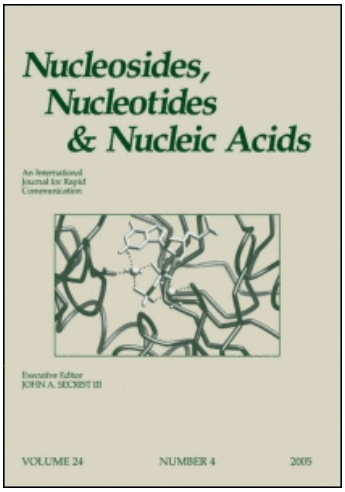

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Effect of 2-Amino Substitution on the Antiviral Effects of 5-Ethyl-2'-Deoxyuridine and (E)-5-(2-bromovinyl)-2'-Deoxyuridine and Their Incorporation into DNA

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EFFECT OF 2-AMINO SUBSTITUTION ON THE ANTIVIRAL EFFECTS OF 5-ETHYL-2'-DEOXYURIDINE AND (E)-5-(2-BROMOVINYL)-2'-DEOXYURIDINE AND THEIR INCORPORATION INTO DNA

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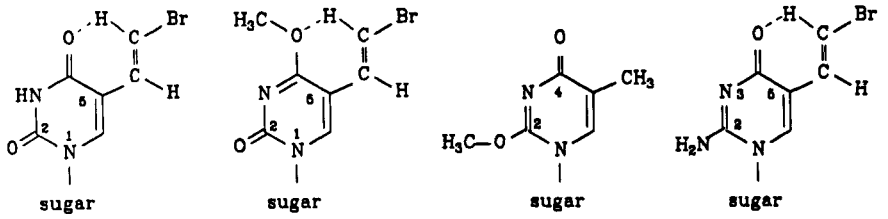
Abstract. The 2-amino derivatives of 5-ethyl-2'-deoxyuridine (EDU) and (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) have been synthesized and evaluated for anti-herpesvirus activity. They were at least 1000-fold less effective against herpes simplex virus replication than the parent compounds EDU and BVDU. The 5'-triphosphates of the 2-amino substituted EDU, BVDU and thymidine derivatives were also synthesized and examined on their substrate/inhibitor properties against different DNA polymerases. None of the compounds proved markedly inhibitory to HSV-1 DNA polymerase or cellular DNA polymerase α. Nor were they incorporated into the growing DNA chain.

(*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU†) is one of the most potent and selective anti-herpesvirus agents that has ever been developed.[1-4] Various analogues of BVDU have been synthesized in attempts to elucidate the structure-function relationship and to find more potent and selective molecules.[5] None of these analogs has proved to be more selective than the parent compound BVDU against herpes simplex virus type 1 (HSV-1).

Structural modifications included changes in both the pyrimidine base and the sugar moiety of BVDU. In most cases[5] the 5-vinyl substituent was modified. Since incorporation of BVDUMP into viral DNA is believed to be the main factor in the anti-herpesvirus activity[2-6], substitutions

†Abbreviations: BVDU, (E)-5-(2-bromovinyl)-2'-deoxyuridine; BVDUTP, 5'-triphosphate of BVDU; EDU, 5-ethyl-2'-deoxyuridine; EDUTP, 5'-triphosphate of EDU; HSV, herpes simplex virus.



(1) (2) (3) (4)

FIG. 1. Structure of BVDU (**1**), O^4-methyl-BVDU (**2**), O^2-methyl-thymidine (**3**) and 2-amino-BVDU (**4**). (Conformation of the O^4-methyl group with respect to the potential base-pair has been determined as *syn* in O^4-methyl-thymidine.[10] Conformation of the 2-bromovinyl side chain has been determined from the BVDU crystal structure.[11]

TABLE 1. Antiviral activity of the test compounds in primary rabbit kidney cell cultures

