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Structure-based Design, Synthesis, and Evaluation of 2'-(2-Hydroxyethyl)-2'-Deoxyadenosine and Its 5'-Diphosphate as Novel Ribonucleotide Reductase Inhibitors

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Abstract

Analysis of the recently solved X-ray crystal structures of yeast ribonucleotide reductase I (RnrI) in complex with effectors and substrates led to the discovery of a conserved water molecule located at the active site that interacted with the 2' hydroxy of the nucleoside ribose. In this study 2'-(2-hydroxyethyl)-2'-deoxy-adenosine **1** and its 5'-diphosphate **2** were designed and synthesized to see if the conserved water molecule could be displaced by a hydroxymethylene group, to generate a novel of inhibitors of this enzyme towards the development of potential anti-neoplastic agents. In this paper, we report the synthesis of these two adenosine analogs **1** and **2**, and the co-crystal structure of adenosine diphosphate analog **2** bound with RnrI enzyme which displaces the conserved water as hypothesized.

Keywords

structure-based design; ribonucleotide reductase; nucleoside; nucleotide; crystallography

Introduction

Ribonucleotide reductases (RNR) catalyze the reduction of ribonucleotides to deoxyribonucleotides, which are essential precursors of DNA synthesis. RNR is crucial for rapidly proliferating cells and thus represents an attractive target for anticancer and antiviral chemotherapy.[1,2] Previously, we had solved the three dimensional X-ray structures of yeast ribonucleotide reductase I (RnrI) in complex with dGTP-ADP, dTTP-GDP, AMPNP-CDP, AMPPNP-UDP, and gemcitabine diphosphate[3,4]. In all the yeast RnrI structures bound with effectors-substrates we observed a conserved water molecule located at the active site that interacted with the 2' hydroxy of the ribose, carbonyl of G247, NH₂ of N426, and the amide of L427 (Figure 1A). Inspired by this observation, 2'-(2-hydroxyethyl)-2'-deoxy-adenosine **1** and its 5'-diphosphate **2** (Figure 1B) were subsequently designed as potential cytotoxic agents and RnrI enzyme inhibitors correspondingly. The designing rationale was that the hydroxy ethyl moiety is linked to the 2' carbon of the ribose, where its

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hydroxyl group can chemically mimic this conserved water molecule bound at the RnrI active site in an effort to enhance binding affinity. Herein, we report the synthesis of these two adenosine analogs **1** and **2**, and the evaluation of these compounds including the crystal structure of yeast RnrI enzyme in complex with adenosine diphosphate analog **2**.

Results and Discussion

Chemistry

In order to synthesize 2'-deoxyadenosine analog **1**, an efficient synthetic route was designed starting from adenosine (Scheme 1). Selective protection of its 3'- and 5'-hydroxys with a cyclic disiloxane group was achieved by reacting adenosine with 1,3-dichloro-1,1,3,3-tetra-isopropylidisiloxane in anhydrous pyridine in 59% yield.[5-7] Chemoselective thioacylation of **3** in the presence of 4-dimethylaminopyridine (DMAP) afforded ester **4** in 89% yield. This was followed by radical-induced reductive cleavage of the ester in the presence of allyltributylstannane to give 2'-allyl-2'-deoxy adenosine derivative **5** in 85% yield.[5-7] Under these conditions only minor amounts of 3',5'-*O*-(1,1,3,3-tetra-isopropylidisilox-1,3-diyl) 2'-deoxyadenosine and 3',5'-*O*-(1,1,3,3-tetra-isopropylidisilox-1,3-diyl)adenosine byproducts were formed. No corresponding β configuration isomer of the allyl group at the 2' position was observed, and the α configuration was confirmed by NOESY NMR spectra, as strong NOEs were observed between H-1' and allylic protons, H-2' and H-3', and H-2' and H-8 of the adenine heterocyclic ring. Next dihydroxylation of olefin **5** was performed using osmium tetroxide to give **6** as a mixture of diastereomeric diols (molar ratio: 1:0.7 based on ^1H NMR analysis), this mixture was carried directly forward into the next reaction and subsequent cleavage of this vicinal diol with NaIO_4 afforded the corresponding aldehyde **7** in 79% yield as a single product.[8] Reduction of aldehyde **7** to primary alcohol **8** was achieved using sodium borohydride in methanol. Final deprotection using tetra-*n*-butylammonium fluoride (TBAF) of **8** provided target molecule 2'-(2-hydroxyethyl)-2'-deoxy-adenosine **1** in 83% yield.

To make its corresponding diphosphate analog **2** of compound **1** a modified synthetic approach was required due to the equivalence of the two primary alcohols found in **1**, which complicated the phosphorylation procedure and made direct conversion of **2** from **1** problematic in test reactions. This problem was solved by using intermediate **8** from the previous synthesis and employing orthogonal protecting strategy for the 2' hydroxyl ethyl group (scheme 2). Alcohol **8** was first trityl protected using trityl chloride in the presence of DCM and DMAP to give protected product **9** in 88% yield. Subsequent deprotection of the silyl protecting group in **9** by TBAF yielded diol **10** in 87% yield. Selective tosylation of the primary alcohol group of **10** gave desired 5'-substituted product **11** in 44% yield, along with minor amounts of the 3'-substituted and 3', 5'-disubstituted products. Deprotection of the trityl group of **11** was then achieved using mild acid conditions of 80% AcOH in water at 50°C for 4h to afford compound **12** in excellent yield. These conditions were found favourable over standard trityl deprotection conditions of 1% TFA in DCM containing 5% triisopropyl silane as significant detosylation occurred in test reactions. Nucleophilic diphosphorylation of tosylate **12** with tris(tetra-*n*-butylammonium) hydrogen pyrophosphate in acetonitrile was performed according to the protocol of Poulter to give 2'-deoxyadenosine diphosphate analog **2** in 38% yield.[9] Finally, the tetra-*n*-butylammonium salt form of **2** was exchanged to the ammonium salt using DOWEX AG 50W-X8 ion exchange resin to provide **2** in a suitable form for subsequent biological evaluation studies.

