Letters

Deoxyribonucleoside 2′- or 3′-Mixed Disulfides: Prodrugs To Target Ribonucleotide Reductase and/or To Inhibit HIV Reverse Transcription

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Abstract: Herein, we report the design, synthesis, and biological effects of nucleosides bearing a disulfide function on the sugar ring as prodrugs of potentially active mercaptonucleotides that can target ribonucleotide reductase or reverse transcriptase. We show that cytidine derivatives efficiently reduce dNTP pools in human CEM/SS cells and that 3′-deoxythymidin-3′-yl methyl disulfide is able to interfere with both cellular dNTP synthesis and HIV reverse transcription.

Introduction. Incorporation of a thiol function in nucleosides, nucleotides or oligonucleotides has led to a number of analogues possessing interesting biological properties.[1] For example, 2′,3′-dideoxy-3′-mercaptonucleoside 5′-triphosphates (T, C, A, G) irreversibly terminated DNA chain elongation by AMV (avian myeloblastosis virus) and HIV (human immunodeficiency virus) reverse transcriptases (RT).[2,3] Recently, we have demonstrated that another nucleotide possessing a thiol function on the sugar, 2′-deoxy-2′-mercaptouridine 5′-diphosphate, strongly inactivates in vitro *E. coli* ribonucleotide reductase.[4] Ribonucleotide reductases are essential enzymes that catalyze the de novo synthesis of 2′-deoxyribonucleoside 5′-(di or tri)phosphates.[5] As a common feature, these enzymes use a radical chemistry to perform the reduction of the four natural substrates. Class I ribonucleotide reductases found in eukaryotes and *E. coli* are composed of two homodimeric subunits named R1 and R2. The large R1 subunit contains substrate and allosteric binding sites in addition to five redox active cysteines. Subunit R2 harbors a stable free tyrosyl radical near an essential iron center. Binding of a substrate triggers electron and proton transfers from R2 to R1, leading to abstraction of H3′ from C3′ of the sugar ring (Scheme 1).

Because ribonucleotide reductase (RNR) is absolutely required for DNA replication and repair, it is considered to be one of the important targets for anticancer and antiviral drugs. Hydroxyurea (HU), which reduces the essential tyrosyl radical, has been widely used over the past 35 years as an anticancer drug.[6] Chemical modifications of the natural substrates at the 2′-position have also led to a number of mechanism-based inhibitors that display anticancer activities.[7] For example, 2′-deoxy-2′,2′-difluorocytidine (gemcitabine) is used in pancreatic carcinoma chemotherapy.[8] To become pharmacologically active, these nucleoside analogues must be phosphorylated to the corresponding diphosphates by cellular kinases. Inhibition of RNR is also of importance in HIV chemotherapy because 2′,3′-dideoxynucleoside 5′-triphosphates (ddNTPs) compete with 2′-deoxyribonucleoside 5′-triphosphates (dNTPs) at the active site of HIV RT. Decreasing the concentrations of natural dNTPs favors incorporation of ddNTPs into DNA and thus potentiate the antiviral activity of the ddN. This has been demonstrated in vitro and in vivo with HU and ddI.[9]

To prepare stable precursors of bioactive mercaptonucleotides, we have synthesized methyl,[10,11] propyl,[12] and symmetrical disulfides[12] of pyrimidine 2′- or 3′-deoxyribonucleosides **1**−**4**, **6**−**8**, and **10** (Figure 1) and shown that these nucleosides can be quantitatively and rapidly reduced at room temperature with dithiothreitol to give the corresponding thiols, for example, **5** and **9**. In this paper, we report the synthesis of the corresponding xylonucleoside methyl disulfides **11** and **12** (Figure 1) and show that only the cytidine disulfides **3**, **4**, and **7** and the 3′-deoxythymin-3′-yl methyl disulfide **8** deplete dNTP pools. Conversely, their uridine and xylo analogues **1**, **2**, **6** and **11**, **12**, respectively, do not affect these pools. Moreover, mercaptonucleosides **5** and **9** are ineffective. Inhibition of the target RNR probably occurs after intracellular reduction of the disulfide bond combined with phosphorylation to the corresponding 5′-diphosphate. The nucleoside analogue **8** exhibits unusual bioactivities by interfering both with the biosynthesis of dNTP and presumably with HIV reverse transcription. These results also show that nucleoside disulfides are interesting prodrugs of bioactive mercaptonucleotides.

