H, d, J = 6 Hz), 7.79 (1 H, d, J = 6 Hz), 7.0 (4 H, m), 6.93 (2 H, b), 5.03 (2 H, bs), 3.78 (3 H, s); IR (cm^{-1}) 3480, 3360, 1675, 1630, 1595, 1550, 1445, 1400, 1360, 1310, 1240, 1195, 1090, 1000. Anal. ($C_{16}H_{13}N_3O_3$) C, H, N.

1-(2-Aminobenzyl)-6-methoxy-7-aminoisoquinoline-5,8-dione (55). From **52** was prepared **55**. It was a dark red crystalline solid (ligroin): yield 54%; mp 135 °C (subl) and 198 °C; NMR δ 8.83 (1 H, d, J = 6 Hz), 7.77 (1 H, d, J = 6 Hz), 7.50 (4 H, bm), 7.17 (2 H, bs), 4.03 (2 H, s); IR (cm^{-1}) 3405, 3320, 1620, 1590, 1520, 1430, 1240, 1040. Anal. ($C_{17}H_{15}N_3O_3$) C, H, N.

1-(4-Nitrophenyl)-6-(1-piperidinyl)-7-bromoisoquinoline-5,8-dione (61). Piperidine (0.1 mL) was added to **31** (0.1 g) in DMF (5 mL). The dark purple mixture was diluted with H_2O and extracted with $CHCl_3$. Concentration of the extract gave **61** as a violet crystalline solid (ether): yield 83%; mp 168–170 °C; NMR δ 9.06 (1 H, d, J = 6 Hz), 8.35 (2 H, d, J = 9 Hz), 8.0 (1 H, d, J = 6 Hz), 7.63 (2 H, d, J = 9 Hz), 3.6 (4 H, m), 1.78 (6 H, m); IR (cm^{-1}) 2950, 2860, 1670, 1640, 1570, 1540, 1510, 1340. Anal. ($C_{20}H_{16}BrN_3O_4$) C, H, N.

A similar procedure was used to prepare the quinones **57–60, 62,** and **63.**

1-(4-Nitrophenyl)-6-(methylamino)-7-bromoisoquinoline-5,8-dione (57). From **31** and methylamine was prepared **57**. It was a red solid (ether): yield 80%; mp 245 °C subl; NMR (DMSO-d_6) δ 9.0 (1 H, m), 8.3 (2 H, d, J = 9 Hz), 7.97 (1 H, m), 7.67 (2 H, d, J = 9 Hz), 3.3 (3 H, s); IR (cm^{-1}) 3360, 1670, 1640, 1600, 1500, 1340, 740. Anal. ($C_{16}H_{10}BrN_3O_4$) C, H, N.

1-(3-Nitrophenyl)-6-(methylamino)-7-bromoisoquinoline-5,8-dione (58). From **32** was obtained **58** as a red solid (ether): mp 250 °C dec; NMR (DMSO-d_6) δ 9.03 (1 H, d, J = 6 Hz), 8.3 (2 H, m), 7.97 (1 H, d, J = 6 Hz), 7.76 (2 H, m), 3.26 (3 H, s); IR (cm^{-1}) 3360, 1670, 1600, 1520, 1340, 740, 670. Anal. ($C_{16}H_{10}BrN_3O_4$) C, H, N.

1-(2-Nitrophenyl)-6-(methylamino)-7-bromoisoquinoline-5,8-dione (59). From **33** was prepared **59**. It was a dark red unstable solid (ether): yield 75%; mp 195 °C dec; NMR (DMSO-d_6) δ 8.99 (1 H, d, J = 6 Hz), 8.23 (1 H, dd, J = 7.5, 1.5 Hz), 7.97 (1 H, d, J = 6 Hz), 7.83 (2 H, td, J = 7.5, 1.5 Hz), 7.4 (1 H, dd, J = 7.5, 1.5 Hz), 3.3 (3 H, s); IR (cm^{-1}) 3360, 1670, 1590, 1510, 1340, 740. Anal. ($C_{16}H_{10}BrN_3O_4$) C, H, N.

1-Benzyl-6-(methylamino)-7-bromoisoquinoline-5,8-dione (60). From **49** was obtained **60** as a red solid (ether): yield 85%; mp 95–98 °C; NMR (DMSO-d_6) δ 8.87 (1 H, d, J = 6 Hz), 7.75 (1 H, d, J = 6 Hz), 7.23 (5 H, s), 4.75 (2 H, s), 3.23 (3 H, s); IR (cm^{-1}) 3360, 1675, 1640, 1600, 1520, 1300, 1110, 700, 690. Anal. ($C_{17}H_{13}BrN_2O_2$) C, H, N.

1-(2-Nitrophenyl)-6-(dimethylamino)-7-bromoisoquinoline-5,8-dione (62). From **33** was prepared **62**. It was a dark purple crystalline solid (ether–ligroin): yield 77%; mp 127 °C; NMR δ 8.97 (1 H, d, J = 6 Hz), 8.26 (1 H, dd, J = 7.5, 1.5 Hz), 7.93 (1 H, d, J = 6 Hz), 7.67 (2 H, td, J = 7.5, 1.5 Hz), 7.33 (1 H, dd, J = 7.5, 1.5 Hz), 3.23 (6 H, s); IR (cm^{-1}) 1670, 1630, 1570, 1510, 1340, 1210. Anal. ($C_{17}H_{12}O_4N_3Br$) C, H, N.

1-(4-Nitrophenyl)-6-(methylamino)-7-azidoisoquinoline-5,8-dione (63). From **34** was obtained **63** as a dark blue crystalline solid (THF): yield 50%; mp 200 °C dec; IR (cm^{-1}) 3360, 2120, 1665, 1600, 1510, 1340. Anal. ($C_{16}H_{10}N_6O_4$) C, H, N.

Acknowledgment. We express our grateful appreciation to the late Mr. John L. Davenport for his generous financial support and his abiding interest. This publication is dedicated to his memory.