Viruses	Minimum inhibitory concentration* (μg/ml)			
	Test compounds			
	2-amino-EDU	2-amino-BVDU	EDU	BVDU
HSV-1 (KOS)	>400	20	0.7	0.02
HSV-1 (F)	>400	70	0.7	0.02
HSV-1 (McIntyre)	>400	70	1	0.02
HSV-2 (G)	>400	150	0.7	2
HSV-2 (196)	>400	300	2	70
HSV-2 (Lyons)	>400	300	2	40
Vaccinia virus	>400	>400	1	20
Vesicular stomatitis virus	>400	>400	>400	>400
TK^- HSV-1 (B-2006)	>400	>400	>400	300
TK^- HSV-1 (VMW-1837)	>400	>400	>400	150

*Required to reduce virus-induced cytopathogenicity by 50%.
The minimum cytotoxic concentration, required to cause a microscopically detectable alteration of normal cell morphology was ≥400 μg/ml for the test compounds.

affecting base-pairing ability should decrease the efficacy of the compound. For example, alkyl substitution at the oxygen atom in position 4 of dTTP changes hydrogen bonding pattern in such a way that the nucleotide becomes markedly less efficient as a substrate for the DNA polymerase enzymes.[7,8] Concomitantly, O^4-ethyl-BVDU (Fig. 1) is about 100-times less potent against HSV-1 than the parent BVDU.[9]

Another example for the modified base-pairing pattern is the O^2-substitution. O^2-Alkyl-dTTPs are almost as poor substrates for the DNA polymerase enzymes as the O^4-alkyl-dTTPs.[8] In 2-amino substituted BVDU the same quinoidal structure is present as in O^2-alkyl-BVDU[9] (Fig. 1). 2-Amino-BVDU has been evaluated against herpesviruses.[9] Practically no activity was observed. We wondered whether the inactivity of 2-amino-BVDU as an anti-herpesvirus compound[9] was related to the inefficient action of the triphosphate derivative as substrate for HSV-1 DNA polymerase. In addition to 2-amino-BVDU, the 2-amino derivatives of thymidine and 5-ethyl-2'-deoxyuridine (EDU), a known antiherpetic agent[12,13] were prepared and evaluated against different herpes simplex virus strains. To study their substrate/inhibitor properties for different DNA polymerases, the 5'-triphosphate derivatives have also been synthesized.

RESULTS AND DISCUSSION

Table 1 shows the antiviral effects of the novel 2-amino-substituted analogs of EDU and BVDU. 2-Amino-EDU and 2-amino-BVDU were at least 1000-fold less effective against HSV-1 and HSV-2 than the parent nucleosides EDU and BVDU. Against vaccinia virus, vesicular stomatitis virus and the TK^- strains of HSV-1, the 2-amino-EDU and 2-amino-BVDU were devoid of any antiviral activity.

Replacement of the keto group by an amino group in C-2 of the pyrimidine base causes changes in the heteroaromatic structure. ^{13}C-NMR data (see Experimental) were assigned as characteristics of the quinoidal structure (Fig. 1), as in the case of *iso*deoxycytidine. Thus, N^3 is deprotonated as in O^4-alkyl-thymidines. Nevertheless, O^4-alkyl-thymidine can base-pair with adenine of the template, with a single hydrogen bond between N^3(T) as an acceptor and N^6(A) as a proton donor.[10] Deprotonated N^3 of both the O^2-alkyl- and the 2-amino-BVDU [(*E*)-5-(2-bromovinyl)*iso*deoxycytidine] is assumed to be also able to act as a

TABLE 2. Inhibitory effect of 2-amino derivatives of dTTP, EDUTP and BVDUTP on HSV-1 DNA polymerase and calf thymus DNA polymerase α

Test compounds	IC_{50} (μM)*	
	HSV-1 DNA polymerase	Calf thymus DNA polymerase α
2-amino-dTTP	> 500	> 500
2-amino-EDUTP	> 500	> 500
2-amino-BVDUTP	379	388

*50% Inhibitory concentration.

