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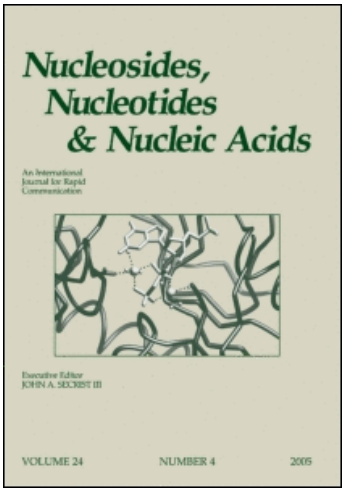

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
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Synergistic Effect of 5-Nitro-2'-deoxyuridine with Ganciclovir Against Human Cytomegalovirus *In Vitro*

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To cite this Article Rao, T. Sudhakar , Rando, Robert F. , Huffman, John H. and Revankar, Ganapathi R.(1995) 'Synergistic Effect of 5-Nitro-2'-deoxyuridine with Ganciclovir Against Human Cytomegalovirus *In Vitro*', Nucleosides, Nucleotides and Nucleic Acids, 14: 9, 1997 — 2008
To link to this Article: DOI: 10.1080/15257779508010719
URL: http://dx.doi.org/10.1080/15257779508010719

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NUCLEOSIDES & NUCLEOTIDES, 14(9&10), 1997-2008 (1995)

SYNERGISTIC EFFECT OF 5-NITRO-2'-DEOXYURIDINE WITH GANCICLOVIR AGAINST HUMAN CYTOMEGALOVIRUS *IN VITRO*

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ABSTRACT: In this paper we describe a practical synthesis of 5-nitro-2'-deoxyuridine (**4**) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-nitrouracil (**11**). These compounds were then evaluated for their ability to inhibit the growth of human cytomegalovirus (HCMV, strain AD169) in MRC-5 cells using a plaque reduction assay. Compound **11** was unable to inhibit the growth of HCMV at the highest concentration tested (100 μg/mL). However, compound **4** (5-NO_2-dU) exhibited marginal activity against HCMV *in vitro* in a dose-dependent manner with a 50% inhibitory concentrations (IC_{50}) of 1 to 5 μg/mL. Combinations of 5-NO_2-dU with ganciclovir synergistically inhibited HCMV induced cell killing in culture.

Combination chemotherapy has been used as a therapeutic approach to viral and malignant diseases. For viral infections, use of combination therapy employing drugs having different viral targets or agents which have similar mechanisms but different sites of toxicity have been explored.[1] This approach may enhance the antiviral efficacy, reduce drug toxicity and eliminate the emergence of drug-resistant strains[2] observed using either of the compounds alone.

Certain pyrimidine 2'-deoxyribonucleosides, substituted at the 5-position by electron-withdrawing groups (e.g. iodo, fluoro, trifluoromethyl, etc.) have shown significant antiviral properties.[3,4] Because of its electron withdrawing nature, the nitro group has been recognized as a biologically important functionality and the inhibition of viral growth by 5-nitro-2'-deoxyuridine (5-

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NO_2-dU) is well documented.[5-8] Although the antiviral effect of 5-NO_2-dU is primarily against vaccinia virus, it has also been shown to inhibit herpes simplex virus type 1 (HSV-1).[5] The antiviral effect of 5-NO_2-dU has been attributed to the inhibition of thymidylate synthetase.[5]

Although the chemical[9-11] and enzymatic[12] syntheses of 5-NO_2-dU are described in the literature, none of these methods are practical for the preparation of larger quantities of 5-NO_2-dU. Hubbard et al.[11] have reported the preparation of anomeric 3',5'-di-*O*-toluoyl-5-NO_2-dU by a direct condensation reaction involving bis-silylated 5-nitrouracil and the α-halogenose (**2**) in the presence of $ZnCl_2$. Although the formation of the nucleoside products in this reaction is claimed to be quite high with the β-anomer predominating (as analyzed by ^{1}H NMR), in our hands the α:β anomeric ratio was found to be 1:2. However, in the absence of $ZnCl_2$ we realized an anomeric ratio of 1:1. These anomers migrate as a single component on TLC and the resolution of α- and β-anomers is not described by Hubbard et al.[11] We now report a convenient route for the resolution of these anomers into the pure components, as well as the first synthesis of 2'-arafluoro-5-NO_2-dU (**11**). Evaluation of 5-NO_2-dU in combination with ganciclovir (DHPG, the drug of choice for the treatment of HCMV infection) synergistically inhibited the growth of HCMV in culture and appears to be more useful than either of the compounds used alone.