2′-Deoxy-2′-methylenecytidine and 2′-Deoxy-2′,2′-difluorocytidine 5′-Diphosphates: Potent Mechanism-Based Inhibitors of Ribonucleotide Reductase

C. H. Baker, J. Banzon, J. M. Bollinger, and J. Stubbe*

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

V. Samano and M. J. Robins*

Department of Chemistry, Brigham Young University, Provo, Utah 84602

B. Lippert, E. Jarvi, and R. Resvick

Merrell Dow Pharmaceuticals, Inc., 2110 E. Galbraith Road, Cincinnati, Ohio 45215-6300. *Received November 21, 1990*

It has been found that 2′-deoxy-2′-methyleneuridine (MdUrd), 2′-deoxy-2′-methylenecytidine (MdCyd), and 2′-deoxy-2′,2′-difluorocytidine (dFdCyd) 5′-diphosphates (MdUDP (1) MdCDP (2) and dFdCDP (3), respectively) function as irreversible inactivators of the *Escherichia coli* ribonucleoside diphosphate reductase (RDPR). **2** is a much more potent inhibitor than its uridine analogue **1**. It is proposed that **2** undergoes abstraction of H3′ to give an allylic radical that captures a hydrogen atom and decomposes to an active alkylating furanone species. RDPR also accepts **3** as an alternative substrate analogue and presumably executes an initial abstraction of H3′ to initiate formation of a suicide species. Both **2** and **3** give inactivation results that differ from those of previously studied inhibitors. The potent anticancer activities of MdCyd and dFdCyd indicate a significant chemotherapeutic potential. The analogous RDPR of mammalian cells should be regarded as a likely target and/or activating enzyme for these novel mechanism-based inactivators.

Introduction

The most potent drug available for the treatment of adult acute leukemia is 1-(β-D-arabinofuranosyl)cytosine (araC).[1,2] Problems associated with araC arise from its rapid deamination to the therapeutically inactive uridine derivative and its ineffectiveness against solid tumors. Novel 2′-substituted cytidine derivatives have been synthesized recently and their antitumor activity investigated in the hope of finding an efficatious drug. Ueda and coworkers[3] and Samano and Robins[4] have recently reported

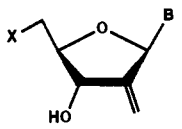
(1) Abbreviations used are as follows: araC, 1-(β-D-arabinofuranosyl)cytosine; MdCyd, 2′-deoxy-2′-methylenecytidine; MdUrd, 2′-deoxy-2′-methyleneuridine; dFdCyd, 2′-deoxy-2′,2′-difluorocytidine; MdCDP (2), MdUDP (1), and dFdCDP (3) are the corresponding nucleoside 5′-diphosphates, respectively; RDPR, ribonucleoside diphosphate reductase; XdNDP, 2′-deoxy-2′-halonucleoside 5′-diphosphate; TEAB, triethylammonium bicarbonate.

(2) Creasey, W. A.; Papac, R. J.; Markiw, M. E.; Calabresi, P.; Welch, A. D. *Biochem. Pharmacol.* **1966**, *15*, 1417.

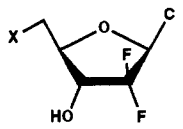
(3) (a) Takenuki, K.; Matsuda, A.; Ueda, T.; Sasaki, T.; Fujii, A.; Yamagami, K. *J. Med. Chem.* **1988**, *31*, 1064. (b) Ueda, T.; Matsuda, A.; Yoshimura, Y.; Takenuki, K. *Nucleosides Nucleotides* **1989**, *8*, 743.

0022-2623/91/1834-1879$02.50/0

the synthesis of 2′-deoxy-2′-methylenecytidine (MdCyd), and Takenuki et al.[3a] showed in preliminary studies that it is active at low concentrations against mouse and human leukemic cell lines as well as human carcinoma cell lines.



MdUrd B = U, X = OH
MdUDP (1) B = U, X = PP
MdCyd B = C, X = OH
MdCDP (2) B = C, X = PP



dFdCyd X = OH
dFdCDP (3) X = PP

Hertel et al.[5] and Sunkara et al.[6a] have prepared 2′-deoxy-2′,2′-difluorocytidine (dFdCyd), and preliminary results have indicated that the diphosphate of dFdCyd inactivates the ribonucleotide reductase from calf thymus[6a] and CCRF-CEM cells[6b] in a time- and concentration-dependent manner in vitro. Heinemann et al.[7] and Sunkara et al.[6a] have determined that dFdCyd has good activity against human leukemic cells and solid tumors in mice. Both MdCyd and dFdCyd appear to inhibit DNA biosynthesis, but their mechanisms of cytotoxicity remain to be established.

We had considered[4] that allylic delocalization of a 3′ radical intermediate by a 2′-deoxy-2′-methylene group could have interesting mechanistic consequences with ribonucleotide reductases. Two potential targets for dFdCyd have been suggested on the basis of recent metabolic studies in a number of cell lines.[6,8] In CHO cells accumulation of large amounts of the metabolite dFdCTP implicated DNA polymerase as a potential target. In human leukemic cell line K562[8] and other cell lines,[6] ribonucleotide reductase was implicated as a target. These results and previous in vitro studies that documented the ability of 2′-halogenated (Cl, F) nucleotide derivatives to function as potent irreversible inhibitors of ribonucleotide reductases[9] prompted us to examine the nucleotides of MdCyd and dFdCyd with the purified *Escherichia coli* ribonucleoside 5′-diphosphate reductase (RDPR). This communication reports that the corresponding 5′-diphosphates of both MdCyd and dFdCyd, as well as 2′-deoxy-2′-methyleneuridine 5′-diphosphate (MdUDP), are potent mechanism-based inhibitors of RDPR. With both of the modified dCDP analogues, time-dependent irreversible inactivation of RDPR is observed and is accompanied by cytosine release. Some unusual properties of these inhibitors that distinguish them from the previously well-studied 2′-halo-2′-deoxynucleotides and 2′-azido-2′-deoxynucleotides have also been found. Given that the *E. coli* reductase is the prototype for its mammalian counterpart and on the basis of the results reported in this communication, ribonucleotide reductase should be given serious consideration as a likely chemotherapeutic target for these cytotoxic nucleosides.