Co-crystal studies

To examine if compound **2** has the potential to bind to ribonucleotide reductase, **2** was soaked into crystals of yeast RnrI and the crystals were subject to X-ray crystallographic

analysis. The 2Fo-Fc electron density map clearly shows that the effector dGTP is bound at the S-site (specificity site) and the 2'-(2-hydroxyethyl)-2'-deoxy-adenosine compound **2** is bound at the catalytic site (C-site) (Figures 2A, B). The effector dGTP in the present structure binds similarly to that observed in the dGTP-ADP structure[3]. Both loop1 and loop2 also have the same conformation except that in the dGTP-compound **2** structure, part of loop2 (N291-R293) was disordered and could not be modelled because no electron density was observed (Figure 2B). Data collection and refinement statistics for compound structure **2** are shown in table 1.

The comparison of the dGTP-compound **2** structure with the dGTP-ADP structure reveals that as desired the water molecule occupying the catalytic site in all ScRnr1 structures[3,4] is displaced by the OH group of the hydroxyethyl moiety of **2**. In fact, ADP portion of **2** fits into the catalytic pocket with minimal disruption (Figure 3). The OH of the hydroxyethyl group makes several hydrogen bonds at the C-site, which involve residues G247 O, N426 Nδ2, and L427 N. These same residues also form second sphere hydrogen bonds to the 2'OH of ADP through the conserved water molecule in the ScRnr1-ADP structure (Figure 4 and Table 2). However, the 2' OH of ADP substrate makes two additional interactions with residues S217 and C218 which compound **2** cannot make due to the hydroxyethyl substitution. The ribose group and the phosphates of **2** and ADP binds similarly to ScRnr1 as described before (Figures 4)[3,4]. There are some differences in the way the adenine base of compound **2** interacts with ScRnr1. In the dGTP-ADP structure, the N1 atom of the base makes two hydrogen bonds to the side chains of Q288 and R293 (Figure 4B), while in the dGTP-compound **2** structure, Q288 only forms a van der Waals interaction with the base and we do not observe the pi-stacking interaction between the guanidinium group of R293 and the adenine base as R293 is disordered in our dGTP-compound **2** structure (Figure 4A).

Enzyme inhibition tests were performed on **2**. The specific activity of ScRNR was 96 nmol/mg/min where Rnr2Rnr4 is in 5 molar excess. However, there was no detectable inhibition even at 0.5 M concentration of **2**. This demonstrated **2** to be a poor inhibitor of ScRnr1 with respect to ADP. This is presumably due to the decreased interactions described above that outweigh the benefit of the displacing the conserved water molecule with the hydroxyl ethyl group. However, inhibition with respect to CDP reduction has to be conducted. Cytotoxicity tests were performed on compound **1** as **2** is phosphorylated and thus unlikely to be able to penetrate cells. These tests showed low level cytotoxic activity presumably due to low target enzyme inhibition and/or poor cellular activation by cellular nucleotide kinases.

Conclusion

Described herein we have been able to design, synthesize and observe a novel inhibitor of ScRnr1 that binds competitively to the active site. This molecule binds in a similar mode to the substrate and displaces the conserved water molecule as designed. Comparing the interactions (hydrogen bonds, van der Waals interactions, ion pairs) made by compound **2** with that by ScRnr1-ADP (Table 2), we conclude that: 1) the 2'OH modified to hydroxyethyl group lost two hydrogen bond interactions within the C-site. 2) the fact that substrate ADP base makes additional hydrogen bonds is caused by the absence of both residue R293 (due to disordered in the electron density map) and some water molecules in the Rnr1-compound **2** structure (Table 2). Thus, though displacement of conserved waters is often considered as highly desirable for inhibitor design in this case the displacement does not overcome the loss of other interactions producing an overall weak inhibitor. However, we believe this co-crystal structure provides valuable information for guiding the design of a second generation of competitive Rnr inhibitors, which can regain some of the lost interactions and generate new enzyme inhibitor interactions to create higher affinity inhibitors.

Experimental Section

All regular and anhydrous solvents were purchased from Aldrich and Fisher and used as received. All chemicals were from Aldrich. Thin layer chromatography (TLC) analysis was done on Merck silica gel 60F₂₅₄ plates and the spots were visualized under a UV lamp. IR spectra were obtained using a Perkin Elmer 1600 series FTIR spectrometer. ¹H and ¹³C NMR spectra were recorded at 500 MHz on a Varian Inova NMR instrument except the ¹³C NMR spectrum of **7** was recorded at 300 MHz on a Bruker ARX instrument. Mass spectra were recorded on a Bruker Esquire LC/MS using ESI.

Analytical RP-HPLC (method A and B) was conducted on an Agilent 1100 HPLC system with an Alltech platinum EPS C18 column (100Å, 5 µm, 4.6 × 150 mm) with precolumn 4.6 × 10 mm, flow rate 1.0 mL/min and a gradient of solvent A (water with 0.1% TFA) and solvent B (acetonitrile): (Method A): 0-2.00 min 100% A; 2.00-17.00 min 0-100% B (linear gradient); 17.00-19.00 min 100% B; (Method B): 0-2.00 min 30% B; 2.00-17.00 min 30-100% B (linear gradient); 17.00-19.00 min 100% B; Analytical RP-HPLC (method C and D) was conducted on a Shimadzu HPLC system with a Phenomenex C18 column (100Å, 3 µm, 4.6 × 50 mm), flow rate 1.0 mL/min and a gradient of solvent A (water) and solvent B (acetonitrile) (method C): 0-2.00 min 100% A; 2.00-8.00 min 0-100% B (linear gradient), 8.00-9.00 min 100% B; (method D): 0-2.00 min 90% B; 2.00-7.00 min 90-100% B (linear gradient), 7.00-9.00 min 100% B.

