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# Nucleosides and Nucleotides. 176. 2'-Deoxy-2'-hydroxylaminocytidine: A New Antitumor Nucleoside That Inhibits DNA Synthesis Although It Has A Ribonucleoside Structure

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**Abstract**: The design and synthesis of potential antitumor antimetabolites 2'-deoxy-2'-hydroxylaminouridine (2'-DHAU) and -cytidine (2'-DHAC) are described. We found that 2'-DHAC in neutral solution generated 2'-aminoxy radicals at room temperature. 2'-DHAC inhibited the growth of L1210 and KB cells, with $IC_{50}$ values of 1.58 and 1.99 μM, respectively, more potently than 2'-DHAU, with $IC_{50}$ values of 34.5 and 27.3 μM, respectively. 2'-DHAC was effective against 9 human cell lines, with $IC_{50}$ values in the micromolar range. The in vivo antitumor activity of 2'-DHAC was also examined using the mouse leukemia P388 model, which gave a T/C value of 167%. Phosphorylation of 2'-DHAC by uridine/cytidine kinase was essential for its cytotoxicity, as suggested by a competition experiment using several common nucleosides. Inhibition of DNA synthesis was the predominant mechanism of action of 2'-DHAC, although it has a *ribo*-configuration. 

*Keywords*: Antiproliferarive Agent; DNA; Nucleosides

Hydroxylamine derivatives have interesting chemical properties. They can be readily reduced to amines and readily oxidized to nitrons. Additionally, the oxidation of hydroxylamine by ceric sulfate [1,2] and OH-radicals [3] produces $NH_2O\cdot$ radicals. Therefore, if such a substituent can be introduced into the sugar moiety of a nucleoside, the result may be a unique nucleoside with a variety of biological activities. Previously, Tronchet *et al.* reported the synthesis of some nucleosides with hydroxylamine derivatives in the expectation of anti-HIV activity [4-7].

For progress in anticancer chemotherapy, we need more effective and selective anticancer agents against solid tumors. Recently, some nucleosides, such as gemcitabine [8], 1-(2-deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine (DMDC) [9-12], (2'*E*)-1-(2-deoxy-2-fluoromethylene-β-D-*erythro*-pentofuranosyl)cytosine (FMDC) [13], 1-(2-*C*-cyano-2-deoxy-β-D-*arabino*-pentofuranosyl)cytosine (CNDAC) [14-16], and 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd) [17-20], have been shown to have potent antitumor activity against not only leukemias and lymphomas, but also several solid tumors. These nucleosides have unique chemical structures at their sugar moieties, which may correlate with their antitumor properties. Therefore, the introduction of a substituent with a unique chemical nature to the sugar moiety of certain nucleosides is an interesting approach to a new type of

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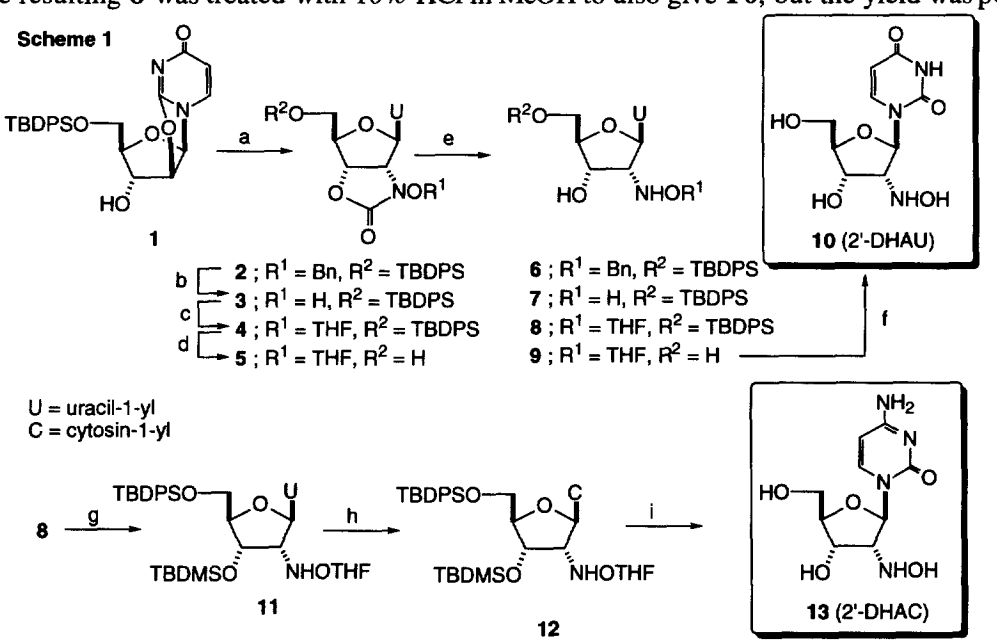
antitumor nucleoside. In this communication, the synthesis of 2'-deoxy-2'-hydroxylaminouridine (2'-DHAU) and -cytidine (2'-DHAC), detection of the 2'-NHO• radicals of 2'-DHAC in neutral solution by ESR, and their antitumor activity in vitro and in vivo are described.