Experimental Section

Materials. 2′-Deoxy-2′,2′-difluorocytidine[5] was a gift of Eli Lilly Co., and the corresponding 5′-diphosphate[6] (3) was a gift of Merrell Dow Pharmaceuticals. 2′-Deoxy-2′-methylenecytidine and 2′-deoxy-2′-methyleneuridine were prepared by Samano and Robins.[4] *E. coli* RDPR was isolated as previously described.[10] The B_1 subunit had a specific activity of ~1000 μmol min^{-1} mg^{-1} and the B_2 subunit ~6300 μmol min^{-1} mg^{-1}. Thioredoxin (specific activity of 29 μmol min^{-1} mg^{-1}) and thioredoxin reductase (specific activity of 489 μmol min^{-1} mg^{-1}) were isolated as previously described.[11,12] *E. coli* alkaline phosphatase, NADPH, and ATP were obtained from Sigma Chemical Co. Triethyl phosphate, phosphorous oxychloride, and carbonyldiimidazole were obtained from Aldrich Chemical Co. All other compounds were of reagent grade. NMR spectra were recorded on a Varian XL-300 spectrometer and chromatographic separations were effected by using a Beckman HPLC with an Alltech Econosil 10 μ C-18 reverse-phase column.

Preparation of Nucleotides from Nucleosides. The standard procedure of Yoshikawa et al. was employed.[13] Typically 2′-deoxy-2′-methyleneuridine (16.1 mg, 67 μmol) was dried in a microsynthesis vial in a dessicator containing P_2O_5 in vacuo overnight. Freshly distilled $PO(OEt)_3$ (167 μL) was added to the nucleoside, and subsequent to dissolution the reaction mixture was cooled to 5 °C. $POCl_3$ (12.5 μL, 134 μmol, freshly distilled) was added and the reaction mixture was stirred at 5 °C for 15 h. The reaction was then quenched with ice–water and neutralized with 1 N NaOH until the pH remained constant at 7.5. The neutralized solution was then loaded onto a DEAE-Sephadex A-25 column (1.7 × 40 cm) and eluted with a 500 mL × 500 mL linear gradient from 0 to 0.4 M triethylammonium bicarbonate (TEAB). The desired material eluted at ~0.2 M TEAB and was concentrated in vacuo to give 35 μmol (52% yield) of the desired 2′-deoxy-2′-methyleneuridine 5′-monophosphate. The contaminating inorganic phosphate was removed by Ba^{+2} precipitation as follows: The nucleotide (35 μmol) was dissolved in H_2O (4 mL) and the pH adjusted to 8.4 (1 N NaOH). Aqueous $BaCl_2$ (1M, 200 μL) was added, the precipitated barium phosphate pelleted by centrifugation, and the nucleotide-containing supernatant decanted. The pellet was washed by stirring with H_2O (1 mL), separated by centrifugation, and the wash combined with the first supernatant. Ethanol (20 mL) was added to the combined supernatants (5 mL) and the solution allowed to stand at −20 °C overnight. The nucleotide salt had precipitated and was isolated by centrifugation. The Ba^{+2} salt of the nucleoside monophosphate was converted to the protonated form by using Dowex 50W-X8 (H^+). Conversion of the monophosphate to diphosphate (1) by the standard procedure of Hoard and Ott[14] gave yields of 40–50%: ^{1}H NMR (300 MHz, D_2O) δ 7.66 (d, 1 H, J_{5-6} = 8.3 Hz, H-6), 6.66 (br s, 1 H, H-1′), 5.88 (d, 1 H, J_{5-6} = 8.3 Hz, H-5), 5.64 (br s, 1 H, 2′-methylene), 5.44 (br s, 1 H, 2′-methylene), 4.92 (m, 1 H, H-3′, $J_{3'-4'}$ = 5.0 Hz), 4.22 (m, 2 H, H-5′,5″), 4.01 (m, 1 H, H-4′, $J_{3'-4'}$ = 5.0 Hz).

The 2′-deoxy-2′-methylenecytidine 5′-monophosphate was prepared under similar conditions, but incubation was for only 1 h at 5 °C. Subsequent to workup the monophosphate was recovered in 30% yield. The yield for the second phosphorylation to give 2 was also 30%: ^{1}H NMR (300 MHz, D_2O) δ 7.64 (d, 1 H, J_{5-6} = 7.4 Hz, H-6), 6.71 (br s, 1 H, H-1′), 6.05 (d, 1 H, J_{5-6} = 7.4 Hz, H-5), 5.59 (br s, 1 H, 2′-methylene), 5.37 (br s, 1 H, 2′-methylene), 4.87 (m, 1 H, H-3′), 4.21 (m, 2 H, H-5′,5″), 4.02 (m, 1 H, H-4′). No impurities were observed by NMR and HPLC analysis of 1 and 2, and their treatment with alkaline phosphatase as described under the section entitled Cytosine Release from Incubation of RDPR with MdCDP showed only MdUrd and MdCyd, respectively.