Results and Discussion. Methyl disulfides of xylonucleosides such as the uracil and cytosine nucleosides **11** and **12** could, after phosphorylation, interfere with the cysteine residue 439 at the active site of RNR (Scheme 1). We synthesized these nucleosides in 90% and 84% yields, respectively, through the reaction of the corresponding 2-(trimethylsilyl)ethyl sulfides[13] with dimethyl(methylthio)sulfonium tetrafluoroborate in the

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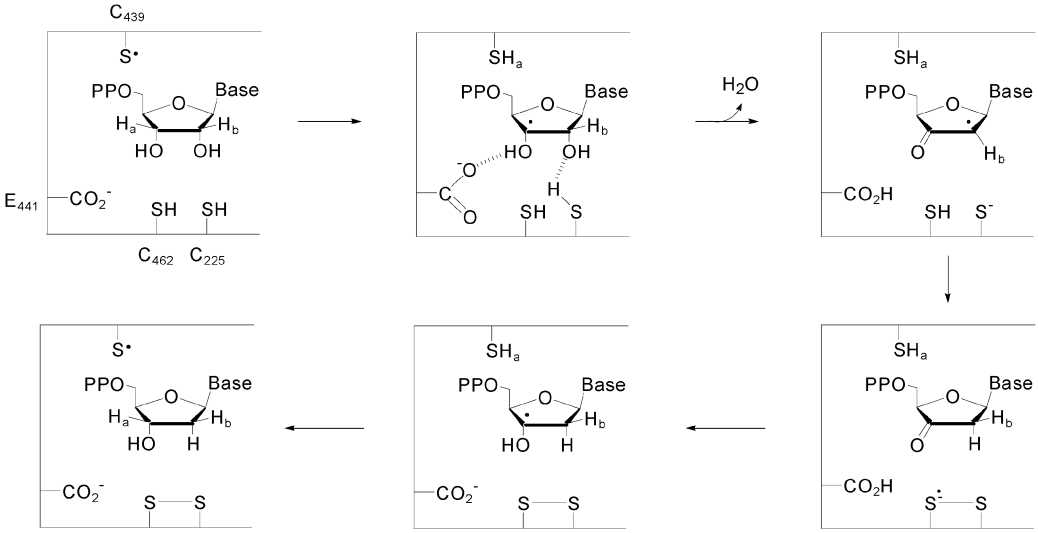
Scheme 1. Postulated Catalytic Mechanism of Reduction of the Natural Ribonucleoside 5′-Diphosphates by RNR[5]



presence of methyl disulfide in excess at room temperature (see Supporting Information).

The effects of the prepared disulfides and mercaptonucleosides **5** and **9** on cellular RNR were evaluated indirectly by measuring dNTP pools in the human lymphoblastoid cell line CEM/SS. Interestingly, studies using intact cells take into account the intracellular phosphorylation of the nucleosides. Quantification of dNTPs was performed using an enzymatic assay based on the catalytic elongation of radiolabeled oligonucleotide primers annealed to complementary templates.[14] In the first experiments, CEM/SS cells were incubated for 24 h with 200 μM of nucleoside disulfides. After cell numeration, the dATP content present in methanolic cell extracts was determined.[14] This nucleotide pool is indeed the most depleted during RNR inhibition.[15,16] Neither the uridine derivatives **1**, **2**, and **6** nor the xylonucleosides **11** and **12** modified dATP pools in CEM/SS (data not shown). We performed a dose-dependent analysis of the active compounds and reported the results in Figure 2. The mixed cytidine disulfides **3** and **4** were found to be equally efficient in lowering dATP levels in CEM/SS (inhibitory concentration or concentration required to deplete by 50% the intracellular dATP concentration $IC_{dATP-50} \cong 50$ μM). The symmetrical cytidine disulfide **7** and the thymidine derivative **8** exhibited $IC_{dATP-50}$ close to 200 μM. By contrast, 2′-deoxy-2′-mercaptocytidine **5** and 2′-deoxy-3′-mercaptothymidine **9** did not affect intracellular dATP concentration. Proliferation of CEM/SS measured after 24 h was decreased by 30% and 15% in the presence of compounds **3**, **4**, **7** and compound **8,** respectively, at 200 μM. This suggests, but does not demonstrate directly, that these nucleoside analogues inhibit RNR activity. Figure 3 depicts the pattern of depletion of the four dNTPs by compound **4**. Compound **3** gave a similar profile, while **7**, **8**, and HU showed less potency (see Supporting Information). Data for HU were in agreement with the literature.[16] The purine pools were the most depleted by nucleoside disulfides **3**, **4**, **7**, and **8**, as is the case with HU, strongly suggesting that these nucleoside analogues target RNR. The nucleoside thiol function can be easily released under mild reduction conditions; therefore, inhibition of the enzyme is likely to occur after intracellular reduction of the disulfide and