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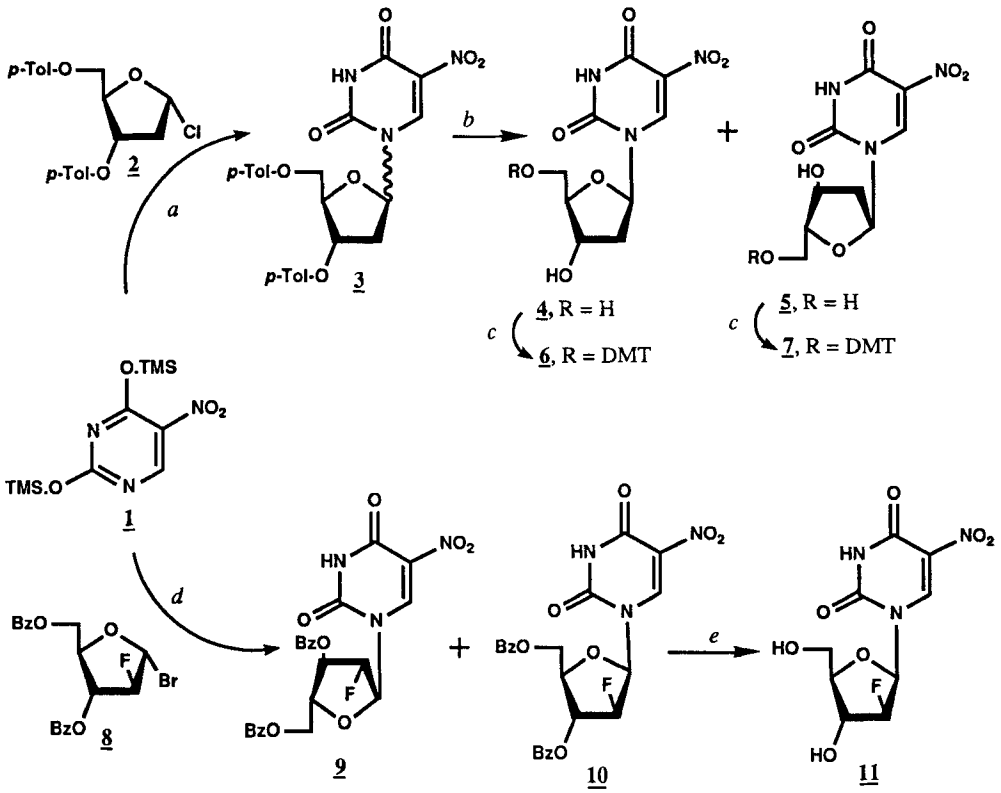
Glycosylation of the trimethylsilyl-5-nitrouracil (**1**) with 1-chloro-2-deoxy-3,5-di-*O*-(*p*-toluoyl)-α-D-*erythro*-pentofuranose[13] (**2**) in anhydrous $CHCl_3$ gave a reaction mixture from which a glycosylated product was isolated by flash chromatography over silica gel in a 94% yield (Scheme 1). Although this product (**2**) was homogeneous by tlc procedures, ^{1}H NMR spectrum of **2** indicated the presence of two components. The protecting toluoyl groups of **3** were removed by the treatment with NaOMe/MeOH/pyridine. The isolated product (84% yield) which appeared to be homogeneous by tlc procedures, was again found to be a mixture of anomers (**4** and **5**) by ^{1}H NMR analysis. As our efforts were not successful in resolving these anomers either at blocked or deblocked nucleoside stage, an attempt was made to separate these anomers as 5'-*O*-dimethoxytrityl derivatives. Thus, the mixture containing **4** and **5** was tritylated by the treatment with 4,4'-dimethoxytrityl chloride in pyridine. The resulting trityl derivatives were readily resolved on a flash silica gel column and isolated in 38% (β-anomer, **6**) and 36% (α-anomer, **7**) yields. Removal of the dimethoxytrityl group from **6** and **7** by the treatment with 80% AcOH afforded pure anomers of 5-nitro-2'-deoxyuridine (**4** and **5**) in good yields. The anomeric configuration of **4**



Reaction conditions: *a*, $CHCl_3$, room temperature (RT); *b*, MeOH/NH_3, RT; c, DMT-Cl in pyridine, RT; *d*, CH_3CN, RT for 3 days, then reflux for 2 days; *e*, $NaOCH_3$ in MeOH, RT.

