 quenched reaction, but at $+45$ Da (data not shown). Once the first cysteine is removed, both the normal series seen in the MS/MS for the acetamide modified peptide (peaks at 831, 702, 645, 588 and 517 Da, Figure 4) and a series which remains at $+43$ Da ($+45$ for the NaB^2H_4 quenched reaction) can be seen. After the cleavage of the second cysteine, only the normal mass peak remains (a fragment at 357 Da). The label derived from the sugar fragment of F_2CTP is thus attached to either C731 or C736. This method is not quantitative, and nothing can be said about the relative extent of labeling at each site. As predicted by the MALDI, the sugar fragment has a mass of 101 Da (43 Da larger than acetamide) and incorporates two deuteriums when the inactivation is quenched with NaB^2H_4 .

The MS/MS of the major labeled peak at 2004 Da is more complex than that at 2063 Da (Figure 5). The expected y series for the C-terminal peptide is still evident, -16 Da relative to the diacetamide modified C-terminal peptide (Figure 1S). Each of these peaks shows several closely related series, fragments that are -17 or -18 Da relative to each y ion (the y^* series that has lost ammonia, and the y^0 series that has lost water) and often show a fragment at -35 Da, representing the loss of both water and ammonia. It is unclear why the intensities of these secondary fragments are enhanced relative to the corresponding series in the 2020 Da and 2063 Da (Figure 4) peptides. Additionally, after the loss of the aspartate before C731 (giving the peak at 975), the peaks visible until the loss of the second cysteine, do not correlate with any simple mass series. This result indicates some irregular fragmentation of the peptides in this mass range. The nature of this fragmentation is not readily apparent. The final tripeptide fragment after cleavage of C736 (PIK) is the same in this spectrum as in those for the 2020 and 2063 species. The PSD for the 2005 Da peak in the NaB^2H_4 spectra is similar, but shifted $+1$ Da

relative to the 2004 Da spectra and the primary y series is of even lower abundance relative to the y^0 and y^* series (data not shown).

This data is consistent with modification at C₇₃₁ and C₇₃₆. If both acetamides are missing, a fragment of mass 100 derived from F₂CTP would account for the observed mass ($2020 - 2 \times 58 + 100 = 2004$). Our interpretation of this data is that this modification is a 100 Da fragment that is covalently modifying both cysteines, providing an internal crosslink. Cleavages in the backbone in between the two cysteines would not actually fragment the peptide if they were connected through a side-chain crosslink and could account for the lack of regular fragments in this region.

Interpretation of MALDI and MS/MS data

The peptides associated with masses at 2004 and 2063 Da represent cysteine-modified C-terminal peptides of RTPR. Modification is evident at both C₇₃₁ and C₇₃₆. The mass at 2004 Da is consistent with the replacement of both acetamides with a fragment of 100 Da that is covalently linked to both cysteines. The peptide at 2063 is consistent with the same peptide, however it has one cysteine labeled with acetamide and one with a sugar fragment of 101 Da. The NaB²H₄ quenched sample shows that one hydrogen on the 100 Da fragment comes from NaBH₄ and that two on the 101 Da fragment come from NaBH₄.

Scheme 2 outlines a model consistent with these data. A furanone equivalent could react with the C-terminal cysteines through conjugate addition, either with one cysteine leaving a compound with an α,β unsaturated ketone, or with both cysteines giving rise to a saturated ketone. Reduction with NaBH₄ would provide the structure with the correct mass and number of hydrogens delivered from NaBH₄. Other alternative schemes that can be envisioned are inconsistent with the NaB²H₄ labeling experiment.

Change in Conformation of RTPR subsequent to labeling by F₂CTP determined by SDS PAGE

Recent studies with of class Ia RNRs inactivated by F₂CDP and analyzed by SDS PAGE subsequent to inactivation, revealed that ~30% of the α migrated as a protein of larger molecular weight.(23–25) Inactivation reaction mixtures of RTPR were therefore analyzed by SDS-PAGE to detect any changes in conformation related to the inactivation process (Figure 6). Samples with and without DTT were analyzed at time 0 and 5 min subsequent to the inactivation and with and without boiling of the 5 min sample. All inactivations quenched at 5 min showed the presence of a new major band at ~100 kDa representing 25% of the overall intensity, with a corresponding reduction in the intensity of the wt-RTPR band at 75 kDa. Assuming 75%–80% of the RTPR is active (47,53–55), this new feature represents ~33% of the RTPR. We suggest that it is an altered protein conformation that is generated regardless of the presence or absence of DTT in the assay buffer or whether or not the sample is boiled prior to sample loading. Samples at 5 min also showed a second new minor band (<5% intensity).