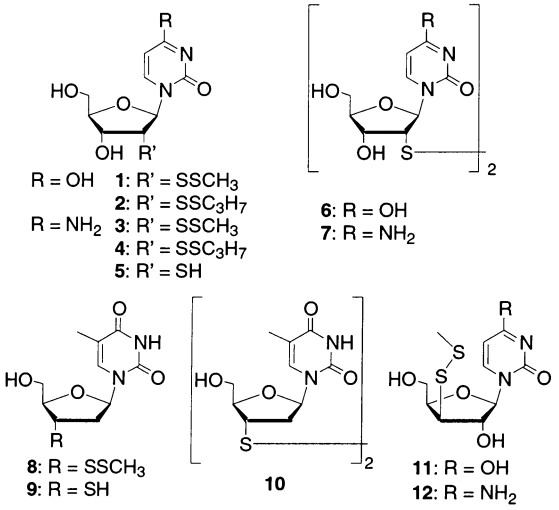


Figure 1. Structures of the studied disulfides.

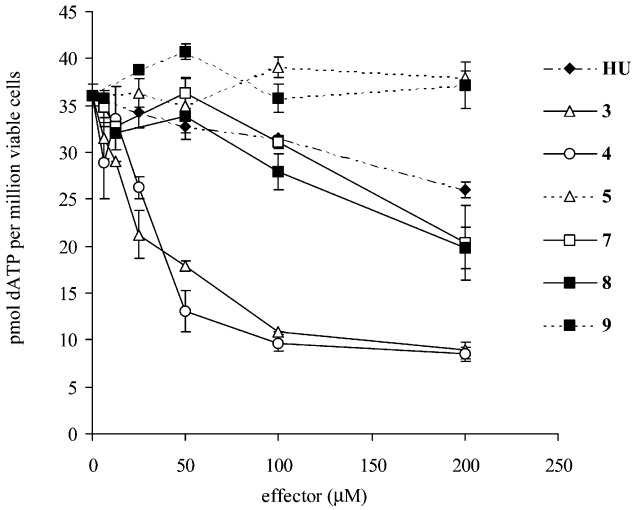


Figure 2. Dose-dependent effect of nucleosides **3**, **4**, **7**, and **8** on the dATP pool in CEM/SS after 24 h of incubation. Data represent the mean ± SD of at least two independent experiments.

Table 1. Anti-HIV-1 and -HIV-2 Activities in CEM Cells and Inhibitory Effects on the Proliferation of Human T-Lymphocyte Cells (CEM, Molt4/C8), Murine Leukemia Cells (L1210), and Murine Mammary Carcinoma Cells (FM3A) of the Methyl Disulfides **1**, **3**, and **8**, the Symmetrical Disulfide **10**, and the Mercaptonucleosides **5** and **9**

	EC_{50}[a] (μM)		IC_{50}[b] (μM)			
compd	HIV-1 (III_B) (CEM)	HIV-2 (ROD) (CEM)	CEM	Molt4/C8	L1210	FM3A
1	>65	>65	160 ± 40	220 ± 25	320 ± 25	>650
3	>65	>65	82 ± 2	255 ± 10	350 ± 35	>650
5	>340	>340	305 ± 35	650 ± 40	670 ± 7	>675
8	10 ± 3	37 ± 14	155 ± 40	25 ± 4	37 ± 6	280 ± 135
9	>390	>390	>770	>770	>770	>770
10	50 ± 40	60 ± 30	>390	325 ± 90	>390	>390
AZT	0.004 ± 0.001	0.004 ± 0.002	>375	>375		
tenofovir	4.2 ± 0.0	3 ± 1	>350	>350	>350	>350

[a] 50% effective concentration or compound concentration required to protect cells against the cytopathicity of HIV by 50%. [b] 50% inhibitory concentration or compound concentration required to inhibit cell proliferation by 50%.

phosphorylation to give the corresponding mercaptonucleoside 5′-diphosphate.

