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Synthesis and Biological Activity of a Bivalent Nucleotide Inhibitor of Ribonucleotide Reductase

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Abstract—A novel nucleotide inhibitor (ADP-S-HBES-S-dGTP) of mouse ribonucleotide reductase was designed to span the active site and the allosteric specificity site of the enzyme. The inhibitor contains ADP and dGTP moieties which are linked by 1,6-hexane-(bis-ethylenesulfone), and has a K_i value of 12 μM.

Introduction

Ribonucleotide reductase (RR) catalyzes the reduction of ribonucleotides to 2′-deoxyribonucleotides, the rate determining step in de novo DNA synthesis.[1,2] It is thus an important target for antiviral and cancer chemotherapeutic agents.[3]

Mammalian RR, a Class I enzyme, has two subunits, mR1 and mR2. The active site and allosteric regulation sites are located in the mR1 subunit, while a tyrosyl radical, required for efficient substrate turnover, is located in the mR2 subunit. The minimal functional form of the enzyme is the heterodimer $R1_2R2_2$. In *Escherichia coli* R1, which is homologous to mR1 (Pender et al., submitted for publication), the active site and allosteric specificity site lie near the $R1_2$ dimer interface, such that the straight-line distance between GDP in the active site in one R1 monomer and dTTP in the specificity site in the other, measured from the centers of the bases, is 14 Å[4] (Fig. 1). This proximity may account for the interesting functional interplay between the two sites that is related to the maintenance of the four cellular dNTP pools. Thus, for example, binding of dGTP in the specificity site induces ADP reductase, exclusively, while binding of dTTP induces GDP reductase.[5]

Bivalent ligands have recently attracted attention in the design of chemotherapeutic agents because of their potential for increased binding affinity to biological receptors via a chelating effect.[6,7] The proximity of the active site and allosteric specificity site in RR led us to design a potential bivalent nucleotide inhibitor for this enzyme, **7**, which contains ADP and dGTP moieties linked via their 8-positions by 1,6-hexane-(bis-ethylenesulfone) (HBES). The length of this spacer is 14.7 Å.

Synthesis

The synthesis of **7** was accomplished by Michael addition reaction of the vinyl sulfone thioether **3** with 8-mercapto-dGTP **6** (Scheme 2). Compound **3** was prepared in three steps from ADP sodium salt (Scheme 1), using published protocols[8,9] for the synthesis of 8-bromo-ADP **1** and 8-mercapto-ADP **2**. Compound **2** (1.3 mg, 3 μmol, in 500 μL water) was added to HBVS (1,6-hexane-(bis-vinylsulfone), 11 mg, 45 μmol, Pierce Chemical Company) in 50 μL of DMSO. The pH value of the reaction mixture was adjusted to pH 7.5–8 with 1 M ammonia water. The reaction was maintained at 37 °C for 24 h, yielding **3** (85%) and small amounts of the symmetrical diadduct **4** (5%).

8-Bromo-dGTP **5** was prepared from dGTP sodium salt according to the published protocol.[10] Preparation of **6** from **5** was complicated by hydrolysis of the triphosphate moieties of both **5** and **6** under the conditions normally used for replacement of the 8-Br group by an 8-SH group on a guanosine nucleus.[11] Nevertheless, careful optimization of $Na_2S_2O_3$ concentration, temperature, and reaction time afforded an acceptable yield of **6** in one step from **5**, avoiding the necessity of a longer synthetic route, with anticipated lower overall yield, via conversion of 8-Br-dGMP to 8-mercapto-dGMP and