3',5'-O-(1,1,3,3-Tetraisopropylidisilox-1,3-diyl)adenosine (**3**)

To 3.97 g (14.85 mmol) of adenosine suspended in 100 mL of anhydrous pyridine was added 4.75 mL (14.85 mmol) of 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane (TPDS-Cl₂), and the mixture was stirred at room temperature under argon for 3 h. Pyridine was evaporated and the residue was suspended in ethyl acetate (150 mL), the organic phase was washed with saturated NaHCO₃ (3 × 75 mL) and evaporated. The resulting residue was purified by column chromatography on silica (eluent: chloroform to 10% methanol/chloroform). Evaporation of appropriate fractions gave 4.5 g (59%) of **3** as a white powder. TLC: R_f = 0.58 (methanol/chloroform = 1/10) (v/v). IR (neat): 3322, 3146, 2944, 1643, 1598, 1033, 989, 883 cm⁻¹. ¹H NMR (DMSO-*d*₆): δ 8.22 (s, 1H, ⁸CH), 8.08 (s, 1H, ²CH), 7.35 (br s, 2H, NH₂), 5.88 (d, *J* = 1.0 Hz, 1H, *H*-1'), 5.64 (d, *J* = 4.6 Hz, 1H, *OH*-2', exchangeable with D₂O), 4.79 (dd, *J* = 5.1 and 8.5 Hz, 1H, *H*-3'), 4.52 (dd, *J* = 4.2 and 4.4 Hz, 1H, *H*-2'), 4.06 (dd, *J* = 3.4 and 12.7 Hz, 1H, CH^a-5'), 4.00 (ddd, *J* = 2.7, 2.9, and 8.5 Hz, 1H, *H*-4'), 3.93 (dd, *J* = 2.7 and 12.7 Hz, 1H, CH^{a'}-5'), 1.04 (m, 28H, 4 × *i*-Pr). ¹³C NMR (DMSO-*d*₆): δ 156.51, 152.89, 149.08, 139.70, 119.71, 89.81, 81.21, 74.10, 70.25, 61.23, 17.83, 17.67, 17.67, 17.64, 17.47, 17.38, 17.36, 17.30, 13.20, 12.92, 12.70, 12.52. Mass spectrum (ESI) *m/z* (MNa)⁺ 532.3; (M)⁻ 508.3. HPLC purity (method A): 97.3%, *t*_R = 15.4 min.

2'-O-Phenoxythiocarbonyl-3',5'-O-(1,1,3,3-tetraisopropylidisilox-1,3-diyl)adenosine (**4**)

To **3** (3.59 g, 7.04 mmol) was added 60 mL of anhydrous methylene chloride, 4-dimethylaminopyridine (DMAP) (1.72 g, 14.08 mmol) and phenoxythiocarbonyl chloride (PTC-Cl) (1.14 mL, 8.45 mmol). The solution was stirred at room temperature for 18 h. The solvent was evaporated and the residue was purified by column chromatography on silica (eluent: chloroform to 3% methanol/chloroform). Evaporation of appropriate fractions gave 4.04 g (89%) of **4** as a white powder. TLC: R_f = 0.64 (methanol/chloroform = 1/10) (v/v). IR (neat): 3312, 3144, 2944, 1771, 1679, 1645, 1601, 1198, 1033, 883 cm⁻¹. ¹H NMR (DMSO-*d*₆): δ 8.29 (s, 1H, ⁸CH), 8.05 (s, 1H, ²CH), 7.50 (t, *J* = 7.8 Hz, 2H(Ph)), 7.42 (br s, 2H, NH₂), 7.35 (t, *J* = 7.3 Hz, 1H(Ph)), 7.17 (d, *J* = 8.1 Hz, 2H(Ph)), 6.50 (d, *J* = 5.4 Hz, 1H, *H*-2'), 6.35 (s, 1H, *H*-1'), 5.52 (dd, *J* = 3.2 and 5.6 Hz, 1H, *H*-3'), 4.00 (m, 3H, *H*-4' and

CH_2-5'), 1.06 (m, 28H, $4 \times i\text{-Pr}$). ^{13}C NMR (DMSO- d_6): δ 194.11, 156.71, 153.34, 153.02, 149.01, 141.13, 130.36, 127.35, 121.99, 119.71, 86.63, 84.32, 81.51, 70.05, 61.00, 17.75, 17.70, 17.62, 17.57, 17.46, 17.36, 17.28, 13.37, 13.11, 12.84, 12.72, 12.66. Mass spectrum (ESI) m/z (MNa) $^+$ 668.4; (M) $^-$ 644.0. HPLC purity (method A): 98.5%, t_R = 17.5 min.

2'-Allyl-2'-deoxy-3',5'-O-(1,1,3,3-tetraisopropylidisilox-1,3-diyl)adenosine (5)