Since 2′-deoxy-2′-mercaptouridine 5′-diphosphate inactivates purified RNR,[4] we assume that uridine derivatives are not efficiently phosphorylated in cells. This base-dependent reactivity of nucleosides toward RNR has already been observed with 2′-azido-2′-deoxycytidine and 2′-azido-2′-deoxyuridine.[17] To gain insight into the activation step of **3**, **4**, **7**, and **8**, the experiments were carried out using a CEM cell line deficient in deoxycytidine kinase activity. The inhibitory potential of the disulfides **3**, **4**, and **7** was abolished in CEM/dCK$^-$ cells, suggesting that deoxycytidine kinase was responsible for the phosphorylation of these cytidine derivatives (data not shown). By contrast, the disulfide **8** was still effective in CEM/dCK$^-$, depleting the dATP pool by 30% at 200 μM.

2′,3′-Dideoxy-3′-mercaptoribonucleosides 5′-triphosphates (T, C, A, G) have been reported as highly effective terminators of HIV reverse transcription.[2] 2′,3′-Dideoxy-3′-mercaptocytidine showed protection against the HIV-1 strain ($HTLV\text{-}III_B$) in MT4 cells (ED_{50} = 20 μM), and the thymidine 3′-symmetrical disulfide **10** was found to be inactive.[3] However, an anti-HIV effect of **10** has been reported in infected MT4 cells (ED_{50} = 16 μM) with cell toxicity (CD_{50} = 34 μM).[18]

The prepared thymidine 3′-methyl disulfide **8** can be assessed as a prodrug of the corresponding potentially bioactive 3′-mercaptonucleoside 5′-triphosphate. Its effects on HIV-1 and HIV-2 strains were evaluated[18,19] and compared to those of the 2′-methyl disulfides **1** and **3** and to those of 3′-deoxy-3′-mercaptothymidine **9** and its symmetrical disulfide **10** (Table 1). Compounds **1** and **3** were devoid of anti-HIV-1 activity (EC_{50} > 65 μM) in infected CEM cells. In contrast, under the same conditions, the thymidine 3′-methyl disulfide **8** reduced HIV-1 multiplication by 50% at 5−10 μM on cultures of CEM cells, with an IC_{50} on CEM cells around 150 μM. A 4-fold lower effect was observed on HIV-2 (ROD). The thymidine methyl disulfide **8** also appeared more inhibitory to HIV than the corresponding thiol and symmetrical disulfide.[3,4]

The cytostatic activities of the methyl disulfides **1**, **3**, and **8** were evaluated on human T4-lymphocyte CEM and Molt4/C8 cells, murine leukemia (L1210), and murine mammary carcinoma cells (FM3A) (Table 1). The uridine and cytidine derivatives **1** and **3** showed antiproliferative effects against CEM cells with an IC_{50} of about 160 and 80 μM, respectively, but were less cytostatic against the other cell lines ($IC_{50} \geq 200$ μM).

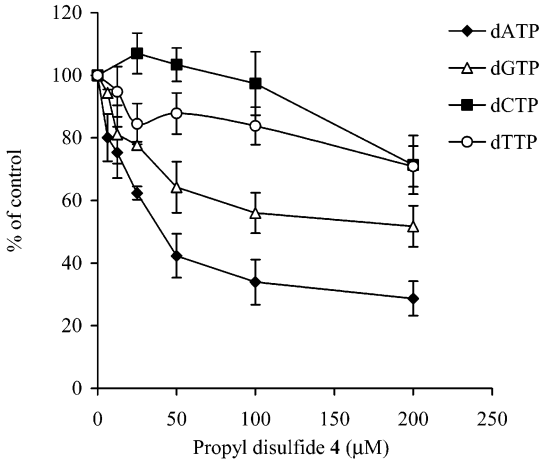


Figure 3. Dose-dependent effect of nucleoside **4** on the level of the four dNTPs. CEM/SS cells were incubated for 24 h at 37 °C in the presence or absence of **4**. The values of no-drug controls for dATP, dGTP, dCTP and dTTP were 35.8, 16.7, 11.8, and 22.9 pmol per million viable cells, respectively. Values represent the mean ± SD of two independent experiments performed in duplicate.