Scheme 1

and **5** was assigned on the basis of $J_{1',2'}$ coupling constants observed for the anomeric proton in the ^1H NMR spectrum.[14] The β-anomer (**4**) displayed a triplet for the anomeric proton with a coupling constant of 6.0 Hz, whereas the α-anomer (**5**) exhibited a doublet of doublets with a coupling constant of 1.2 Hz. Thus, it is evident that the isolation and purification of 5-NO_2-dU *via* its 5'-*O*-dimethoxytrityl derivative is a practical method for the large scale preparation.

The key intermediate required for the synthesis of 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-nitrouracil (**11**) was 2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-α-D-arabinofuranosyl bromide (**8**), which was prepared as reported.[15] Condensation of **8** with **1** in CH_3CN gave an anomeric mixture of 1-(2-deoxy-2-

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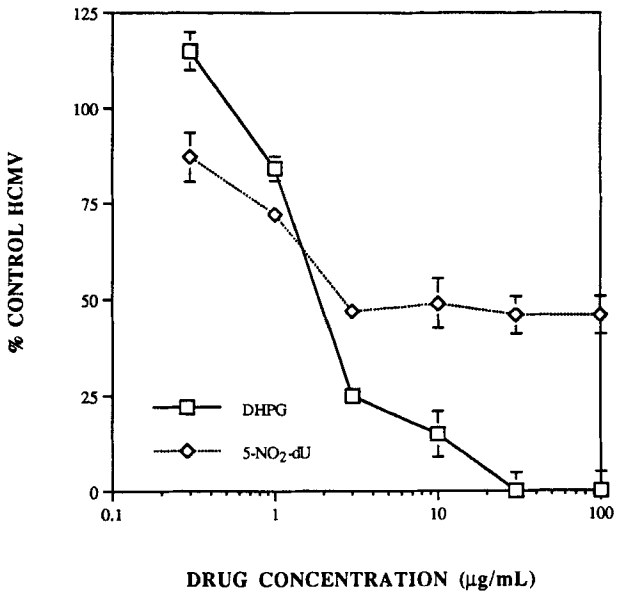

Figure 1. Dose response curve of 5-NO_2-dU and DHPG on HCMV production in a plaque reduction assay. The data presented are the average of quadruplicate experiments in which the number of plaques are normalized to virus infected untreated MCR-5 cells (% control plaques).

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fluoro-3,5-di-*O*-benzoyl-D-arabinofuranosyl)-5-nitrouracil (**9** and **10**). The pure β-anomer (**10**) was obtained in a 52% yield by triturating the purified reaction mixture with MeOH:CH_2Cl_2 (5:1). Debenzoylation of **10** with NaOMe/MeOH gave hitherto unknown arafluoro nucleoside **11** in a 75% yield. Compound **11** was fully characterized by spectral and elemental analyses.

5-NO_2-dU was evaluated in comparison with ganciclovir (DHPG) for its ability to inhibit the growth of human cytomegalovirus (HCMV, strain AD169) in MRC-5 cells using a plaque reduction assay. The results of these assays indicated that 5-NO_2-dU was active against HCMV in a dose-dependent manner (Figure 1) with a 50% inhibitory concentration (IC_{50}) values ranging from 1 to 5 μg/mL. It should be noted that while the IC_{50} value of 5-NO_2-dU was comparable to that obtained for DHPG, the dose response curve was shallow and at higher concentrations appeared to plateau at approximately 55-60% inhibition. Compound **11** was also assayed for antiviral activity against HCMV at three different concentrations (1, 20 and 100 μg/mL), and was found to be inactive (data not shown).