(4) Samano, V.; Robins, M. J. *Synthesis*, in press.
(5) Hertel, L. W.; Kroin, J. S.; Misner, J. W.; Tustin, J. M. *J. Org. Chem.* **1988**, *53*, 2406.
(6) (a) Sunkara, P. S.; Lippert, B. J.; Snyder, R. D.; Jarvi, E. T.; Farr, R. A. *Proc. Am. Assoc. Cancer Res.* **1988**, *29*, 1289. (b) Heinemann, V.; Xu, Y.-Z.; Chubb, S.; Sen, A.; Hertel, L. W.; Grindey, G. B.; Plunkett, W. *Mol. Pharmacol.* **1990**, *38*, 567.
(7) Heinemann, V.; Hertel, L. W.; Grindey, G. B.; Plunkett, W. *Cancer Res.* **1988**, *48*, 4024.
(8) (a) Plunkett, W.; Gandhi, V.; Chubb, S.; Nowak, B.; Heinemann, V.; Mineishi, S.; Sen, A.; Hertel, L. W.; Grindey, G. B. *Nucleosides Nucleotides* **1989**, *8*, 775. (b) Gandhi, V.; Plunkett, W. *Cancer Res.* **1990**, *50*, 3675.
(9) Stubbe, J. *Adv. Enzymol.* **1989**, *63*, 349.
(10) Salowe, S. P.; Stubbe, J. *J. Bacteriol.* **1986**, *165*, 363.
(11) Laurent, T. C.; Moore, E. C.; Reichard, P. *J. Biol. Chem.* **1964**, *239*, 3436.
(12) Pigiet, V. P.; Conley, R. R. *J. Biol. Chem.* **1977**, *252*, 6367.
(13) Yoshikawa, M.; Kato, T.; Takenishi, T. *Tetrahedron Lett.* **1967**, 5065.
(14) Hoard, D. E.; Ott, D. G. *J. Am. Chem. Soc.* **1965**, *87*, 1785.

Kinetic Analysis of the Mechanism-Based Inhibitors. Time-dependent inactivations were performed to investigate the inhibitory effects of MdUDP (1), MdCDP (2), and dFdCDP (3) on RDPR. A typical assay mixture contained in a final volume of 90 μL: 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.0 mM NADPH, 1.25 mM [^{14}C]CDP (specific activity = 7×10^5 cpm/μmol), 1.6 mM ATP, 4.6 μM thioredoxin, and 0.29 μM thioredoxin reductase. A typical inactivation mixture contained in a final volume of 100 μL at 25 °C: 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 0.5 mM NADPH, 12 μM thioredoxin, 0.78 μM thioredoxin reductase, 1.25 μM B_1 and 1.25 μM B_2, and variable concentrations of the inhibitors (1, 2, and 3). At various times, 10 μL was removed from the inactivation mixture and added to 90 μL of the assay mixture. The resulting assay mixture was incubated for 15 min at 25 °C. Reactions were stopped by placing the mixture in a boiling water bath for 1 min. The conversion of CDP to dCDP was measured by the method of Steeper and Steuart.[15] Time points taken and concentrations of the inhibitors used varied with the nucleoside diphosphates under investigation and are indicated in the appropriate figure legends. In all cases, control experiments were performed with no inhibitor in the incubation mixture.

Irreversible Inactivation. A typical inactivation study contained in a final volume of 450 μL: 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 0.5 mM NADPH, 12 μM thioredoxin, 0.79 μM thioredoxin reductase, 2.84 μM B_1, 2.53 μM B_2, and 0.33 mM 2'-deoxy-2'-methyleneuridine 5'-diphosphate (1) (incubation time 20 min). An identical control was run in which the inhibitor solution was replaced with H_2O. The reaction mixture (5 μL) was assayed for activity as described above and then 400 μL was loaded onto a Sephadex G50 column (1.2 × 24.5 cm) equilibrated in 50 mM HEPES, 15 mM $MgSO_4$, and 1.0 mM EDTA (pH 7.6). One-mL fractions were collected, and the protein-containing fractions were pooled and assayed for RDPR activity. The protein also was analyzed by UV spectroscopy to look for an absorbance at 320 nm. A similar experiment with dFdCDP was carried out with use of 40 μM inhibitor with an incubation time of 10 min prior to Sephadex G50 chromatography.

Cytosine Release from Incubation of RDPR with MdCDP. In a total volume of 100 μL was incubated 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 1 mM NADPH, 28 μM thioredoxin, 0.85 μM thioredoxin reductase, 25 μM B_1, 25 μM B_2, and 0.75 mM MdCDP (2). A control was run under identical conditions in which H_2O replaced inhibitor solution. Both mixtures were incubated at 25 °C for 1 h and then treated with 50 μL containing 1 unit of alkaline phosphatase. One third of the resulting solution (50 μL) was diluted to 150 μL with RDPR assay buffer and injected into a reverse-phase C18 column (4.6 mm × 25 cm). The products were eluted from the column at a flow rate of 1 mL/min, by using 100% 5 mM potassium phosphate buffer (pH 6.8) for 10 min, followed by a linear gradient over 10 min from 0% to 10% methanol, and isocratic elution at 10% MeOH for an additional 10 min. Product, retention time (min), and yield (nmol) for the experiment were, respectively, cytosine, 4.6, 78.9; and MdCyd, 14, 7.5; and for the control MdCyd, 14, 75. The quantitation was determined by collecting the peak and recording its UV spectrum: cytosine λ_{max} (pH 6.8) = 264 nm (ϵ = 6600 M^{-1} cm^{-1}) or λ_{max} (pH 1.6) = 274 nm (ϵ = 9000 M^{-1} cm^{-1}); MdCyd λ_{max} (pH 6.8) = 278 nm (ϵ = 8900 M^{-1} cm^{-1}).

Change in Absorbance at 320 nm. Evidence for Furanone Production. In a total volume of 400 μL in a cuvette was mixed 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.60 mM ATP, 0.1 mM NADPH, 7 μM thioredoxin, 0.21 μM thioredoxin reductase, 25 μM B_1, and 25 μM B_2. The reaction was started by addition of 0.5 mM MdCDP and the solution scanned repeatedly from 190 to 820 nm.

Cytosine Release from Incubation of RDPR with dFdCDP. In a total volume of 100 μL was incubated 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1.0 mM EDTA, 1.60 mM ATP, 1.0 mM NADPH, 0.045 mg of thioredoxin, 0.004 mg of thioredoxin reductase, 2.5 nmol B_1, 2.5 nmol B_2, and 125 μM dFdCDP (3, 12.5 nmol). The dithiothreitol was removed by using a Penefsky column.[16] The reaction mixture was incubated for 30 min at 25 °C and the reaction stopped by placing the reaction mixture in a boiling water bath for 1 min. The protein that precipitated was removed by centrifugation. Alkaline phosphatase (1 unit) in 50 μL of Tris-HCl (pH 8.5) was then added to the reaction mixture that was incubated for 1 h at 37 °C. The reaction was stopped as described above. The cytosine was quantitated by removing 10- or 20-μL aliquots from this reaction mixture, diluting to 150 μL with HEPES buffer, and analyzing by HPLC as described above. A standard curve for cytosine release was made by injecting known amounts of cytosine and cutting and weighing each chart peak area on an analytical balance. Cytosine (5.85 nmol) was recovered. A control experiment using B_2 whose tyrosyl radical had been reduced with hydroxyurea (met B_2) showed no cytosine production.