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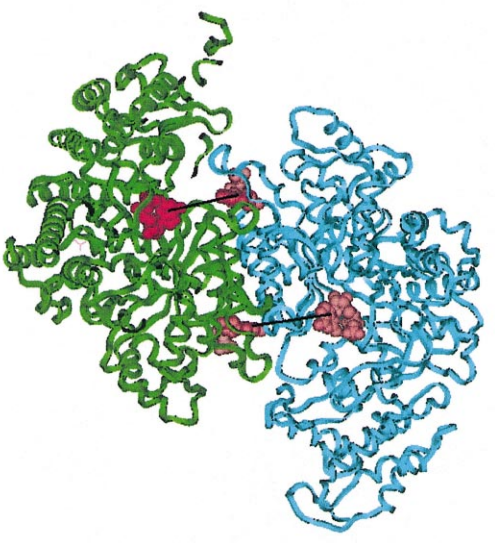
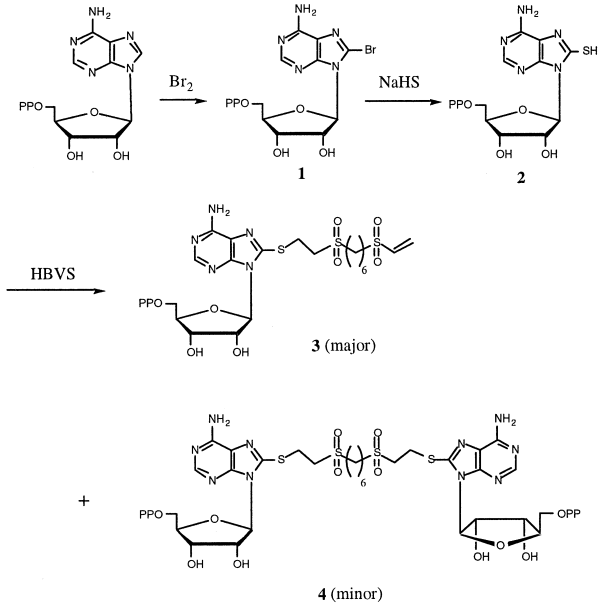
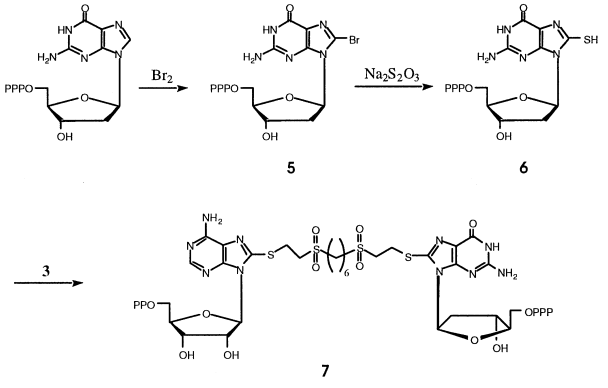


Figure 1. Structure of *Escherichia coli* R1 dimer with dTTP at the specificity site and GDP at the active site.[4] The straight line shows the distance between the base-ring centers of nucleotides in these two sites.



Scheme 1. Synthesis of 8-substituted ADP derivatives.



Scheme 2.

two phosphorylation reactions. One μmole of 8-Br-dGTP was dissolved into 700 μL of butanol:ethanol: water (4:2:1), and 200 μmol of sodium thiosulfate was added.[12] The reaction mixture was kept at 100 °C for 25 min to give **6** in 40% yield, determined following HPLC purification. Compounds **3** (0.6 μmol) and **6** (0.06 μmol) were combined in 10 μL of ammonia water (pH 8). Incubation at 37 °C for 36 h afforded **7** in 60% yield. Excess **3** was reisolated for repeat use in synthesizing additional **7**.

All new compounds were purified to homogeneity by RP-HPLC (Synchropak C_{18}, Micra Scientific Inc.) using a 0 to 5% acetonitrile in 20 min gradient containing 0.01 M triethylammonium acetate (pH 4.6). The flow rate was 1 mL/min. Under these conditions, **6** (elution time, 16 min) was baseline resolved from 8-mercapto-dGDP (13 min), 8-Br-dGDP (18 min) and **5** (20 min). Each compound was characterized by UV spectrum,[13,14] total phosphate analysis[15] and electrospray ionization mass spectrometry (ESI-MS). The structure of **7** was confirmed by MALDI-TOF mass spectrometry.