To a solution of **4** (0.646g, 1mmol) in 10 mL of anhydrous toluene in a quartz tube, α,α' -azoisobutyronitrile (AIBN) (82.5 mg, 0.5 mmol) and allyltributylstannane (1.54 mL, 5 mmol) were added. The solution was degassed with argon for 20 min, then the quartz reaction tube was put inside a UV reactor (the light source RPR-3500, Southern N. E. Ultraviolet Co.). The reaction vessel was rotated inside the UV reactor for 15 h. After completion of the reaction (checked by TLC and MS). Solvent was evaporated and the residue was purified by column chromatography on silica (eluent: chloroform to 3% methanol/chloroform). Evaporation of appropriate fractions gave 0.451 g (85%) product as a white powder. TLC: R_f = 0.77 (methanol/chloroform = 1/10) (v/v). IR (neat): 3279, 3142, 2944, 1676, 1600, 1119, 1033, 883 cm^{-1} . ^1H NMR (DMSO- d_6): δ 8.28 (s, 1H, ^8CH), 8.10 (s, 1H, ^2CH), 7.31 (br s, 2H, NH_2), 5.92 (d, J = 4.6 Hz, 1H, $H-1'$), 5.76 (m, 1H, $\text{CH}=\text{}$), 5.07 (dd, J = 1.5 and 17.1 Hz, 1H, $\text{CH}^{\text{a}}=\text{}$), 5.04 (dd, J = 2.9 and 5.1 Hz, 1H, $H-3'$), 4.94 (dd, J = 1.5 and 10.3 Hz, 1H, $\text{CH}^{\text{a}}'=\text{}$), 3.93 (m, 3H, $H-4'$ and CH_2-5'), 3.10 (m, 1H, $H-2'$), 2.52 (m, 1H, $\text{CH}^{\text{b}}(\text{all})$), 2.21 (m, 1H, $\text{CH}^{\text{b}}'(\text{all})$), 1.05 (m, 28H, $4 \times i\text{Pr}$). ^{13}C NMR (DMSO- d_6): δ 156.56, 152.98, 149.34, 140.18, 136.22, 119.69, 117.38, 87.05, 84.13, 72.77, 63.29, 46.02, 30.69, 17.85, 17.69, 17.68, 17.67, 17.51, 17.42, 17.39, 17.36, 13.23, 13.10, 12.74, 12.67. Mass spectrum (ESI) m/z (MNa) $^+$ 556.3; (M) $^-$ 532.2. HPLC purity (method B): 95.2%, t_R = 16.0 min.

2'-(2,3-Dihydroxypropyl)-2'-deoxy-3',5'-O-(1,1,3,3-tetraisopropylidisilox-1,3-diyl)adenosine (6)

Allyl adenosine derivative **5** (0.534 g, 1 mmol) and *N*-methylmorpholine *N*-oxide (NMO) (0.152 g, 1.3 mmol) were suspended in H_2O /acetone (20 mL, 1/3, v/v), followed by the addition of osmium tetroxide (2.54 mg, 0.01 mmol). The reaction mixture was stirred at room temperature overnight. After removing the solvent, the crude material was chromatographed on silica gel (eluent: chloroform to 5% methanol/chloroform) to afford 0.49 g (86%) diastereomeric products (molar ratio: 1:0.7 based on ^1H NMR analysis) as a white noncrystalline solid. TLC: R_f = 0.46 (methanol/chloroform = 1/10) (v/v). IR (neat): 3320, 3154, 2944, 1643, 1602, 1120, 1032, 884 cm^{-1} . ^1H NMR (DMSO- d_6) (major isomeric product): δ 8.27 (s, 1H, ^8CH), 8.10 (s, 1H, ^2CH), 7.31 (br s, 2H, NH_2), 5.98 (d, J = 3.9 Hz, 1H, $H-1'$), 5.04 (dd, 1H, J = 7.6 and 7.1 Hz, $H-3'$), 4.53 (t, J = 5.6 and 5.4 Hz, 1H, OH), 4.48 (m, 1H, OH), 3.93 (m, 3H, $H-4'$ and CH_2-5'), 3.51 (m, 2H, CH_2), 3.23 (m, 1H, CH), 3.14 (m, 1H, $H-2'$), 1.73 (m, 1H, CH^{a}), 1.60 (m, 1H, CH^{a}'), 1.05 (m, 28H, $4 \times i\text{Pr}$). (minor isomeric product): δ 8.25 (s, 1H, ^8CH), 8.10 (s, 1H, ^2CH), 7.31 (br s, 2H, NH_2), 6.01 (d, J = 5.6 Hz, 1H, $H-1'$), 4.95 (m, 1H, $H-3'$), 4.59 (d, J = 5.4 Hz, 1H, OH), 4.48 (m, 1H, OH), 3.93 (m, 3H, $H-4'$ and CH_2-5'), 3.29 (m, 2H, CH_2), 3.22 (m, 1H, CH), 3.07 (m, 1H, $H-2'$), 2.03 (m, 1H, CH^{a}), 1.22 (m, 1H, CH^{a}'), 1.06 (m, 28H, $4 \times i\text{Pr}$). ^{13}C NMR (DMSO- d_6): δ 156.80, 153.21, 153.15, 149.71, 149.47, 140.25, 119.92, 119.88, 88.27, 87.74, 84.94, 83.87, 72.76, 72.60, 70.73, 69.09, 66.94, 66.82, 63.49, 63.21, 44.45, 43.37, 30.39, 29.88, 18.10, 17.95, 17.93, 17.91, 17.76, 17.68, 17.66, 17.65, 17.61, 13.62, 13.45, 13.30, 13.09, 12.97, 12.92, 12.87. Mass spectrum (ESI) m/z (MNa) $^+$ 590.4; (M) $^-$ 566.2. HPLC purity (method A): 100%, t_R = 16.4 min.