proton acceptor since O^2-alkyl-dTTPs could be incorporated into DNA by polymerases.[8] From a recent publication, however, it appears that dATP cannot be incorporated opposite an *iso*deoxycytidine.[14] Since the 2-amino compounds did not exert any marked activity against herpesviruses, they must have failed at either the virus-induced thymidine kinase or HSV DNA polymerase level. As a consequence of the altered base-pairing ability, the inhibitory effects at the DNA polymerase level may have been negatively influenced. Therefore, the effects of the 5'-triphosphates of 2-amino-dThd, 2-amino-EDU and 2-amino-BVDU on HSV-1 DNA polymerase and calf thymus DNA polymerase α were examined. The compound concentrations required to achieve 50% inhibition of the DNA polymerases are listed in Table 2.

Neither 2-amino-dTTP nor 2-amino-EDUTP proved inhibitory to HSV-1 DNA polymerase at concentrations up to 500 μM. Also, 2-amino-BVDUTP was virtually inactive against HSV-1 DNA polymerase. Furthermore, none of the test compounds proved inhibitory to calf thymus DNA polymerase α. This is also reflected by the minimum cytotoxic concentrations that were higher than 400 μg/ml (Table 1). Thus, the 2-amino-dUTPs have no effect on DNA polymerase reactions. This is in marked contrast with the behavior of the parent triphosphates: K_i of HSV-1 DNA polymerase for BVDUTP is 0.25 μM, while the K_m for dTTP is 0.66 μM. Similarly, K_i of DNA polymerase α for BVDUTP is 3.6 μM, whereas the K_m for dTTP is 5.3 μM.[15] Similarly to EDUTP, BVDUTP is incorporated into DNA by HSV-1 DNA polymerase[16] as well as by the bacterial Klenow DNA polymerase.[13,17-20]

TABLE 3. Substitution of dTTP by the test compounds in the replication of poly(dA-dT) by Klenow DNA polymerase

Triphosphate	Incorporation of [^{3}H]dAMP					
	1 hr		2 hr		4 hr	
	pmol	%	pmol	%	pmol	%
dTTP	557	100	626	100	814	100
2-amino-dTTP	17	0.2	20	1.1	5	0
2-amino-EDUTP	19	0.3	16	0.5	0	0
None	16	-	13	-	15	-

The reaction mixture contained [^{3}H]dATP and either dTTP or 2-amino-dTTP or 2-amino-EDUTP. When calculating % incorporation of the triphosphates, background pmol incorporation (only [^{3}H]dATP present) was subtracted from pmol incorporation measured in each reaction.

TABLE 4. Substitution of dTTP by the test compounds in the replication of activated calf thymus DNA by Klenow DNA polymerase

Triphosphate	Incorporation of [^{3}H]dAMP					
	1 hr		2 hr		4 hr	
	pmol	%	pmol	%	pmol	%
dTTP	223	100	335	100	637	100
2-amino-dTTP	13	0	16	0	19	0.2
2-amino-EDUTP	14	0.5	15	0	21	0.5
None	13	-	19	-	18	-

The reaction mixture contained [^{3}H]dATP, dCTP, dGTP and either dTTP or 2-amino-dTTP or 2-amino-EDUTP as substrates. Calculation of % incorporation was performed as described in the footnote to Table 3.

TABLE 5. Substitution of dGTP by the test compounds in the replication of poly(dI-dC) by Klenow DNA polymerase

Triphosphate	Incorporation of [^{3}H]dCMP					
	1 hr		2 hr		4 hr	
	pmol	%	pmol	%	pmol	%
dGTP	615	100	800	100	839	100
2-amino-dTTP	11	0.7	10	0.4	9	0.3
2-amino-EDUTP	6	0	5	0	8	0
None	7	-	7	-	7	-

TABLE 6. Substitution of dCTP by the test compounds in the replication of poly(dI-dC) by Klenow DNA polymerase

Triphosphate	Incorporation of [^{3}H]dGMP					
	1 hr		2 hr		4 hr	
	pmol	%	pmol	%	pmol	%
dCTP	308	100	489	100	492	100
2-amino-dTTP	6	0.3	5	0.2	3	0
2-amino-EDUTP	5	0	4	0	4	0
None	5	-	4	-	4	-

TABLE 7. Substitution of dTTP by the test compounds in the replication of poly(dA-dT) by Klenow DNA polymerase at pH 8.8.