Previous synthetic methods for introducing hydroxylamine derivatives into the sugar moiety of nucleosides mainly involve reducing oximes with certain reducing agents. However, when we tried to reduce of a 2'-oxime of uridine derivatives [21], none of the desired hydroxylamino derivatives with a *ribo*-configuration were obtained [7]. Recently, Sebesta *et al.* reported a new synthetic method for preparing 2'-alkoxyaminouridines via an intramolecular nucleophilic substitution of 2,2'-*O*-anhydrouridine derivatives [22]. Using this method, we can synthesize the target nucleosides 2'-DHAU and 2'-DHAC (Scheme 1). Uridine was converted to 5'-*O*-TBDPS-2,2'-*O*-anhydrouridine (**1**), which was subsequently treated with *N,N'*-carbonyldiimidazole followed by *O*-benzylhydroxylamine. The resulting 3'-*O*-(benzyloxyamino)carbonyl derivative, without purification, was converted into the corresponding 2'-benzyloxyamino-2'-*N*, 3'-*O*-carbonyl derivative **2** in good yield [22]. When **2** was treated with $Cs_2CO_3$ in MeOH, the cyclic carbamate was effectively cleaved to give 2'-*O*-benzyloxyamino-2'-deoxyuridine derivative **6** in good yield. However, hydrogenation of the benzyl group of **6** using 10% Pd-C catalyst under a $H_2$ atmosphere did not give the desired 2'-hydroxylamino derivative **7**. On the other hand, hydrogenation of **2** under the same conditions gave *N*-hydroxyl derivative **3** in 79% yield. Since the cyclic carbamate moiety of **3** was not cleavable using $Cs_2CO_3$ in MeOH, the *N*-hydroxyl group was again protected with a tetrahydrofuranyl group to give **4**, which would be deprotected in the final stage of the reaction sequence. The 5'-*O*-silyl group of **4** was deprotected with tetrabutylammonium fluoride to give **5**, and the cyclic carbamate moiety of **5** was cleaved with $Cs_2CO_3$ in MeOH to give **9**. Finally, the THF group was removed with 10% HCl in EtOH to give the desired 2'-deoxy-2'-hydroxylaminouridine (**10**, 2'-DHAU)[1] as a hydrochloride. After hydrolysis of the cyclic carbamate of **4**, the resulting **8** was treated with 10% HCl in MeOH to also give **10**, but the yield was poor.

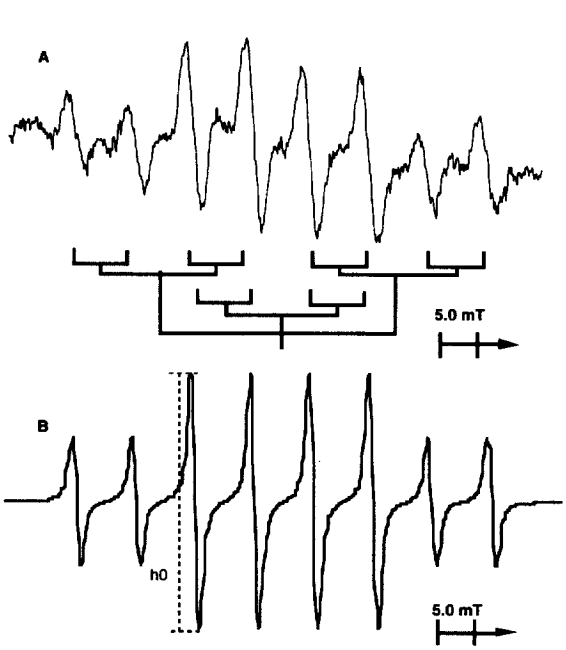
**Scheme 1**




Reagents and conditions: (a) see ref. 22; (b) 10% Pd-C, EtOH, rt, 79%; (c) DHF, PPTS, THF, rt, quant; (d) TBAF, THF, rt, 79%; (e) $Cs_2CO_3$, MeOH, 75% for **6**, 85% for **7**, 85% for **9**; (f) 10% HCl/EtOH, 0 °C, 96%; (g) TBDMSCl, imidazole, DMF, rt, quant; (h) 1) TPSCl, DMAP, $Et_3N$, MeCN, 0 °C to rt; 2) 28% $NH_4OH$, rt, 91%; i) 30% HCl/EtOH, rt, 91%.