2'-(2-Formylmethyl)-2'-deoxy-3',5'-O-(1,1,3,3-tetraisopropylidisilox-1,3-diyl)adenosine (7)

Dihydroxylation product **6** (0.334 g, 0.588 mmol) and sodium periodate (0.138 g, 0.647 mmol) were suspended in H_2O /dioxane (9 mL, 1/3, v/v), the reaction mixture was stirred at

room temperature overnight. The solvent was evaporated, the resulting solid residue was suspended in minimum amounts of ice-water, filtration was performed and the filter cake was dried to give 0.25 g (79%) product. TLC: R_f = 0.65 (methanol/chloroform = 1/10) (v/v). IR (neat): 3323, 2944, 1724, 1614, 1035, 883 cm^{-1} . ^1H NMR (DMSO- d_6): δ 9.70 (s, 1H, CHO), 8.26 (s, 1H, ^8CH), 8.10 (s, 1H, ^2CH), 7.33 (br s, 2H, NH_2), 5.92 (d, J = 4.4 Hz, 1H, $H-1'$), 5.15 (dd, J = 2.4 and 5.1 Hz, 1H, $H-3'$), 3.94 (m, 3H, $H-4'$ and CH_2-5'), 3.56 (m, 1H, $H-2'$), 2.94 (ddd, J = 1.2, 8.1, and 18.1 Hz, 1H, CH^a), 2.70 (dd, J = 6.1 and 18.1 Hz, 1H, CH^a), 1.05 (m, 28H, $4 \times i\text{Pr}$). ^{13}C NMR (DMSO- d_6): δ 201.81, 156.60, 152.94, 149.30, 140.41, 119.75, 87.29, 83.91, 72.41, 63.14, 41.97, 40.68, 17.84, 17.68, 17.66, 17.36, 17.29, 17.27, 13.13, 13.07, 12.76, 12.66. Mass spectrum (ESI) m/z (MNa) $^+$ 558.3. HPLC purity (method A): 97.1%, t_R = 16.4 min.

2'-(2-Hydroxyethyl)-2'-deoxy-3',5'-O-(1,1,3,3-tetraisopropylidisilox-1,3-diyl)adenosine (8)

To a suspension of aldehyde **7** (0.25 g, 0.46 mmol) in methanol (5 mL) sodium borohydride (0.088 g, 2.33 mmol) was added, the reaction mixture was stirred at room temperature overnight. The solvent was evaporated, the residue was purified by column chromatography on silica (eluent: chloroform-4% methanol/chloroform) to yield 0.24 g (97%) of **8**. TLC: R_f = 0.62 (methanol/chloroform = 1/10) (v/v). IR (neat): 3280, 3140, 2943, 1681, 1602, 1121, 1036, 883 cm^{-1} . ^1H NMR (CDCl_3): δ 8.31 (s, 1H, ^8CH), 8.22 (s, 1H, ^2CH), 6.40 (br s, 2H, NH_2), 6.34 (s, 1H, $H-1'$), 5.67 (br s, 1H, OH), 4.62 (t, J = 8.1 Hz, 1H, $H-3'$), 4.25 (dd, J = 1.7 and 12.9 Hz, 1H), 4.04 (dd, J = 2.7 and 13.2 Hz, 2H), 3.98 (dt, J = 2.2 and 8.8 Hz, 1H, $H-4'$), 3.89 (m, 1H), 2.66 (m, 1H, $H-2'$), 2.14 (m, 1H, CH^a), 1.75 (m, 1H, CH^a), 1.05 (m, 28H, $4 \times i\text{Pr}$). ^{13}C NMR (CDCl_3 , 300 MHz): δ 155.27, 152.04, 147.59, 137.42, 119.99, 87.74, 82.54, 67.80, 60.66, 59.94, 47.19, 27.01, 16.93, 16.80, 16.76, 16.73, 16.56, 16.50, 16.40, 12.84, 12.49, 12.29, 12.09. Mass spectrum (ESI) m/z (MNa) $^+$ 560.3; (M) $^-$ 536.2. HPLC purity (method A): 98.0%, t_R = 16.3 min.

2'-(2-Hydroxyethyl)-2'-deoxy-adenosine (1)

3',5'-protected adenosine **8** (0.160 g, 0.298 mmol) was dissolved in THF (5 mL), followed by the addition of tetra-*n*-butylammonium fluoride (TBAF) (0.156 g, 0.596 mmol). The reaction was stirred at room temperature overnight. The solvent was evaporated, the residue was purified by column chromatography on silica (eluent: ethyl acetate-15% methanol/ethyl acetate), 0.73 g (83%) product was obtained as a white powder. TLC: R_f = 0.16 (methanol/ethyl acetate = 1/4) (v/v). IR (neat): 3292, 3133, 2954, 1673, 1608, 1342, 1074, 996, 728 cm^{-1} . ^1H NMR (CD_3OD): δ 8.35 (s, 1H, ^8CH), 8.20 (s, 1H, ^2CH), 6.05 (d, J = 9.3 Hz, 1H, $H-1'$), 4.47 (d, J = 5.4 Hz, 1H, $H-3'$), 4.17 (m, 1H, $H-4'$), 3.88 (dd, J = 3.2 and 12.5 Hz, 1H, CH^a), 3.79 (dd, J = 3.2 and 12.5 Hz, 1H, CH^a), 3.54 (t, J = 6.4 Hz, 2H, CH_2), 3.09 (m, 1H, $H-2'$), 2.03 (m, 1H, CH^b), 1.52 (m, 1H, CH^b). ^{13}C NMR (CD_3OD): δ 157.72, 153.65, 150.30, 141.99, 121.10, 91.73, 90.34, 74.31, 64.31, 60.85, 47.33, 28.41. Mass spectrum (ESI) m/z (MNa) $^+$ 318.1; (M) $^-$ 294.0. HPLC purity (method A): 100%, t_R = 6.64 min.