Since the quantitation of cytosine was close to stoichiometric with respect to B_1 (2 active sites per subunit), the dFdCyd was also quantitated subsequent to the above reaction. The inactivation mixture (40 μL) was loaded onto a Dowex anion exchange column in the borate form[15] (0.8 × 5 cm) and washed with 16 mL of H_2O. The nucleoside passed directly through the column. The effluent was concentrated to 550 μL and analyzed by reverse-phase HPLC (retention time dFdCyd, 23 min). A standard curve was also prepared by using dFdCyd for quantitation purposes. The recovery of dFdCyd was 6.1 nmol.

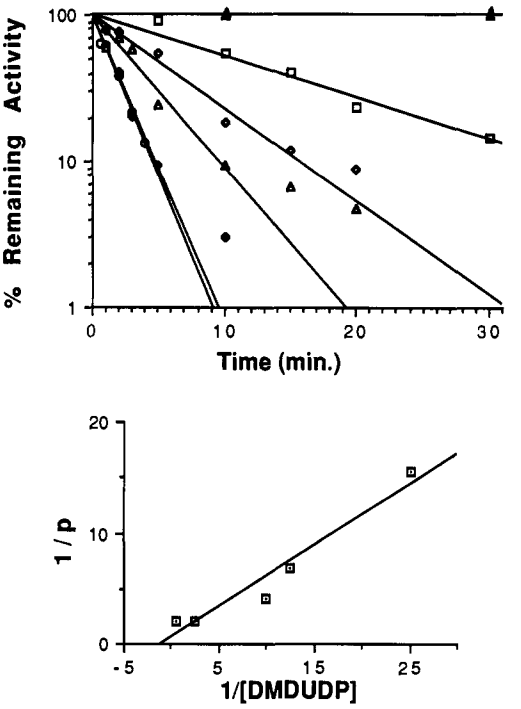


Figure 1. Time-dependent inactivation of RDPR by MdUDP (1). RDPR (1.25 μM) was incubated with varying concentrations of 1: ▲, 0 mM; □, 0.04 mM, ◇, 0.08 mM; △, 0.1 mM; ○, 0.23 mM; ◆, 0.42 mM. Aliquots were removed at various times and assayed for activity. The insert is a replot of the reciprocals of the slopes vs the reciprocals of the inhibitor concentrations: K_i = 0.83 mM, k_{inact} = 0.015 s^{-1}.

Results

Inactivation of *E. coli* RDPR by MdCDP and MdUDP. Incubation of varying concentrations of MdUDP (1) with *E. coli* RDPR and then assaying for enzymatic activity by dilution into a solution containing a large excess of CDP resulted in the time-dependent inactivation shown in Figure 1. A replot of the data (Figure 1, insert) gave a K_i = 0.83 mM and K_{inact} = 0.015 s^{-1}. A similar experiment was carried out with MdCDP (2) with the results indicated in Figure 2. In contrast with the uridine derivative, the rate of inactivation is much more rapid at

(15) Steeper, J. R.; Steuart, C. D. *Anal. Biochem.* **1970**, *34*, 123.

(16) Penefsky, H. S. *J. Biol. Chem.* **1977**, *252*, 2891.

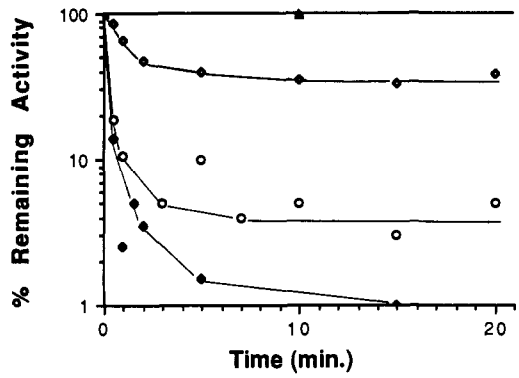


Figure 2. Time-dependent inactivation of RDPR by MdCDP (2). RDPR (1.25 μM) was incubated with varying concentrations of 2: ▲, 0 μM; ◇, 10 μM; ○, 40 μM; ◆, 70 μM. Aliquots were removed at various times and assayed for activity.

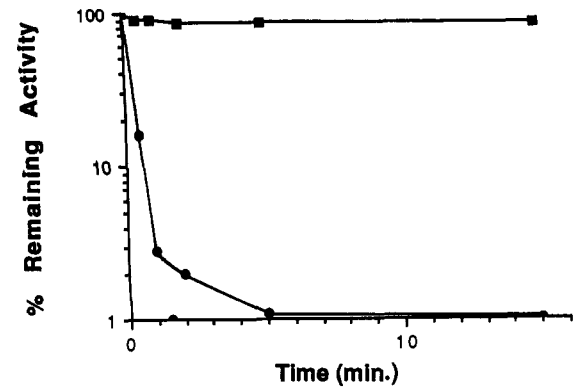


Figure 3. Dithiothreitol protects RDPR from inactivation by MdCDP (2). RDPR (1.25 μM) was incubated with 2 (100 μM) in the presence of (■) or absence (●) of DTT under standard assay conditions. The enzyme was assayed for activity at various times.