Inactivation of C₁₁₉S RTPR by [1'-³H]- F₂CTP

Studies of the class Ia RNRs suggested that an active site cysteine involved directly in nucleotide reduction (C₂₂₅ in *E. coli* RNR) is alkylated by the sugar moiety of F₂CDP.(24, 25) C₁₁₉S RTPR, a mutant of corresponding α face cysteine implicated in labeling in the class Ia RNR), was therefore inactivated by [1'-³H]- F₂CTP and analyzed by SEC. The results demonstrated 0.33 eq. of radiolabel co-eluting with the protein under denaturing conditions. This number contrasts with 0.47 eq. labeling for the wt-RTPR and suggests that this cysteine might in part represent a site of labeling, not identified in the peptide mapping analysis described above.

In a separate experiment, the small molecule fraction of the inactivation mixture with C₁₁₉S RTPR was examined, subsequent to quenching at 2 min with NaBH₄ to reduce any transient ketone containing species. The phosphates of the nucleotides were removed with alkaline phosphatase and the small molecules were analyzed by RP-HPLC. Three regions of radioactivity were identified: one broad region (15–19 min, 26% of radioactivity) and two sharper peaks (22.2 min, 43% of radioactivity; 23.2 min, 11% of radioactivity). The peak at 22.2 min co-eluted with 2'-deoxy-2'-fluorocytidine, while the peak at 23.3 min co-eluted with F₂C. The presence of a compound with a mass equal to 2'-fluorocytidine was confirmed by high resolution ESI MS for the 22.2 min peak. Trapping of this species has interesting mechanistic implications discussed below.

DISCUSSION

We previously reported SF kinetic experiments on the inactivation of RTPR with AdoCbl by F₂CTP. The results demonstrated that 0.7 eq./RTPR of cob(II)alamin was formed with a k_{obs} of 50 s⁻¹. RFQ EPR experiments further revealed that cob(II)alamin was exchange coupled to a thiyl radical (1.4 spins/RTPR) and that over the next 255 ms that the amount of total spin remained unchanged and a new radical that was weakly exchange coupled to cob(II)alamin was generated. We now propose that this initially formed radical is **13** (Scheme 3) and that it partitions into two major pathways (Scheme 3, A and B). Pathway A results in covalent modification of RTPR by the sugar moiety of F₂CTP and is the focus of this paper, while pathway B results in production of 0.25 to 0.3 of a stable cytidine nucleotide radical and is the focus of the accompanying paper.(27) These two pathways account for 0.75–0.8 eq. of F₂CTP consumed in the inactivation study, which is sufficient for complete RTPR inactivation, given that our previous pre-steady state experiments have indicated that only 75–80% of our recombinant protein is active.(47,53–55)

Similarities of the mechanism of inactivation of RTPR by F₂CTP and the Class Ia (E. coli and human with $\beta 2$ or p53 $\beta 2$) RNRs by F₂CDP

First, in all RNRs that we have examined, inhibition by a gemcitabine nucleotide requires only a single nucleotide/ α for complete inactivation.(24,25) In class I RNRs that have complex quaternary structures ($\alpha_n\beta_m$ where $n = 2, 4$ or 6 and $m = 2, 4$ or 6), complete inactivation by one F₂CDP/ α results from increased affinity of the $\alpha_n\beta_m$ subunits due to the inactivation in one $\alpha\beta$ pair. Second, despite the stoichiometry of the inhibition reaction, there are at least two pathways responsible for inactivation: one involving covalent modification and one involving destruction of the cofactor (pathways A and B in Scheme 3). Three, there are ~0.5 labels from the sugar moiety of [1'-³H] F₂CTP that become covalently attached to α . The attachment site (s) is (are) chemically labile making identification of these sites challenging. Four, in all cases the sugar modified α subunit of RNR migrates on SDS PAGE gel as a protein of larger molecular weight than the unmodified RNR. Five, all RNRs inactivated by F₂CD(T)Ps are accompanied by release of ~ one cytosine and loss of two fluoride ions. The data in sum, suggests that the mechanism of inactivation of RNRs by gemzar nucleotides do in fact follow the paradigm for other 2'-substituted nucleotide mechanism based inhibitors (Scheme 1). The loss of the second fluoride ion, however, makes the mechanism of F₂CD(T)P more complex than the generic mechanism for the 2'-monosubstituted deoxynucleotide mechanism based inhibitors (Scheme 1). However, we propose that the predominant mechanism of loss of the second fluoride is also likely to be similar in all RNRs (Scheme 3).