The cytosine counterpart **13** was synthesized from **8**. Protection of the 3'-hydroxyl group of **8** with a *tert*-butyldimethylsilyl group gave **11**, which was converted into a cytosine nucleoside **12** in the usual manner, followed by deprotection of **12** with 30% HCl in EtOH to give 2'-deoxy-2'-hydroxylaminocytidine (**13**, 2'-DHAC) as a dihydrochloride.[2]

Since hydroxylamine produces aminoxy radicals, the ability of 2'-DHAC to generate aminoxy radical was checked. 2'-DHAC (20 mM) was dissolved in phosphate buffer (pH 7.0, 200 mM) and its ESR spectrum was measured at room temperature. As shown in Figure 1A, this signal showed a hyperfine structure consisting of 8 lines with a signal intensity of 1:1:2:2:2:2:1:1. The hyperfine coupling constants of this signal were assigned to a primary triplet of 1.36 mT (aN) with secondary splits into a 1.36 mT (aHα) doublet and a 0.68 mT (aHβ) doublet, indicating that this compound has two protons at its α- and β-positions. The computer-simulated spectrum is constructed as shown in Figure 1B; this spectrum also supports the aminoxy radical structure as shown in Figure 1C. The half-life of this radical was also measured. The aminoxy radical was rapidly generated as soon as crystalline 2'-DHAC was dissolved in the buffer. The maximum signal intensity was reached in about 15 min and the half-life was about 35 min (data not shown). Therefore, this radical was assumed to be produced by the release of atomic hydrogen.



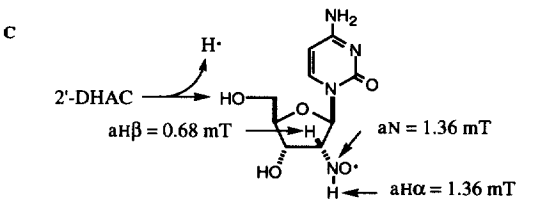




**Fig. 1.** **A**: ESR spectrum of phosphate buffer (pH 7.0, 200 mM) containing 2'-DHAC (20 mM). The ESR spectrum was recorded 10 min after 2'-DHAC was dissolved in the phosphate buffer. **B**: Computer-simulated spectrum for the aminoxy radical derived from 2'-DHAC. aN =1.36 mT, aHα = 1.36 mT, aHβ = 0.68 mT and line width = 0.1 mT were used as the simulation parameters. **C**: The mechanism of aminoxy radical generation from 2'-DHAC in neutral pH solution.

1) Physical data for 2'-DHAU: mp 160-162 °C; $^1$H-NMR (500 MHz, MeOH-$d_4$) δ 7.97 (d, 1 H, H-6, $J_{6,5}$ = 8.1 Hz), 6.41 (d, 1 H, H-1', $J_{1',2'}$ = 6.8 Hz), 5.77 (d, 1 H, H-5, $J_{5,6}$ = 8.1 Hz), 4.64 (dd, 1 H, H-3', $J_{3',2'}$ = 6.2, $J_{3',4'}$ = 3.0 Hz), 4.20 (dd, 1 H, H-2', $J_{2',1'}$ = 6.8, $J_{2',3'}$ = 6.2 Hz), 4.15 (ddd, 1 H, H-4', $J_{4',3'}$ = 3.0, $J_{4',5'a}$ = 2.9, $J_{4',5'b}$ = 2.9 Hz), 3.83 (dd, 1 H, H-5'a, $J_{5'a,4'}$ = 2.6, $J_{gem}$ = 12.2 Hz), 3.75 (dd, 1 H, H-5'b, $J_{5'b,4'}$ = 2.9, $J_{gem}$ = 12.2 Hz). Anal. calcd for $C_9H_{13}N_3O_4$·HCl·0.2$H_2O$: C, 36.12; H, 4.85; N, 14.04. Found: C, 36.45; H, 4.76; N, 13.71.