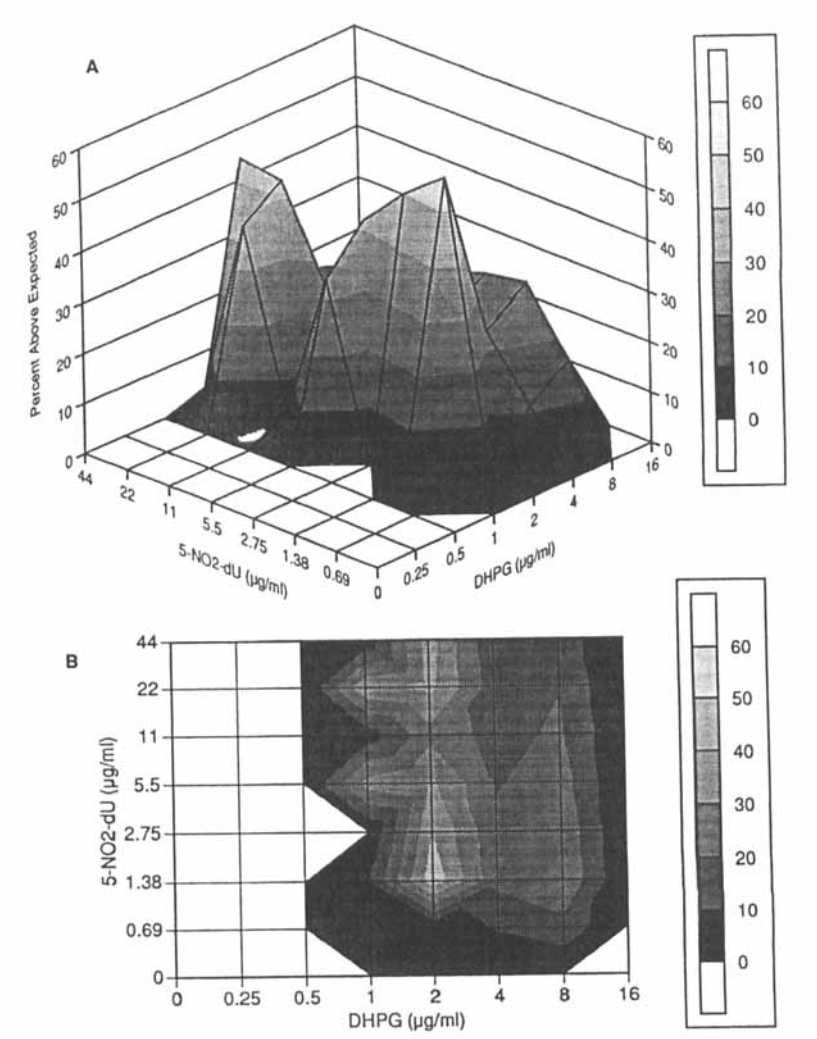

Figure 2. Synergy plot (**A**) and contour plot (**B**) of 3D analysis of the anti-HCMV activity of combinations of 5-NO_2-dU and DHPG in MRC-5 cells (99.9% confidence).

To ascertain the effectiveness of simultaneous administration of two agents with potentially different anti-HCMV mechanisms of action, we examined the combined activity of 5-NO_2-dU and DHPG as described in experimental section. The resultant data from this experiment were plotted as a three-dimensional graph to produce a dose-response surface of the synergy plot (99.9% confidence limits) as shown in Figure 2A. The contour plot of the three-dimensional graph is shown in Figure 2B. The three-dimensional representation

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of the data plotted in Figure 2A clearly show antiviral activity above that expected for additive drug interactions, which would have yielded a flat dose-response plain. This indicates a synergistic anti-HCMV activity obtained when 5-NO_2-dU and DHPG were used in combination. In addition, there was no observed cytotoxicity to the MRC-5 cell layer at any concentration of combined drugs.

When applied to confluent cultures of MRC-5 cells and scored visually for toxic effects, 5-NO_2-dU had no effect on cell morphology at the highest concentration tested (TC_{50} value > 100 μg/mL, data not shown). The ability of 5-NO_2-dU to inhibit cell growth was then monitored using log phase growing cells. It has been reported by De Clercq et al.[5] that cells in exponential growth preferentially utilize deoxyuridine (dU) for the synthesis of thymidine monophosphate (TMP) *de novo*. In such proliferating cells, the incorporation of dU will be suppressed by any compound, such as 5-NO_2-dU, that interferes with thymidylate synthetase.[5] Therefore, as expected, in the growth inhibition experiment 5-NO_2-dU had a pronounced effect on cell proliferation with a median toxic concentration (TC_{50}) of approximately 10 μg/mL (data not shown).

In summary, we have succeeded in preparing anomerically pure 5-NO_2-dU and 2'-arafluoro-5-NO_2-dU in good yields. We have analysed the *in vitro* anti-HCMV activity of 5-NO_2-dU alone and in combination with DHPG. While the data indicate that 5-NO_2-dU is marginally effective in reducing the HCMV replication in culture on its own, when administered in combination with DHPG in a plaque reduction assay, 5-NO_2-dU caused a synergistic enhancement of the overall antiviral efficacy of the two agents. Thus, 5-NO_2-dU appears to be a good candidate for further evaluation using combination therapy with DHPG in the murine cytomegalovirus model.