Proposed mechanisms of covalent modification of RTPR

The mechanism of covalent labeling of RTPR is complex and many possibilities have been considered. Our current model in Scheme 3 proposes that **13** is common in all pathways. Evidence in support of this intermediate comes from the trapping of 2'-deoxy-2'-fluorocytidine

subsequent to the inactivation of C₁₁₉S-RTPR with F₂CTP using NaBH₄. To account for the majority of labeled-RTPR, pathway A Scheme 3, C₁₁₉ or C₄₀₈ are proposed to directly attack C2' of **13**. This attack would prevent escape of the sugar moiety from the active site and allow the trapped sugar to reside in the active site long enough to lose tripolyphosphate. Subsequent alkylation could then occur at C1' or C5' (**16**, Scheme 3), providing an explanation for our inability to isolate well-behaved peptide adducts (Figure 1). In the class Ia RNR inactivation studies, mutagenesis studies suggest that the C₁₁₉ equivalent is involved in covalent labeling. (23–25) In the case of RTPR, at most 30% of the covalent modification could arise from this cysteine. Additional labeling could potentially come from C₄₀₈. In pathway B, instead of attack by C₁₁₉ or C₄₀₈, attack by an active site H₂O is proposed leading to **14**. The evidence that supports this proposal is described in the accompanying manuscript.

Our characterization of the radiolabeled C-terminal peptide of RNR, suggests that covalent modification is more complex than indicated in Scheme 3. A portion (10–15%) of the covalent labeling requires an additional pathway as the mass spectrometric data suggests that F₂CTP is converted to a furanone (Scheme 2) that alkylates one or both of the C-terminal cysteines of RTPR. It is difficult to rationalize how these adducts could be generated from **15**, Scheme 3. A proposal for the generation of the furanone is shown in Scheme 4. This mechanism starts with the common intermediate **13**. However, in this pathway **13** is reduced by a mechanism similar to that proposed for CTP reduction, involving intermediates **17** and **18**. This intermediate can now lose HF to generate **19**, which subsequent to electron transfer from C₄₀₈ would rapidly eliminate cytosine. Intermediate **20** can only be alkylated by the C-terminal cysteines as the active site cysteines involved in nucleotide reduction are in a disulfide. Once the first alkylation occurs, tripolyphosphate can eventually be released and the second alkylation can occur.

SUMMARY

The mechanism of F₂CTP inactivation of RTPR is complex even though it is stoichiometric. Perhaps most surprising is that the remarkable number of similarities in the mechanism of inactivation shared between the class II RNR described in this and the accompanying manuscript and the class I RNRs. Understanding the details of this simpler system may help to unravel class I RNRs, targets of this antitumor agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

RNR	ribonucleotide reductase
eq	equivalent
Gemzar TM , F ₂ C	2',2'-fluoro-2'-deoxycytidine
F ₂ CDP	2',2'-difluoro-2'-deoxycytidine 5'-diphosphate
F ₂ CTP	2',2'-difluoro-2'-deoxycytidine 5'-triphosphate
RTPR	ribonucleotide triphosphate reductase

AdoCbl	adenosylcobalamin
SF	stopped flow
RFQ	rapid freeze quench
SA	specific activity
dCK	deoxycytidine kinase
TR	thioredoxin
TRR	thioredoxin reductase
TEAB	triethyl ammonium bicarbonate
PEP	phosphoenolpyruvate
SEC	size exclusion chromatography
PSD	Post-source decay
HMDS	hexamethyldisilazide
α	RNR large subunit
β	RNR small subunit

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FIGURE 1.