2) Physical data for 2'-DHAC: mp 170-172 °C; $^1$H-NMR (500 MHz, MeOH-$d_4$) δ 8.40 (d, 1 H, H-6, $J_{6,5}$ = 8.0 Hz), 6.45 (d, 1 H, H-1', $J_{1',2'}$ = 6.0 Hz), 6.23 (d, 1 H, H-5, $J_{5,6}$ = 8.0 Hz), 4.68 (dd, 1 H, H-3', $J_{3',2'}$ = 6.2, $J_{3',4'}$ = 3.4 Hz), 4.30 (dd, 1 H, H-2', $J_{2',1'}$ = 6.0, $J_{2',3'}$ = 6.2 Hz), 4.23 (ddd, 1 H, H-4', $J_{4',3'}$ = 3.4, $J_{4',5'a}$ = 2.6, $J_{4',5'b}$ = 2.8 Hz), 3.88 (dd, 1 H, H-5'a, $J_{5'a,4'}$ = 2.6, $J_{gem}$ = 12.3 Hz), 3.75 (dd, 1 H, H-5'b, $J_{5'b,4'}$ = 2.8, $J_{gem}$ = 12.3 Hz). Anal. calcd for $C_9H_{14}N_4O_6$·2HCl: C, 32.64; H, 4.87; N, 16.92. Found: C, 32.81; H, 4.92; N, 16.58.

The inhibitory activity of 2'-DHAC and 2'-DHAU against the *in vitro* growth of murine leukemia L1210 and human epidermoid KB cells was evaluated using the MTT assay [23]. 2'-DHAC inhibited the growth of L1210 and KB cells, with $IC_{50}$ values of 1.58 and 1.99 μM, respectively, more potently than 2'-DHAU, with $IC_{50}$ values of 34.5 and 27.3 μM, respectively. Next, we evaluated 2'-DHAC against 10 human tumor cell lines, including three leukemias, one colon, two pancreas, one fibro, one breast, one melanoma, and one hepatoma tumor cell lines in vitro. The results are summarized in Table 1. 2'-DHAC was effective against 9 cell lines with $IC_{50}$ values in the micromolar range and was ineffective against one pancreas adenocarcinoma cell line (PANC-1). We also evaluated the *in vivo* antitumor activity of 2'-DHAC against P388 mouse leukemia cells that had been implanted intraperitoneally into female CDF1 mice. When 2'-DHAC was administered intraperitoneally on days 1-5 consecutively at a dose of 20 mg/kg/day, it showed antitumor activity with a T/C value of 167%, while all of the tumor-bearing mice (6 mice were used as a control) without administration of the drug died on day 10. Since multiple-dose schedules were not tested, we may not have used this drug under optimal conditions.

**Table 1.**
Inhibitory Effects of 2'-DHAC on the Growth of Various Human Tumor Cells in Vitro[a]

| cell line | origin | $IC_{50}$ (μM) |
|---|---|---|
| K562 | chronic myelogenous leukemia | 2.77 |
| HL-60 | promyelocytic leukemia | 5.76 |
| CCRF-CEM | acute lymphoblastic leukemia | 1.73 |
| Colo320DM | colon adenocarcinoma | 8.93 |
| MiaPaCa-2 | pancreas adenocarcinoma | 4.00 |
| PANC-1 | pancreas adenocarcinoma | 69.1 |
| HT-1080 | fibrosarcoma | 2.53 |
| MCF-7 | mammary adenocarcinoma | 1.30 |
| A375 | melanoma | 0.89 |
| HuH7 | hepatoma | 1.47 |

[a]Tumor cell growth inhibitory activity assay in vitro was done following the method [23]. Each tumor cell (2 x $10^3$ cells/well) was incubated in the presence or absence of compounds for 72 h. MTT-reagent was added to each well and plate was incubated for 4 h more, the resulting MTT-formazan was dissolved in DMSO and the OD (540 nm) was measured. Percent inhibition was calculated as follows: % inhibition = [1-OD (540 nm) of sample well/OD (540 nm) of control well] x 100. $IC_{50}$ (mg/mL) was given as the concentration at 50% inhibition of cell growth.

To estimate the metabolic pathway of 2'-DHAC, the inhibitory effect of 2'-DHAC on the growth of KB cells was examined in the presence or absence of common nucleosides, such as uridine, cytidine, adenosine, guanosine, 2'-deoxycytidine, and thymidine. KB cells ($10^4$ cells/mL) were treated with graded concentrations of 2'-DHAC and each nucleoside (final concentration; 100 μM) for 72 h. The inhibitory effect of 2'-DHAC on the growth of KB cells was prevented by the addition of either cytidine or uridine (Fig. 2). Therefore, the antitumor activity of 2'-DHAC may require phosphorylation by uridine/cytidine kinase.