2'-(2-Trityloxyethyl)-2'-deoxy-3',5'-O-(1,1,3,3-tetraisopropylidisilox-1,3-diyl)adenosine (9)

A solution of alcohol **8** (0.24 g, 0.446 mmol), triphenylmethyl chloride (0.62 g, 2.23 mmol) and DMAP (0.27 g, 2.23 mmol) in anhydrous dichloromethane (20 mL) was stirred at room temperature for 7 days, then under reflux for 1 day. The solvent was evaporated and column chromatography of the residue on silica gel (eluent: chloroform-5% methanol/chloroform) gave 0.306 g (88%) of **9**. TLC: R_f = 0.87 (methanol/chloroform = 1/10) (v/v). IR (neat): 3315, 3160, 2944, 1640, 1596, 1465, 1068, 1029, 883 cm^{-1} . ^1H NMR (CDCl_3): δ 8.26 (s, 1H, ^8CH), 7.86 (s, 1H, ^2CH), 7.33 (m, 6H, Trt), 7.22 (m, 9H, Trt), 5.96 (d, J = 4.9 Hz, 1H, $H-1'$), 5.69 (br s, 2H, NH_2), 4.79 (dd, J = 5.9 and 7.3 Hz, 1H, $H-3'$), 3.97 (m, 3H, CH_2-5' and $H-4'$), 3.26 (ddd, J = 3.2, 6.1 and 9.3 Hz, 1H, CH^a), 3.16 (ddd, J = 2.9, 6.4 and 9.3 Hz, 1H, CH^a), 2.95 (m, 1H, $H-2'$), 2.25 (m, 1H, CH^b), 1.75 (m, 1H, CH^b), 1.05 (m, 28H, $4 \times$

*i*Pr). ^{13}C NMR (CDCl_3 , 300 MHz): δ 154.80, 152.47, 149.11, 143.50, 138.54, 127.98, 127.18, 126.39, 119.65, 87.40, 86.24, 83.91, 76.66, 71.00, 62.17, 61.22, 44.39, 25.71, 16.95, 16.85, 16.80, 16.64, 16.54, 16.53, 16.46, 12.81, 12.73, 12.43, 12.17. Mass spectrum (ESI) m/z (MNa^+) 802.5; (M^-) 778.2. HPLC purity (method D): 100%, t_R = 7.65 min.

2'-(2-Trityloxyethyl)-2'-deoxyadenosine (10)

Protected nucleoside **9** (0.306 g, 0.392 mmol) was dissolved in THF (5 mL), followed by the addition of tetra-*n*-butylammonium fluoride (TBAF) (0.205 g, 0.785 mmol). The reaction was stirred at room temperature overnight. The solvent was evaporated, the residue was purified by column chromatography on silica gel (eluent: ethyl acetate-7% methanol/ethyl acetate), 0.184 g (87%) product was obtained. TLC: R_f = 0.32 (methanol/chloroform = 1/10) (v/v). IR (neat): 3323, 3180, 2921, 1638, 1596, 1578, 1063, 705, 697 cm^{-1} . ^1H NMR (CDCl_3): δ 8.29 (s, 1H, ^8CH), 7.70 (s, 1H, ^2CH), 7.28 (m, 15H, Trt), 6.53 (dd, J = 1.5 and 12.0 Hz, 1H, $\text{OH}-5'$), 5.78 (br s, 2H, NH_2), 5.74 (d, J = 9.5 Hz, 1H, $H-1'$), 4.61 (dd, J = 2.9 and 4.9 Hz, 1H, $H-3'$), 4.27 (s, 1H), 3.97 (tt, 1H, CH^a), 3.78 (dt, J = 1.7 and 12.9 Hz, 1H, $\text{CH}^{a'}$), 3.48 (d, J = 5.1 Hz, 1H), 3.35 (m, 1H, CH), 3.29 (d, J = 2.7 Hz, 1H), 3.17 (m, 1H), 3.10 (m, 1H), 1.97 (m, 1H, CH^b), 1.40 (m, 1H, $\text{CH}^{b'}$). ^{13}C NMR (CDCl_3 , 500 MHz): δ 155.88, 152.47, 148.60, 143.37, 140.45, 128.49, 128.07, 127.35, 121.45, 92.45, 88.90, 88.17, 73.95, 63.83, 62.66, 47.73, 24.94. Mass spectrum (ESI) m/z (MNa^+) 560.3; (M^-) 536.0. HPLC purity (method C): 99%, t_R = 5.90 min.

5'-O-Tosyl-2'-(2-Trityloxyethyl)-2'-deoxyadenosine (11)

A solution of silyl deprotected nucleoside **10** (163 mg, 0.303 mmol), tosyl chloride (69 mg, 0.364 mmol) and DMAP (48 mg, 0.394 mmol) in anhydrous dichloromethane (5 mL) was stirred at room temperature for 4 days. The solvent was removed under reduced pressure, the residue was purified by column chromatography on silica gel (eluent: chloroform-5% methanol/chloroform) to yield 93 mg (44%) of **11**. TLC: R_f = 0.60 (methanol/chloroform = 1/10) (v/v). IR (neat): 3373, 3323, 3121, 2926, 1636, 1595, 1578, 1361, 1175, 979, 903, 705, 697 cm^{-1} . ^1H NMR (CDCl_3): δ 8.23 (s, 1H, ^8CH), 7.90 (s, 1H, ^2CH), 7.74 (d, J = 8.3 Hz, 2H, phenyl- H), 7.30 (m, 17H, Trt and phenyl- H), 6.00 (d, J = 9.3 Hz, 1H, $H-1'$), 5.72 (br s, 2H, NH_2), 4.41 (d, J = 4.9 Hz, 1H, $H-3'$), 4.26 (m, 3H, CH_2-5' and $H-4'$), 3.56 (br s, 1H, $\text{OH}-3'$), 3.37 (m, 1H, CH^a), 3.06 (dt, J = 2.7 and 9.5 Hz, 1H, $\text{CH}^{a'}$), 2.90 (m, 1H, $H-2'$), 2.41 (s, 3H, CH_3), 2.06 (m, 1H, CH^b), 1.53 (m, 1H, $\text{CH}^{b'}$). ^{13}C NMR (CDCl_3 , 500 MHz): δ 155.37, 153.07, 150.01, 145.25, 143.30, 139.27, 139.14, 132.40, 130.03, 129.92, 128.46 (m), 128.01 (m), 127.35 (m), 119.96, 88.55 (d), 88.06, 83.62, 73.16, 69.52, 62.45, 47.75 (d), 24.58, 21.98. Mass spectrum (ESI) m/z (MNa^+) 714.3. HPLC purity (method C): 98%, t_R = 6.68 min.