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EXPERIMENTAL

Melting points (uncorrected) were recorded on a Thomas-Hoover capillary melting-point apparatus. Elemental analyses were performed by Quantitative Technologies Inc., Whitehouse, NJ. The presence of water as indicated by elemental analysis was verified by ^1H NMR spectroscopy. Thin layer chromatography (TLC) was performed on aluminum plates coated (0.2 mm) with silica gel $60F_{254}$ (EM Science). The components were visualized by ultraviolet absorbance and 10% H_2SO_4/MeOH spray followed by heating. Whatman silica gel (230-400 mesh, 60Å) was used for flash column chromatography. All solvents and chemicals used were reagent grade and were

not further dried/purified unless otherwise noted. Evaporations were carried out at a temperature ≤ 30 °C and under diminished pressure. Infrared (IR) spectra were recorded in KBr with a Perkin-Elmer 1420 IR spectrophotometer and ultraviolet spectra (UV) were recorded with a Hewlett-Packard 8452 diode array spectrophotometer. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 400 MHz with a Brücker AM400 wide bore NMR spectrometer. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane (internal) (key: s = singlet, d = doublet, t = triplet, m = multiplet and br = broad).

1-(2-Deoxy-3,5-di-O-p-toluoyl-D-erythro-pentofuranosyl)-5-nitrouracil (**3**). A mixture of dry 5-nitrouracil (Aldrich Chemicals, 1.6 g, 10 mmol), 1,1,1,3,3,3-hexamethyldisilazane (HMDS, 15 mL) and $(NH_4)_2SO_4$ (0.1 g) was heated under reflux for 18 h with the exclusion of moisture. Excess HMDS was removed by distillation and the residue was subjected to high vacuum for 6 h. The resulting bis-silyl derivative (**1**) was dissolved in anhydrous $CHCl_3$ (50 mL) and 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-*D-erythro*-pentofuranose (**2**)[13] (4.3 g, 11.0 mmol) was added in two portions at 2 h interval. After stirring the mixture at room temperature for 20 h, it was diluted with CH_2Cl_2 (150 mL) and the organic solution was washed with water (2 × 50 mL). The organic layer was dried (Na_2SO_4) and the solvent was evaporated. The solid thus obtained was purified by chromatography over a silica gel column (2.5 × 25 cm) eluting with CH_2Cl_2 containing 0-1% MeOH. The appropriate fractions were pooled and evaporated to give 4.8 g (94.3 %) of an anomeric mixture (**3**) as an amorphous solid; ^{1}H NMR (DMSO-d_6) δ 2.36, 2.37, 2.39, 2.45 (4s, 12 H, *p*-toluoyl C*H*$_3$), 2.60-3.0 (m, 4 H, C$_2$*H*), 4.46-4.68 (m, 5 H, C$_4$*H*, C$_5$*H*$_2$), 5.19 (t, 1 H, C$_4$*H*), 5.68 (m, 2 H, C$_3$*H*), 6.23 (m, 2 H, C$_1$*H*), 7.21-7.93 (m, 16 H, *p*-toluoyl), 8.95, 9.01 (2s, 2 H, C$_6$*H*), 12.13 (br s, 2 H, N*H*). Anal. Calcd for $C_{25}H_{23}N_3O_9 \cdot 0.5\ H_2O$: C, 57.91; H, 4.60; N, 8.10. Found: C, 57.97; H, 4.49; N, 7.96.

1-(2-Deoxy-D-erythro-pentofuranosyl)-5-nitrouracil (**4** **and** **5**). To a solution of **3** (4.0 g, 7.9 mmol) in a mixture of MeOH (100 mL) and pyridine (40 mL) was added $NaOCH_3$ (1.6 g, 29.5 mmol). The mixture was protected from moisture and stirred at room temperature for 5 h. The reaction was quenched by the addition of excess of amberlite IR-120 (H^+) resin (pH6). The resin was removed by filtration and the resin was washed with MeOH (3 × 50 mL). The combined filtrate and washings were evaporated and the residual solid was