HPLC purification of peptides from RTPR inactivated with $[1\text{'-}^3\text{H}]\text{-F}_2\text{CTP}$, treated with NaBH_4 at 2 min, alkylated with iodoacetamide and digested with trypsin. $A_{214\text{nm}}$ (—), cpm (---). (A) Initial purification, linear gradient (·····) 0.1% TFA, 0–45% CH_3CN over 90 min. Region I, 50–53 min, 18.1% of radioactivity. Region II, 54–59 min, 19.7% of radioactivity. Region III, 60–63 min, 13.9% of radioactivity. Region IV, 63–67 min, 26% of radioactivity. (B) repurification of region I, linear gradient (·····) 10 mM NH_4OAc , pH 6.8, 0–35% CH_3CN over 90 min. 80% recovery of radiolabel.

FIGURE 2.

Full MALDI-TOF analysis of the peptides isolated in Region I from the trypsin digest of RTPR inactivated with F₂CTP and treated with NaBH₄. (A) [1'-³H]-F₂CTP, NaBH₄. (B) [1'-²H,³H]-F₂CTP, NaBH₄. (C) [1'-³H]-F₂CTP, NaBD₄. The features at 1102.5 Da and 1347.7 Da are not from RTPR, as MS/MS fragmentation analysis showed no matches with sequences from RTPR.. Inset shows a zoom of (A) around the 2004 peak to highlight the presence of a peak at 2020 Da.

FIGURE 3.

(A) Expansion of the MALDI spectrum in Figure 2 in the region around the 2004 Da peak. (I) [1'-³H]-F₂CTP, NaBH₄. (II) [1'-²H,³H]-F₂CTP, NaBH₄. (III) [1'-³H]-F₂CTP, NaBD₄. (B) Expansion of the MALDI spectrum in Figure 2 in the region around the 2063 Da peak. (I) [1'-³H]-F₂CTP, NaBH₄. (II) [1'-²H,³H]-F₂CTP, NaBH₄. (III) [1'-³H]-F₂CTP, NaBD₄.

FIGURE 4.

(A) MS/MS of peak at 2063 Da, containing a label derived from F₂CTP. The y series is marked, and corresponds to the C-terminal peptide of RTPR (DLELVDTQDCEGGACPIK). The absolute MWs detected are shifted +43 Da relative to the acetamide labeled C-terminal peptide (Figure S1), suggesting alkylation at cysteine by a group 43Da larger than acetamide. The modification is present to some extent on each cysteine, as indicated by the appearance of the y series of the normal peptide after C₇₃₁ is cleaved (indicated at 831, 702, 645, 588 and 517 Da).

FIGURE 5.

MS/MS of the peak at 2004 Da corresponding to the C-terminal peptide of RTPR (DLELVDQTDCEGGACPIK) containing an internal cysteine-cysteine crosslink derived from F₂CTP. The y ion series is indicated. No regular ions were detected in the mass range 360–970 Da.

FIGURE 6.
SDS PAGE of RTPR inactivated by treatment with F₂CTP.

SCHEME 1.

Proposed mechanism of inactivation of RNR by 2'-substituted mechanism based inhibitors.

SCHEME 2.

Proposal for structures that can be accommodated by the labeling observed in the MS data.

SCHEME 3.

Proposed mechanism of inactivation by RTPR by F_2CTP by the non-alkylative and alkylative pathways.

SCHEME 4.

Proposed mechanism for generation of furanone during the inactivation of RTPR with F₂CTP.

TABLE 1Covalent labeling of RTPR with [1'-³H] and [5-³H]-F₂CTP.

Enzyme	F ₂ CTP Label	TR/TRR/NADPH	Sephadex G-50 ^a	Label eluting with protein (eq./RTPR)
wt	[1'- ³ H]	+	Native	0.46
wt	[1'- ³ H]	–	Denaturing	0.47
wt	[1'- ³ H]	–	Native	0.49
wt	[1'- ³ H]	–	Denaturing	0.44
wt, NaBH ₄ @ 2min	[1'- ³ H]	–	Native	0.33
wt	[5- ³ H]	+	Native	0.17
wt	[5- ³ H]	+	Denaturing	0.13
wt	[5- ³ H]	–	Native	0.15 ^b
wt	[5- ³ H]	–	Denaturing	0.12
wt, NaBH ₄ @ 2min	[5- ³ H]	–	Native	0.03

^aUnder denaturing conditions, 6M guanidinium HCL was added to the inactivation mixture before loading to the Sephadex column and the column was eluted with 2M guanidinium HCL.

^bUnder these conditions, the small molecules were dephosphorylated and analyzed by HPLC and 0.71 eq. of cytosine and 0.18 equiv. of F₂C were found.