The thymidine derivative **8** appeared more cytostatic against Molt4/C8, L1210, and FM3A cells than nucleosides **1** and **3** (IC_{50} = 25, 40, and 280 μM, respectively). The effects of the three methyl disulfides appear to be highly dependent on the cell lines. The cytoxicities of compounds **3** and **8** on CEM/SS cells (MTT) were found to be low (CC_{50} = 330 and 120 μM, respectively).

Conclusion. Modified nucleosides have to be phosphorylated in cells to target RNR or RT in vivo and may act as antitumor or antiviral agents. Unfortunately, many nucleosides, the precursors of active 5′-di or -triphosphates, are poor substrates for nucleoside kinases. Our results validate a prodrug approach using stable mixed disulfides as precursors of easily oxidizable and unstable nucleotides bearing a thiol function on the sugar ring. Such an approach appeared to be previously successful in the eradication of tumor xenografts by targeted delivery of cytotoxic drugs with their immunoconjugates.[20] These drugs have been linked to antibodies via disulfide bonds, which ensures the release of the fully active drugs inside the cell after reduction. We show here that this concept can be applied to thionucleosides. 2′-Deoxycytidine mixed disulfides **3** and **4** were found to deplete dNTP pools in human CEM/SS cells, in agreement with the expected RNR inhibition, whereas the corresponding symmetrical disulfide **7** was less active and the corresponding thiol **5** was inactive.

The observed similar reductions of dNTP pools in the presence of methyl disulfide **3** or its propyl analogue **4** strongly suggest an effect of the corresponding mercaptonucleoside 5′-diphosphate. The corresponding uridine disulfides **1** and **2** did not interfere with dNTP synthesis probably because of lack of phosphorylation. 3′-Deoxythymidin-3′-yl methyl disulfide **8** was able to diminish HIV replication in CEM cells more efficiently than the corresponding thiol and symmetrical disulfide. Disulfide **8**, but not the corresponding thiol **9**, was also shown to interfere with cellular dNTP synthesis and to suppress efficiently L1210 and Molt4 cell proliferation. Natural substrates of class I RNR are ADP, GDP, CDP, and UDP. Compound **8** is a thymidine derivative in the 2′-deoxyribose series. As such, its diphosphate derivative is not expected to be recognized at the substrate binding site. On the other hand, the allosteric site of RNR binds dTTP and compound **8**, at the triphosphate level could mimic dTTP and interfere with RNR activity. dTTP activates GDP reduction and inhibits pyrimidine nucleotide reduction. However, analysis of dNTP pools shows that compound **8** depletes mostly purine nucleotide pools, as observed with the cytidine disulfides **3** and **4**. It would be interesting to determine if compound **8** targets RNR or other nucleotide-metabolizing enzymes.

It has been shown that RNR inhibitors potentiate the antiviral activity of nucleoside analogues.[9] On the basis of these results, new self-potentiated antiviral nucleosides could be developed, acting on RNR at the di- or triphosphate level and on HIV RT as triphosphates, thus enhancing their own antiviral activity by improving the ddNTP/dNTP ratio. To further such an approach, it will be interesting (i) to determine whether the disulfide or the thiol is the main phosphorylated form, (ii) to study the interaction of 3′-deoxythymidin-3′-ylmethyl disulfide 5′-di and 5′-triphosphates with purified RNR, and (iii) to synthesize and study the biological activities of 3′-deoxynucleosides 3′-methyl disulfides toward RNR and HIV RT.

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Supporting Information Available: Experimental details corresponding to the synthesis of the xylonucleosides **11** and **12** and to the biological experiments and dose-dependent effects of nucleosides **3**, **7**, **8**, and hydroxyurea on the level of the four dNTPs. This material is available free of charge via the Internet at http://pubs.acs.org.

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