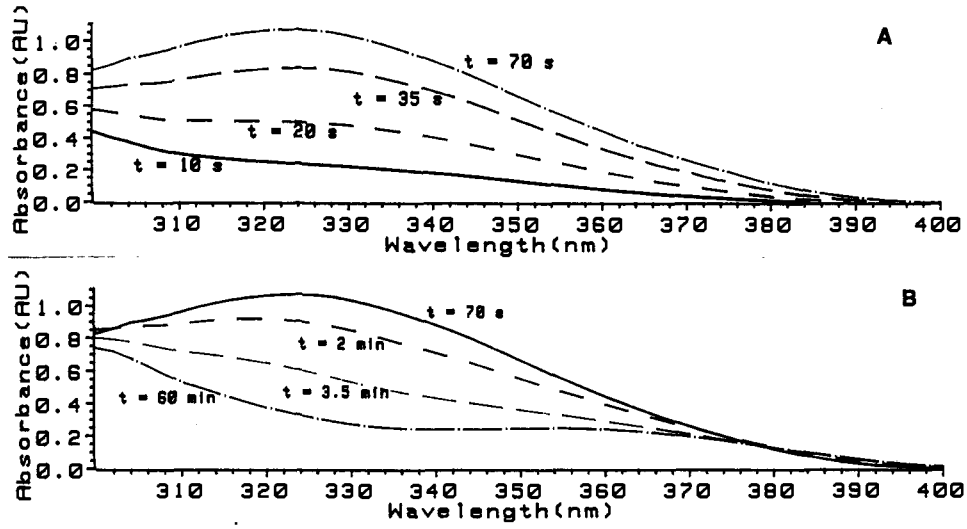
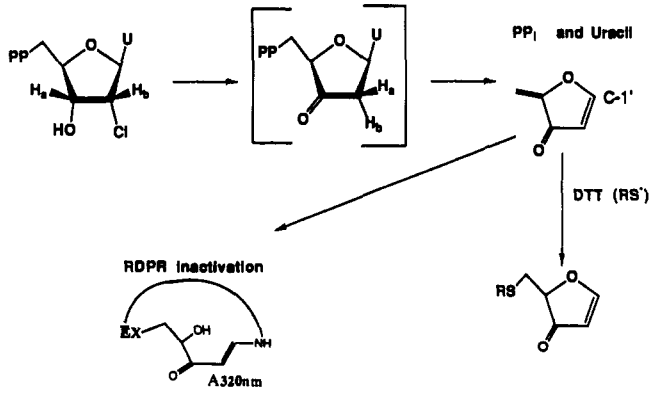


Figure 4. UV–vis difference spectra resulting from incubation of RDPR with MdCDP (2). A: the spectrum at time $t = 0$ was subtracted from the spectra at $t = 10$, 20, 35, and 70 s. B: the spectrum at time $t = 0$ was subtracted from the spectra at $t = 70$ s, 2 min, 3.5 min, and 60 min.

Scheme I



much lower concentrations of inhibitor. At 70 μM MdCDP, the half life of inactivation is <1 min—that is, too fast to measure. The differences in the concentration dependence on the inactivation between these two derivatives may be related to similar differences observed with their corresponding substrates K_m = 220 μM and V_{max} = 870 nmol min^{-1} mg^{-1} for UDP and K_m = 50 μM and V_{max} = 1200 nmol min^{-1} mg^{-1} for CDP.

To test if the inactivation of RDPR is irreversible, the inactivated enzyme mixture was passed through a Sephadex G50 column. No recovery of enzymatic activity was observed. Control experiments run under identical conditions but with H_2O replacing the inhibitor solution in the inactivation mixture revealed 80–100% recovery of activity. This inactivation of RDPR by MdCDP and MdUDP thus appears to be irreversible.

Product Identification and Modification of RDPR Accompanying Inactivation. Previous studies with 2′-deoxy-2′-substituted-nucleotides (2′-substituent = Cl, F, N_3) that are mechanism-based inhibitors of RDPR have revealed that inactivation is accompanied by enzyme-mediated 3′ C–H bond cleavage followed ultimately by elimination of the nucleic acid base and PPi to produce a furanone (Scheme I).[9,17] The furanone covalently modifies RDPR in a process that is accompanied by a change in absorbance on the protein at 320 nm.[18] While the rate of inactivation by 2′-chloro-2′-deoxyuridine 5′-diphosphate (CldUDP) is fast ($t_{1/2}$ = 30 s), under identical conditions the change in absorbance on the protein at 320 nm is very slow ($t_{1/2}$ = 45–60 min). These results have been interpreted to indicate rapid inactivation by nucleophilic attack of a group on the enzyme at the more reactive exocyclic methylene of the 2-methylene-3(2*H*)-furanone. The absorbance increase at 320 nm results from subsequent attack of a lysine residue at the C-1′ position ultimately leading to the putative α,β-unsaturated enamine (Scheme I). Collapse of the 3′-ketodeoxynucleotide to the furanone is believed to occur in solution, not within the

(17) Ator, M. A.; Stubbe, J. *Biochemistry* **1985**, *24*, 7214.
(18) Ashley, G. W.; Harris, G.; Stubbe, J. *Biochemistry* **1988**, *27*, 4305.

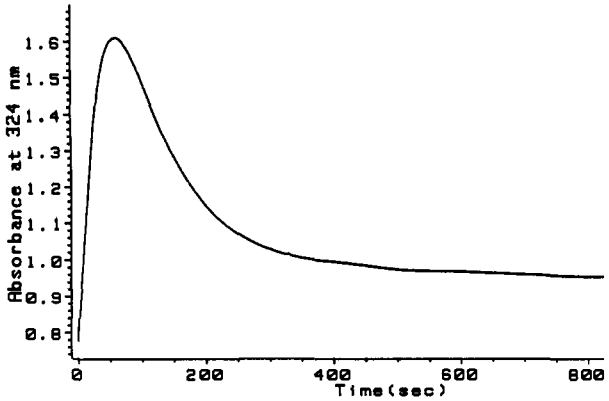


Figure 5. Change in absorbance at 324 nm as a function of time during the inactivation of RDPR by MdCDP (2).

active site, since nucleophiles such as DTT protect the enzyme against inactivation (Scheme I).[9,18]