The effects of 2'-DHAC (20 μM) on DNA and RNA synthesis in L1210 cells was investigated by measuring the incorporation of [$^3$H]-thymidine and -uridine as precursors, respectively, in the presence or absence of 2'-DHAC. As a result, DNA synthesis was predominantly inhibited, and RNA synthesis was inhibited non-negligibly by the addition of 2'-DHAC, as shown in Figure 3. Although its inhibition of RNA synthesis is not likely to be a major mechanism of tumor cell death, it is conceivable that this inhibition may contribute to its antitumor efficacy.

**Fig. 2.**
Competitive effects of nucleosides on the cytotoxicity of 2'-DHAC.

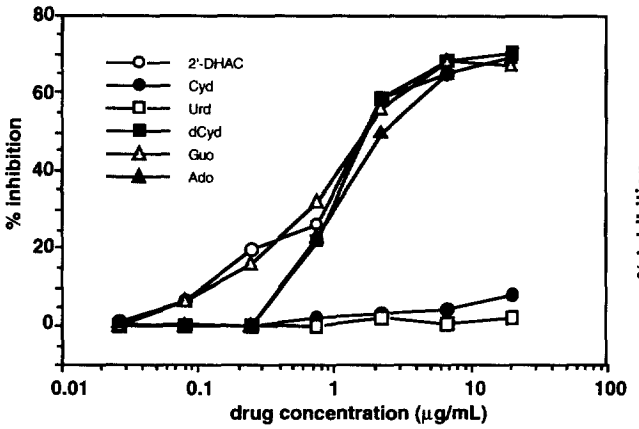
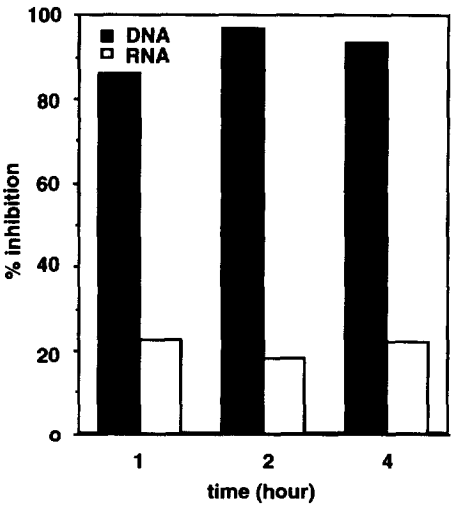



**Fig. 3.**
Effects of 2'-DHAC on the incorporation of thymidine and uridine into macromolecular fractions of L1210 cells.




2'-DHAC may be phosphorylated by uridine/cytidine kinase to its 5'-monophosphate, which would be further phosphorylated by certain nucleotide kinase to its 5'-di- (2'-DHACDP) and -tri- (2'-DHACTP) phosphates. Since 2'-DHAC mainly inhibited DNA synthesis, a consideration of pyrimidine nucleotide metabolism suggested that 2'-DHACDP might inhibit ribonucleoside diphosphate reductase (RDR), which is responsible for the *de novo* biosynthesis of deoxyribonucleotides required for DNA synthesis, which would inhibit DNA synthesis, and 2'-DHACTP would be responsible for inhibiting RNA polymerase. Hydroxyurea has been shown to inhibit RDR by destruction of its tyrosine free radical [24]. Although the sugar structure of 2'-DHAC suggests that it could chelate iron ions, it may have a different mechanism of inhibition, since uridine and cytidine, but not adenosine, guanosine, thymidine, or 2'-deoxycytidine, reverse its tumor cell growth inhibitory activity. Therefore, generation of the aminoxy radical in 2'-DHACDP might be responsible for inhibiting the function of RDR. It has been shown that 2'-chlorouridine 5'-diphosphate and 2'-azidouridine 5'-diphosphate are potent inhibitors of RDR from E. coli [25]. However, these nucleosides do not show cytotoxicity against tumor cells because they might not be substrates of nucleoside kinase. Gemcitabine 5'-diphosphate [12], DMDC 5'-diphosphate [12], and FMDC 5'-diphosphate [13] also potently inhibit RDR. As described above, these nucleosides have strong cell growth inhibitory activity and are substrates of deoxycytidine kinase. 2'-DHAC has a *ribo*-configuration and, interestingly, inhibits DNA synthesis after phosphorylation by uridine/cytidine kinase, a salvage enzyme responsible for ribonucleosides. Since the activity of uridine/cytidine kinase is much higher in various human tumor tissues than in non-neoplastic tissues [26,27], 2'-DHAC would be expected to show its antitumor activity in a tumor-specific manner. Further studies to optimize doses and schedules and to elucidate the mechanism of action of 2'-DHAC are underway.

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