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purified by chromatography over a silica gel column (2.5 × 30 cm). The product was eluted with a gradient of CH_2Cl_2 containing 0-8% MeOH. The appropriate fractions containing the product were pooled and the solvent was evaporated to give 1.8 g (84%) of an anomeric mixture of **4** and **5**; ^{1}H NMR (DMSO-d_6) δ 2.11-2.75 (m, 4 H, $C_{2'}H$, $C_{2''}H$), 3.36-3.93 (m, 4 H, $C_{5'}H_2$), 4.32 (m, 4 H, $C_{3'}H$, $C_{4'}H$), 4.93, 5.25, 5.32, 5.41 (4s, 4 H, O*H*), 6.11 (m, 2 H, $C_{1'}H$), 9.20, 9.51 (2s, 2 H, C_6H), 12.04 (br s, 2 H, N*H*). Anal. Calcd for $C_9H_{11}N_3O_7 \cdot 0.5\ H_2O$: C, 38.30; H, 4.28; N, 14.89. Found: C, 38.48; H, 4.26; N, 14.67.

***1-[5-O-(4,4'-Dimethoxytrityl)-2-deoxy-β-D-erythro-pentofuranosyl]-5-nitrouracil* (6) *and 1-[5-O-(4,4'-Dimethoxytrityl)-2-deoxy-α-D-erythro-pentofuranosyl]-5-nitrouracil* (7).** The anomeric mixture of 1-(2-deoxy-*D-erythro*-pentofuranosyl)-5-nitrouracil (**4** and **5**) (1.6 g, 8.5 mmol) was dried by co-evaporation with anhydrous pyridine (2 × 25 mL). The residue was dissolved in dry pyridine (30 mL) and 4,4'-dimethoxytrityl chloride (2.6 g, 7.6 mmol) was added under an argon atmosphere. After stirring the mixture at room temperature for 4 h, the reaction was quenched by the addition of MeOH (5 mL). The solvent was evaporated and the residue was co-evaporated with toluene (2 × 20 mL) to remove traces of pyridine. The crude product was purified by chromatography over a silica gel column (2 × 20 cm) and the product was eluted with a gradient of CH_2Cl_2 containing 0-3% MeOH. The appropriate fractions containing the pure isomers were collected separately and evaporated to give the pure α- and β-anomers.

β-Anomer (**6**): yield, 1.9 g (38%); mp 126-128 °C; ^{1}H NMR (DMSO-d_6) δ 2.33 (m, 2 H, $C_{2'}H$, $C_{2''}H$), 3.22 (br s, 2 H, $C_{5'}H_2$), 3.76 (s, 6 H, 2OCH_3), 3.90 (br s, 1 H, $C_{4'}H$), 4.21 (br s, 1 H, $C_{3'}H$), 5.36 (s, 1 H, $C_{3'}$O*H*), 6.01 (t, 1 H, J = 5.60 Hz, $C_{1'}H$), 6.85-7.38 (m, 13 H, DMT protons), 8.88 (s, 1 H, C_6H), 12.08 (s, 1 H, N*H*). Anal. Calcd for $C_{30}H_{29}N_3O_9$: C, 62.60; H, 5.08; N, 7.30. Found: C, 62.35; H, 4.99; N, 7.19.

α-Anomer (**7**): yield, 1.8 g (36%); mp 122-124 °C; ^{1}H NMR (DMSO-d_6) δ 2.13 (d, 1 H, $C_{2'}H$), 2.62 (m, 1 H, $C_{2''}H$), 3.05, 3.11 (2m, 2 H, $C_{5'}H_2$), 3.74 (s, 6 H, 2OCH_3), 4.20 (br s, 1 H, $C_{4'}H$), 4.44 (br s, 1 H, $C_{3'}H$), 5.46 (s, 1 H, $C_{3'}$O*H*), 6.17 (dd, 1 H, J = 1.60 Hz, $C_{1'}H$), 6.90-7.39 (m, 13 H, DMT protons), 9.15 (s, 1 H, C_6H), 12.06 (s, 1 H, N*H*). Anal. Calcd for $C_{30}H_{29}N_3O_9 \cdot 1.25\ H_2O$: C, 60.24; H, 5.30; N, 7.03. Found: C, 60.17; H, 5.03; N, 6.84.

General procedure for the removal of 4,4'-dimethoxytrityl group. A solution of the nucleoside **6** or **7** (0.5 g, 1 mmol) in 80% aqueous AcOH (8 mL)

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was stirred at room temperature for 15 min. Acetic acid was evaporated and the residual solid was triturated with ether to afford analytically pure free nucleosides.