To determine whether a similar mechanism of inactivation is occurring with MdCDP (2), three types of experiments were carried out. In the first experiment (Figure 3) 10 mM DTT was shown to protect the enzyme against inactivation. These results are consistent with a reactive species being generated in solution that then functions as a nonspecific alkylating agent. The second experiment utilized HPLC to analyze for cytosine release. Subsequent to inactivation of 2.5 nmol of RDPR with 75 nmol of MdCDP, ~75 nmol of cytosine was produced, and ~7.5 nmol of MdCDP was recovered. The cytosine was identified by its comigration with authentic material on HPLC and by its characteristic λ_{max} shift from 267 to 274 nm (at pH 6.8 and pH 1.6, respectively). A control experiment run in the absence of the B_2 subunit of RDPR revealed no cytosine formation. The third experiment was to establish if inactivation of RDPR was accompanied by a change in absorption on the protein at 320 nm. RDPR (25 μM) was incubated with MdCDP (100 μM) in a complete assay mixture, and the spectral region from 300–820 nm was repeatedly scanned by using a diode array spectrometer. As indicated in Figure 4, over a period of 70 s, the time required for (>90%) enzyme inactivation, a change in absorption at 326 nm was observed. This is in stark contrast to the results with the well-characterized mechanism-based inhibitor CldUDP in which inactivation was much more rapid than the change in absorbance at 320 nm. If one assumes an extinction coefficient of (20 000 M^{-1} cm^{-1}), for a putative furanone species (Scheme I), 2.2 mol of furanone per mole of enzyme was observed. However, much to our amazement and unique in comparison with previous mechanism-based inhibitors, continued scanning of the reaction mixture in the UV–vis region showed a decrease in absorbance in the 326-nm region with time. By 20 min, a new broad absorption band with a much lower extinction coefficient appeared at 366 nm (Figure 4B). The kinetics of the absorbance change at 326 nm are shown in Figure 5. Whereas the release of cytosine and protection against enzyme inactivation by DTT are very similar to results observed with a number of 2'-substituted 2'-deoxynucleotide mechanism-based inhibitors, the rate of increase in absorbance at 326 nm and the subsequent decrease in this absorbance are substantially different from any of the previous compounds.

Inactivation of RDPR by dFdCDP. Incubation of dFdCDP (3) with RDPR resulted in rapid time-dependent

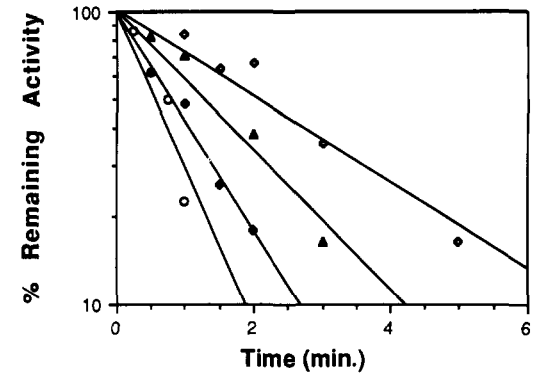


Figure 6. Time-dependent inactivation of RDPR by dFdCDP (3). RDPR (1.25 μM) was incubated with varying concentrations of 3 (○, 20 μM; ◆, 16 μM; ▲, 12 μM, ◇, 8 μM). Aliquots were removed and assayed for activity at the indicated times.

inactivation (Figure 6). With as little as 16-fold excess of inhibitor over enzyme, all activity is lost with a $t_{1/2}$ = 30 s. Chromatography of the inactivated enzyme on a Sephadex G-50 column, indicated no recovery of activity. The inactivation appears to result from either an irreversible modification or a very tight reversible complexation. In contrast to studies with previous 2'-halo-substituted mechanism-based inhibitors, DTT does not protect against inactivation (data not shown). These results are reminiscent of those with 2'-azido-2'-deoxyuridine 5'-diphosphate, a stoichiometric mechanism-based inhibitor.[19] No change in absorbance at 320 nm was observed over 10 s to 1 h. Thus, the mode of action of dFdCDP (3) also appears to be unique in comparison with those of previously well-studied mechanism-based inhibitors.[9]

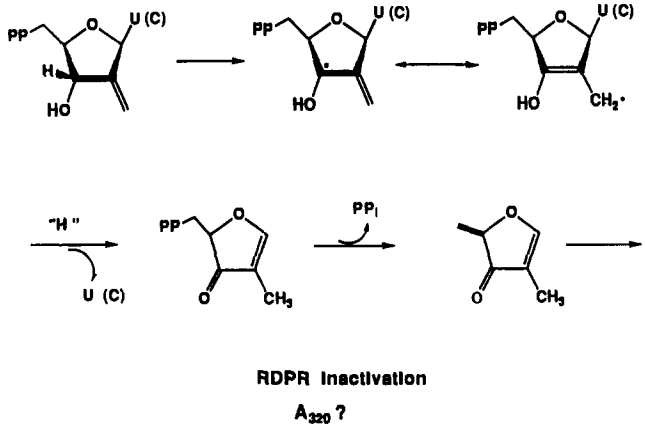
Due to the low number of turnovers required to give complete inactivation, and the inability to achieve larger numbers of turnovers when DTT was used as a protectant, analysis for products in the absence of appropriate radiolabeled nucleotides is difficult. The results of a typical HPLC experiment and control to analyze for cytosine release indicate that with 2.5 nmol of RDPR and 12.5 nmol of dFdCDP (2), 5.85 nmol of a product with the same retention time as cytosine is produced, indicating approximately one turnover per inactivation. The recovered dFdCDP was 6.1 nmol, indicating that cytosine is the only major product. It thus appears that dFdCDP (2) is a stoichiometric inactivator. Detailed analysis of the reaction products and their stoichiometry awaits the synthesis of appropriate isotopically labeled molecules.