Biological Activity Evaluation

Compounds were assayed for as inhibitors using a standard RR activity assay.[16] ADP derivatives were tested for inhibition of dGTP-activated ADP reduction, and dGTP derivatives and **7** were tested for the inhibition of dTTP-activated GDP reduction. K_m values and K_i values were calculated using Lineweaver–Burke and Dixon plots, respectively, giving the results summarized in Table 1. Given the low turnover number of RR (0.18 and 0.28 s^{-1} for ADP and GDP, respectively),[17] it is not unreasonable to assume that K_m and K_d values for substrate are essentially equivalent. With this assumption, the results show that 8-alkylthio substitution at the ADP position is well tolerated in the active site, since both **3** and **4**, which is not expected to bind bivalently, have slightly higher affinity than ADP itself. On the other hand, **1** and **2** bind more weakly, perhaps reflecting unfavorable steric or electronic effects. The allosteric specificity site is somewhat more sensitive to 8-alkylthio substitution. The 8-mercapto-dGTP mono adduct with HBVS, **8**, synthesized from **6** in a manner analogous to

Table 1. K_i values

Nucleotide	K_i (μM)
ADP	34±4[a]
8-Bromo-ADP **1**	77±3
8-Mercapto-ADP **2**	158±8
ADP-8-S-HESVS **3**	22±3
ADP-8-S-HBES-S-8-ADP **4**	19±2
dGTP	1.8±0.1[b]
8-Bromo-dGTP **5**	56±4
8-Mercapto-dGTP **6**	13±2
ADP-8-S-HEVS-S-8-dGTP **7**	12±2
dGTP-8-S-HESVS **8**	9±1

[a] K_m of ADP as substrate.
[b] K_d of dGTP binding to specificity site (ref 17).

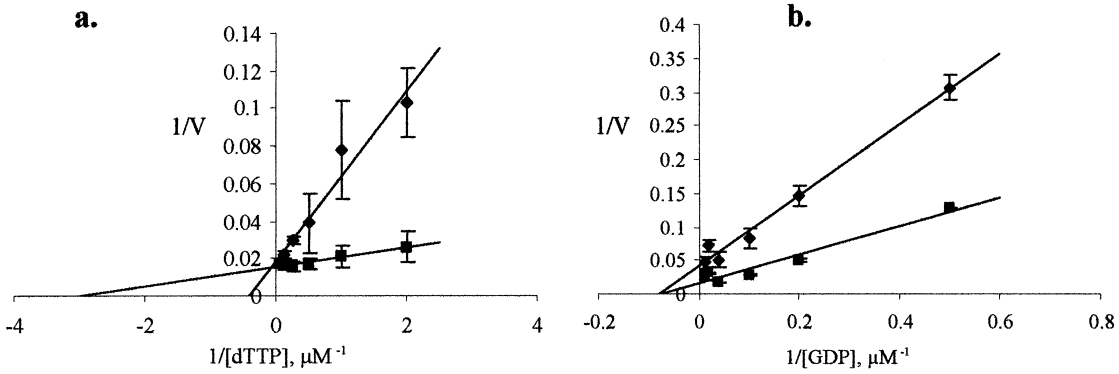


Figure 2. Lineweaver–Burke plots demonstrating the effect of added **7** on GDP reductase, measured as a function of either dTTP concentration (specificity activator, part a), or GDP concentration (substrate, part b). No inhibitor (■); +20 μM of **7** (◆).

2 to **3** conversion but with longer reaction time (36 h), has about a five-fold lower affinity than dGTP itself. 8-Mercapto-dGTP **6**, binds with approximately the same lower affinity, while the 8-Br derivative **5** has markedly lower affinity.

Disappointingly, the designed bivalent inhibitor **7** has approximately the same affinity as **6** and **8**, suggesting that this compound binds only to the allosteric site and not to the active site. This suggestion was confirmed by Lineweaver–Burke plots (Fig. 2) showing that **7** is competitive with dTTP for the specificity site binding, while it is a noncompetitive inhibitor toward GDP.

Nevertheless, the present results provide grounds for optimism that high affinity bivalent inhibitors of RR are attainable. We believe that the failure of **7** to bind bivalently reflects a nonoptimal tether, since the results in Table 1 show that 8-alkylthio substitution is reasonably well-tolerated at both targeted sites. Future experiments will focus on tether optimization, via straightforward modifications of the synthetic route for **7**.

Acknowledgements

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