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-5-nitrouracil (**4**): yield, 0.2 g (89%); mp 153-154 °C; Lit.[12] mp 152-153 °C; IR υ 1525 (NO_2), 1725 (C=O), 3150-3650 (NH, OH) cm^{-1}; UV (MeOH) λmax 238 nm (ε7,700), 304 (9,000); ^{1}H NMR (DMSO-d_6) δ 2.77 (t, 2 H, $C_{2'}H$, $C_{2''}H$), 3.60 (m, 2 H, $C_{5'}H_2$), 3.90 (m, 1 H, $C_{4'}H$), 4.26 (m, 1 H, $C_{3'}H$), 5.10 (t, 1 H, $C_{5'}OH$), 5.18 (d, 1 H, $C_{3'}OH$), 6.06 (t, 1 H, J = 6.0 Hz, $C_{1'}H$), 9.42 (s, 1 H, C_6H), 11.89 (s, 1 H, N_3H). Anal. Calcd for $C_9H_{11}N_3O_7 \cdot 0.25\ H_2O$: C, 38.92; H, 4.17; N, 15.13. Found: C, 38.74; H, 4.11; N, 14.88.

1-(2-Deoxy-α-D-erythro-pentofuranosyl)-5-nitrouracil (**5**): yield, 0.22 g (93%); mp > 250 °C; ^{1}H NMR (DMSO-d_6) δ 2.10 (d, 1 H, $C_{2'}H$), 2.65 (m, 1 H, $C_{2''}H$), 3.43 (m, 2 H, $C_{5'}H_2$), 4.30 (m, 2 H, $C_{3'}H$ and $C_{4'}H$), 4.78 (t, 1 H, $C_{5'}OH$), 5.30 (d, 1 H, $C_{3'}OH$), 6.10 (dd, 1 H, J = 1.2 Hz, $C_{1'}H$), 9.16 (s, 1 H, C_6H), 11.93 (s, 1 H, N_3H). Anal. Calcd for $C_9H_{11}N_3O_7$: C, 39.56; H, 4.06; N, 15.38. Found: C, 39.45; H, 3.98; N, 15.20.

1-(2-Deoxy-2-fluoro-3,5-di-O-benzoyl-β-D-arabinofuranosyl)-5-nitrouracil (**10**). To a solution of the bis-silyl 5-nitrouracil (**1**) (prepared from 0.7 g, 4.45 mmol of 5-nitrouracil) in dry CH_3CN (20 mL) was added a solution of 2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-α-*D*-arabinofuranosyl bromide (**8**) (prepared[15] from 2.2 g, 4.7 mmol of 2-deoxy-2-fluoro-1,3,5-tri-*O*-benzoyl-α-*D*-arabinofuranose) in dry CH_3CN (15 mL). The mixture was stirred at room temperature for 3 days and then heated under reflux for 2 days with the exclusion of moisture. The solvent and the volatile materials were evaporated and the residual foam was purified by chromatography on a silica gel column (2.5 × 25 cm). The nucleoside products were eluted with a gradient of CH_2Cl_2 containing 0-2.5% MeOH to yield 1.5 g of a mixture of α:β anomers (**9** and **10**) in a ratio of 1:5 (as judged by ^{1}H NMR). The foam was triturated with a mixture of MeOH (50 mL) and CH_2Cl_2 (8 mL), and the solid was collected by filtration. The filtrate was evaporated and the residual solid was triturated with MeOH:CH_2Cl_2 (5:1, 20 mL). The solid was collected by filtration and the combined solids were dried to yield 1.2 g (52%) of pure β-anomer (**10**); mp 200-202 °C; ^{1}H NMR (DMSO-d_6) δ 3.17 (m, 2 H, $C_{5'}H_2$), 4.10 (m, 1 H, $C_{4'}H$), 5.55 (d, 1 H, $C_{3'}H$), 5.63 (d, 1 H, $C_{2'}H$), 6.37 (dd, 1 H, $C_{1'}H$), 7.49-7.72 (m, 6 H, benzoyl protons), 8.03, 8.07 (2d, 4 H, benzoyl

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protons), 8.89 (s, 1 H, C_6H), 12.36 (s, 1 H, N_3H). Anal. Calcd for $C_{23}H_{18}FN_3O_9$: C, 55.31; H, 3.63; N, 8.41; F, 3.80. Found: C, 55.12; H, 3.60; N, 8.26; F, 3.79.