Discussion

Extensive studies on the mode of inactivation of RDPR by 2'-halo-2'-deoxynucleotides have provided a model to compare inactivation of RDPR by other 2'-substituted 2'-deoxynucleotide derivatives.[9,17-19] As indicated in Scheme I, incubation of 2'-chloro- or 2'-fluoro-2'-deoxyuridine 5'-diphosphates (CldUDP, FdUDP) results in rapid time-dependent inactivation of the protein accompanied by destruction of the substrate. Isotopic labeling studies have shown that inactivation is initiated by 3' carbon–hydrogen bond cleavage followed by production of a 3'-keto-2'-deoxynucleotide (3'-keto-dUDP). This intermediate subsequently decomposes by successive β-elimination of uracil and inorganic pyrophosphate to produce a 2-methylene-3(2*H*)-furanone. The latter has been shown to be responsible for enzyme inactivation. Inactivation requires five CldUDP's/B_1 of RDPR suggesting that the 3'-keto-CldUDP produced by the enzyme dissociates into solution.[9,17] Nonenzymatic-mediated chemistry generates

(19) Salowe, S. P.; Ator, M. A.; Stubbe, J. *Biochemistry* 1987, *26*, 3408.

Scheme II



the furanone that then acts as a nonspecific alkylating agent. Inactivation is accompanied by a change in absorption at 320 nm on the protein. Model studies have implicated this absorption change to result from nucleophilic attack at the C-1′ carbon to ultimately produce the α,β-unsaturated enamine (Scheme I). Similar studies with 2′-FdUDP indicate that analogous chemistry occurs to that described for 2′-CldUDP. However, in the 2′-fluoro case a partitioning results between the production of the normal dUDP product and production of the 3′-keto-dUDP intermediate.[9]

The previous studies with 2′-X-dUDP's allowed us to formulate the following working hypothesis for interactions of MdUDP (1) and MdCDP (2) with RDPR (Scheme II). The 3′ C–H bond would be cleaved homolytically to leave a nucleotide 3′-radical in which the electron could be delocalized onto the exocyclic 2′-methylene group. The radical could be quenched by hydrogen-atom abstraction from an amino acid residue within the active site or from solution, and the resulting enol could then eliminate uracil (cytosine) and pyrophosphate to generate the methyl-substituted equivalent of the furanone identified from inactivation of RDPR by CldUDP. This model makes predictions that we have begun to test experimentally. As indicated in Figures 1 and 2, the RDPR is rapidly inactivated by both the cytidine (2) and uridine (1) diphosphate derivatives. The former is a much more potent inhibitor and has been investigated in more detail. If the methyl furanone were generated in solution, then dithiothreitol would be expected to protect the enzyme against inactivation. As shown in Figure 3, such is the case. The model (Scheme II) would also predict that cytosine release would accompany inactivation. Incubation of a 30-fold excess of 2 with RDPR resulted in the production of 30 equiv of cytosine and total consumption of 2. As in the case of CldUDP, it appears that 2 is rapidly turned over by RDPR to produce a species that is capable of inactivation of the enzyme by alkylation. As indicated in Figure 2, at least a 30-fold excess of inhibitor over enzyme is required to achieve inactivation. While the kinetics of inactivation of RDPR by 1 appear to be consistent with a rapid reversible complex between RDPR and 1, the kinetics of inactivation of RDPR by 2 are more complex. Inactivation by a methylfuranone from solution would not predict a simple first-order process.

The most intriguing observation made with 2 and RDPR is that the inactivation is accompanied by a rapid increase in absorbance at 326 nm. At first appearance this is consistent with production of a methyl-substituted α,β-unsaturated enamine by analogy with CldUDP chemistry. Replacement of H by CH_3 in the furanone would be expected to red-shift the λ_{max} by 5–10 nm. What is unusual and unprecedented is that the rate of appearance of the λ_{max} at 326 nm is very similar to the rate of inactivation. Furthermore, assuming an extinction coefficient of ϵ = 20 000 M^{-1} cm^{-1} previously established for furanone derived products, the maximum absorbance at 326 nm would correspond to a stoichiometry of labeling by 2.2 mol furanone-like compounds/mol B_1.

Even more intriguing is the observation that when the mixture containing protein and 2 is continually monitored by UV–vis spectroscopy over an additional 30 min, all of the absorbance at 326 nm disappears and a new broad absorption is produced at 366 nm with a substantially decreased extinction coefficient (Figure 4). The kinetics of the formation and decay of this intermediate have not as yet been unraveled. The nature of these species is at present a mystery and awaits further experimentation. These results suggest that RDPR is capable of catalyzing the interconversion of both 1 and 2 to a reactive species capable of enzyme inactivation. As with the 2′-halodNDP's the active species appears to be generated in solution and hence the target of this alkylating reagent in vivo would be RDPR or other proteins (and small or large molecules that can act as nucleophiles in its immediate vicinity). Whether the cytotoxicity of 2 in tissue culture systems is related to the chemistry mediated by RDPR remains to be established. The present studies suggest that RDPR must be considered as a likely target.

The studies with dFdCDP (3) also are intriguing since 3 appears to be a stoichiometric inactivator with 1 equiv of cytosine released for each protomer of RDPR inactivated. With several equivalents of 3 per enzyme, inactivation was too rapid to determine accurate kinetic parameters. Dithiothreitol did not protect the enzyme against inactivation by 3, and no ultraviolet absorbance change at 320 nm was observed. Presumably, abstraction of the 3′ hydrogen atom, loss of at least one fluoride ion, and release of cytosine are involved in formation of the putative inactivating species. No alternative nucleotides appear to be produced. These results are compatible with all chemistry occurring within the active site in the absence of the DTT present in bulk solution. The study of isotopically labeled dFdCDP's with RDPR should provide further insight into the chemistry of this unusual enzyme-catalyzed reaction. At present the results suggest, as previously proposed[6-8] for eucaryotic systems, that ribonucleotide reductase should be given strong consideration as the target of dFdCDP to explain the cytotoxic effects observed in animal cell culture.

Acknowledgment. This work was supported by NIH Grant No. GM 29595 (J.S.) and American Cancer Society Grant No. CH-405 (M.J.R.).