5'-O-Tosyl-2'-(2-hydroxyethyl)-2'-deoxyadenosine (12)

Trityl ether **11** (73 mg, 0.106 mmol) was treated with 80% AcOH in H_2O (10 mL), the reaction was heated at 50 $^\circ\text{C}$ for 4 h. The solution was evaporated under reduced pressure at ambient temperature. The residue was purified by column chromatography on silica gel (eluent: chloroform-10% methanol/chloroform) gave 43.7 mg (92%) of alcohol **12**. TLC: R_f = 0.30 (methanol/chloroform = 1/10) (v/v). IR (neat): 3323, 1678, 1632, 1204, 1139, 1084, 801 cm^{-1} . ^1H NMR (CD_3OD): δ 8.23 (s, 1H, ^8CH), 8.14 (s, 1H, ^2CH), 7.77 (d, J = 8.3 Hz, 2H, phenyl- H), 7.36 (d, J = 8.3 Hz, 2H, phenyl- H), 6.06 (d, J = 9.0 Hz, 1H, $H-1'$), 4.35 (m, 3H, CH_2-5' and $H-3'$), 4.23 (dt, J = 1.5 and 4.6 Hz, 1H, $H-4'$), 3.51 (t, 2H, CH_2), 3.10 (m, 1H, $H-2'$), 2.43 (s, 3H, CH_3), 1.99 (m, 1H, CH^a), 1.52 (m, 1H, $\text{CH}^{a'}$). Mass spectrum (ESI) m/z (MNa^+) 472.1. HPLC purity (method C): 98%, t_R = 4.97 min.

2'-(2-Hydroxyethyl)-2'-deoxyadenosine 5'-Diphosphate (2)

To a solution of nucleoside tosylate **12** (40 mg, 0.089 mmol) in acetonitrile (0.35 mL) tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (120 mg, 0.133 mmol) was added, the reaction solution was stirred at room temperature for 3 days. The resulting reaction solution was purified by preparative silica gel TLC (methanol/chloroform = 20:80), the product was concentrated under vacuum and tetra-*n*-butylammonium cation was exchanged for ammonium by a DOWEX AG 50W-X8 column (100-200 mesh, ammonium form). The eluent was evaporated under reduced pressure to yield 17 mg (38%) of **2**. TLC: R_f = 0.07 (tailed) (methanol/chloroform = 2/8) (v/v). IR (neat): 3193, 1690, 1648, 1606, 1421, 1212, 1081, 924 cm^{-1} . ^1H NMR (CD_3OD): δ 8.61 (s, 1H, ^8CH), 8.21 (s, 1H, ^2CH), 6.17 (d, J = 9.3 Hz, 1H, $H-1'$), 4.67 (d, J = 4.9 Hz, 1H, $H-3'$), 4.22 (m, 3H, CH_2-5' and $H-4'$), 3.53 (m, 2H, CH_2), 3.04 (m, 1H, $H-2'$), 2.03 (m, 1H, CH^a), 1.50 (m, 1H, CH^a). ^{13}C NMR (D_2O , 500 MHz): δ 154.21, 150.85, 108.10, 87.62, 86.35, 72.76, 65.83, 59.13, 45.55, 26.27. Mass spectrum (ESI) m/z (M) $^-$ 453.9.

Rnr1 Enzyme Assay

The RNR assay to evaluate for compound inhibition was performed as described by Xu.[10] Rnr1p was expressed in BL21 (DE3)plys cells at 15°C using IPTG. Bacterial cells were harvested and proteins were precipitated in 40% ammonium sulfate. Rnr1p was purified using peptide affinity chromatography [11]. Rnr2p and Rnr4p were co-expressed in BL21 (DE3) RIL cells at 15°C[12]. Cells were lysed and Rnr2p.Rnr4p complex in the resultant supernatant was purified using cobalt affinity chromatography[12]. Protein concentrations were measured using Bradford reagent and the purity of the proteins was checked using SDS-PAGE. Activity assay mixtures contained 1 μM Rnr1p, 5 μM of Rnr2p.Rnr4p, 20 mM HEPES-KOH (pH 7.4), 200 mM K acetate, 20 mM Mg acetate, 2 mM dGTP, 1 mM [^{14}C] adenosine -5- diphosphate (specific activity: 5784 cpm/nmol), 20 mM FeCl_3 and 20 mM DTT, and this mixture was incubated with different concentrations of **2** (2 : concentrations 500 nM, 1 μM , 50 μM , 250 μM and 500 μM) at 30°C for 0-12 min. The amount of dADP formed at different concentrations of **2** were separated by boronate chromatography and quantified by scintillation counting[13].

Crystallography

Yeast Rnr1 was produced and purified as described previously[3,4] and crystallized from a reservoir solution containing 20–25% PEG 3350, 0.2 M NaCl, 10mM TTP and 100 mM Hepes, pH 7.5. Crystals were incubated for 4 h in reservoir solution containing 20 mM dGTP and the hydroxyethylene nucleotide analogue. Subsequently, crystals were cryogenized by soaking in 25% PEG 3350, 0.2 M NaCl, and 100 mM Hepes, pH 7.5, supplemented with 15% glycerol. Data were collected at BioCARS at the Advanced Photon Source (APS) CAT 14-ID beamline using a Quantum-315 CCD detector (Area Detector Systems Corp.) and the other at APS beamline 23-ID-B (GM/CAT) In order to improve completeness, the two datasets were merged together. The crystals belong to the orthorhombic space group $\text{P}2_12_12$ and were integrated, scaled and merged using HKL2000[14] (see Table 1). The structure was directly determined by the difference Fourier technique (Table 1) using the known PDB entry 2CVX as the initial model. The graphics software O[15] was used for model building, interspersed with refinement using both CNS[16] and REFMAC[17]. The final refinement statistics are summarized in Table 1. The final models were all evaluated with PROCHECK[18] and figures were prepared using Pymol[19]. The atomic coordinates have been deposited into the PDB data bank with RCSB ID code 2ZNG.