1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-nitrouracil (**11**). To a suspension of **10** (0.25 g, 0.5 mmol) in anhydrous MeOH (15 mL) was added a solution of $NaOCH_3$ in MeOH (25%, 0.7 mL, 3.2 mmol). After stirring the reaction mixture at ambient temperature for 1 h, the reaction was quenched by the addition of amberlite IR-120 (H^+) resin (pH6). The resin was removed by filtration, and the resin was washed with MeOH (2 × 15 mL). The combined filtrate and washings were evaporated and the residual solid was purified by chromatography over a silica gel column (2 × 20 cm). The product was eluted with a mixture of CH_2Cl_2:MeOH (9:1). The homogeneous fractions were collected and the solvent evaporated to yield 0.11 g (75%) of pure **11**; mp 202-204 °C; IR υ 1525 (NO_2), 1735 (C=O), 3430-3570 (NH, OH) cm^{-1}; UV (MeOH) λmax 236 nm (ε8,400), 298 (9,600); ^1H NMR (DMSO-d_6) δ 3.58-3.69 (m, 2 H, C_5H_2), 3.89 (m, 1 H, C_4H), 4.28 (m, 1 H, C_3H), 5.17 (tt, 1 H, C_2H), 5.33 (t, 1 H, C_5OH), 5.97 (d, 1 H, C_3OH), 6.15 (dd, 1 H, C_1H), 9.25 (s, 1 H, C_6H), 12.25 (s, 1 H, N_3H). Anal. Calcd for $C_9H_{10}FN_3O_7$: C, 37.12; H, 3.46; N, 14.43; F, 6.52. Found: C, 37.14; H, 3.38; N, 14.18; F, 6.43.

ANTIVIRAL ACTIVITY ASSAYS

Cells and Viruses. Human cytomegalovirus (HCMV, strain AD 169) and MRC-5 cells were obtained from American Type Culture Collection (ATCC), Rockville, MD. MRC-5 cells were grown in Basal Medium Eagle (BME) with 10% FBS, 0.035% $NaHCO_3$ and 50 μg/mL gentamicin. Cells were passaged according to conventional procedures.[16]

Inhibition of HCMV in culture using a plaque reduction assay. Compounds were assessed for their ability to inhibit HCMV in cell culture using a plaque reduction assay essentially as described.[17] In these experiments monolayer cultures of MRC-5 cells were grown to confluence in 24 well tissue culture plates. The growth medium was removed and then 1 mL of virus [AD169 strain of HCMV, 50 plaque forming units (pfu)] in test medium (Dulbecco's modified Eagle medium (DMEM) containing 2% FBS, 1.0% $NaHCO_3$ and 50 μg of gentamicin/mL) was added to each well. The plates were centrifuged at 2200 rpm for 30 min and then the medium was aspirated from

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each well. Individual dilutions (in test medium) of each compound were added to each test well (0.8 mL/well, two wells/dilution). The plates were incubated at 37 °C in a moist atmosphere of 5% CO_2 until virus plaques had formed in the control cells (HCMV infected, untreated). The medium was aspirated from all wells and the cells were stained by adding 0.3 mL of 0.2% crystal violet in 10% buffered formalin in each well. After 15 min the stain was removed, the wells were rinsed with tap water until the rinsed water was clear, and the plates were inverted and dried at ambient temperature. Plaques were counted with a dissecting microscope. Ganciclovir (DHPG, obtained from Syntex Research, Palo Alto, CA) was used as a positive control in all assays.

The plaque reduction assay was also used to assess the effect of agents added in combination on HCMV growth. In these studies both compounds (5-NO_2-dU and DHPG) were prepared in test medium at double the highest concentration of each drug. Each preparation was then diluted by serial 2-fold dilutions in test medium. A uniform volume of 5-NO_2-dU at various concentrations was added to sterile tubes. An equal volume of each concentration of DHPG was then added to each concentration of 5-NO_2-dU. Controls included no drug, various concentrations of 5-NO_2-dU without DHPG or various concentrations of DHPG without 5-NO_2-dU. The ability of 5-NO_2-dU to inhibit virus infection in combination with DHPG was determined using the MacSynergy program as described by Prichard and Shipman.[18]

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Cytotoxicity assays. Stationary uninfected MRC-5 cells were evaluated for visual cytotoxicity on the scoring basis of 0 (no visual cytotoxicity at 20 fold magnification) to 4 (cell sheet nearly destroyed). Each assay was performed in quadruplicate. The resultant data were averaged, graphed and then used to calculate the dose required to reduce cell proliferation by 50% (TC_{50}).

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Received June 8, 1995
Accepted September 7, 1995

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