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A

B

Figure 1.

1A. Ligplot of the ScR1-dGTP-ADP C-site. The color scheme is Carbon-Yellow, Oxygen-Red, Nitrogen-Blue and Sulfur-Green. All hydrogen-bonds are drawn as broken lines. The conserved water molecule is labelled W. B. Novelty designed target molecules 2'-(2-hydroxyethyl)-2'-deoxyadenosine **1** and its 5'-diphosphate **2**.

Scheme 1.

Synthetic route to **1** from adenosine. Reagents and conditions: a. TPDS-Cl₂, Pyridine, 3h, 59%; b. PhOC(S)Cl, DMAP, DCM, RT, 18h, 89%; c. allyl-SnBu₃, AIBN, PhCH₃, UV, RT, 15h, 85%; d. NMO, OsO₄, H₂O/Acetone (1/3), O/N, 86%; e. NaIO₄, H₂O/Dioxane (1/3), RT, 79%; f. NaBH₄, MeOH, RT, O/N, 97%; g. TBAF, THF, O/N, 83%.

Scheme 2.

Synthesis of **2** from **8**. Reagents and conditions: a. TrtCl/DMAP, DCM, 7d at r.t. and 1d at reflux, 88%; b. TBAF/THF, ON, 87%; c. Tosyl chloride/DMAP, DCM, 4d, 44%; d. AcOH/H₂O, 50 °C, 4h, 92%; e. i. (n-Bu₄N⁺)₃

Figure 2. Structure of yeast R1-dGTP-compound complex. A. Overall structure of R1 with effector dGTP at the S-site (specificity site) and inhibitor compound at the C-site (catalytic site, or active site); B. The 2Fo-Fc difference Fourier electron density map contoured at 1 σ showing the effector dGTP (at the S-site) and compound 2 (at the C-site) and loop1 and partially disordered loop2.

Figure 3.
The electrostatic potential surface showing the active binding site on R1. Red indicates negative surface charges, blue indicates positive surface charges, and gray represents uncharged surfaces. Only residues within the van der Waals distance at the C-site are shown.

Figure 4. Schematic diagram of the catalytic site interactions. A. Hydrogen bond interactions formed between R1 and compound **2** at the C-site shown in dashed lines with hydrogen bonding distances. B. Hydrogen bond interactions formed between R1 and ADP at the C-site shown in dashed lines with hydrogen bonding distances.

Table 1Data Collection and Refinement Statistics for Rnr1-compound **2** structure

Data collection	
Space group	P2 ₁ 2 ₁ 2
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	107.53, 116.92, 64.07
Wavelength (Å)	0.90020/1.03320
Resolution (Å)	50.0 – 2.10
Unique / total reflections	47632 / 324956
<i>R</i> _{merge} (%) [*]	16.4 (42.3)
<i>I</i> / (<i>I</i>) [*]	8.5 (2.4)
Completeness (%) [*]	99.3 (96.8)
Redundancy [*]	6.9 (3.8)
Refinement	
Resolution (Å)	50.0 – 2.10
No. reflections	45193
<i>R</i> _{work} / <i>R</i> _{free} ^{a)}	0.254 / 0.290
No. atoms	
Protein	5121
Ligand/ion	67 ^{b)}
Water	135
B-factors	
Protein	53.9
Ligand/ion	62.7
Water	54.2
rms deviations	
Bond lengths(Å)	0.017
Bond angles(°)	1.719

^{*} Highest resolution shell is shown in parentheses.

^{a)} R_{work} and $R_{\text{free}} = \Sigma ||F_{\text{O}}| - |F_{\text{C}}|| / \Sigma |F_{\text{O}}|$, where F_{O} and F_{C} are the observed and calculated structure factor amplitudes. For the calculation of R_{free} , 5% of the reflection data were selected and omitted from refinement.

^{b)} The ligand/ions are dGTP, glycerol and compound.

Table 2Comparison of the Rnr1-compound **2** and Rnr1-ADP interactions.

Complex	Nucleotide atom and residue name	Rnr1 atom and residue name
Rnr1-ADP		
Hydrogen bonds	N1	Q288 Nε2, R293 Nη2
	N3	G246 N (via H ₂ O), G247 N
	N6	P294 O (via H ₂ O)
	2'OH	S217 O, C218 Sγ, G247 O (via H ₂ O), N426 Nδ2 (via H ₂ O), L427 N (via H ₂ O)
	3'OH	C218 Sγ, N426 Nδ2, E430 Oε1, Oε2
van der Waals interactions	Adenine	G246, G247, Q288, R293, A296, L427, C428
	2'OH	S217, C218, G247, N426, L427
	3'OH	N426, C428, E430
Rnr1-compound 2		
Hydrogen bonds	N3	G247 N
	modified 'OH	G247 O, N426 Nδ2, L427 N
	3'OH	C218 Sγ, N426 Nδ2, E430 Oε1, Oε2
van der Waals interactions	Adenine	G246, G247, Q288, A296, L427, C428
	modified 'OH	S217, C218, F219, G247, I248, N426, L427
	3'OH	N426, C428, E430

Only the hydrogen bonds and van der Waals interactions to the bases and 2' (in inhibitor compound, modified hydroxyl), 3' ribose hydroxyls of the nucleotides are shown.