Triphosphate	Incorporation of [^{3}H]dAMP					
	1 hr		2 hr		4 hr	
	pmol	%	pmol	%	pmol	%
dTTP	81	100	90	100	117	100
2-amino-dTTP	8	5.2	8	4.7	6	2.6
2-amino-EDUTP	7	3.9	5	1.2	3	0
None	4	-	4	-	3	-

To determine whether the 2-amino derivatives may be incorporated into DNA, substitution assays were carried out with *E.coli* Klenow DNA polymerase enzyme.

Tables 3 and 4 present the replication of the synthetic template/primer poly(dA-dT) and calf thymus DNA by Klenow DNA polymerase. No definite incorporation of [^{3}H]dAMP into DNA was observed if dTTP was replaced by the 2-amino derivatives of either dTTP or EDUTP.

Incorporation of the 2-amino-dUTP derivatives opposite cytosine or guanine was also determined. Assays were done with poly(dI-dC) as the template/primer. Results are shown in Tables 5 and 6, respectively.

Thus, the 2-amino-dUTPs could not substitute for any natural nucleotide in the replication reactions catalyzed by the Klenow DNA polymerase. Partial protonation at neutral pH of the 2-amino group derivatives may negatively influence this incorporation. Therefore, an assay at

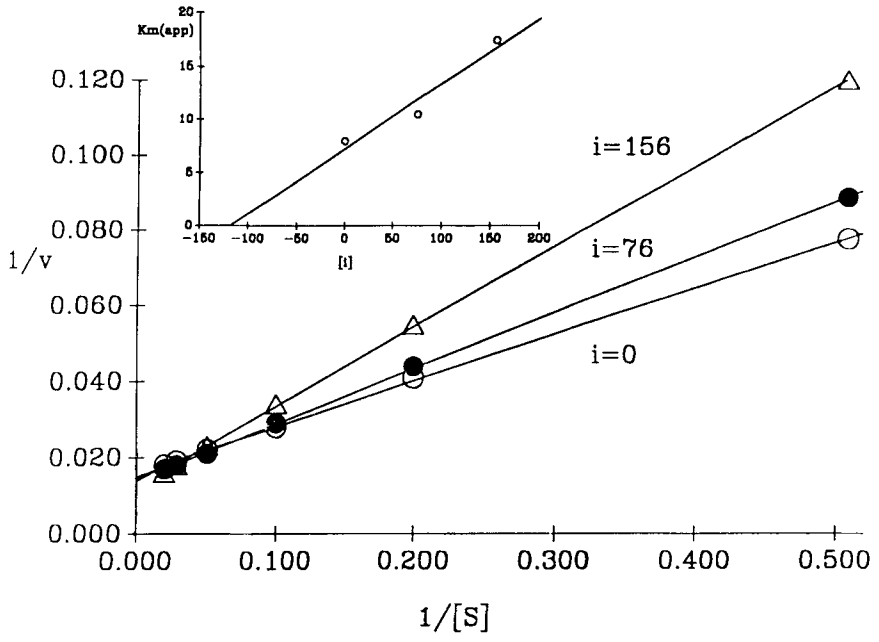


FIG. 2. Double reciprocal plot of replication rate *vs* six concentrations of dTTP in the absence and presence, respectively of 2-amino-dTTP (76 and 156 μM). Reaction mixture was composed as indicated in the Experimental with activated calf thymus DNA as a primer-template and [^{3}H]dTTP as the labelled substrate. Incubations were performed for 30 minutes. Intercept refers to competitive type of inhibition. Insert shows determination of K_i by plotting $K_{m(app)}$ *vs* concentration of the potential inhibitor, 2-amino-dTTP. Intercept gives a K_i of 118 μM. Comparison of K_i to the K_m for dTTP of the Klenow DNA polymerase (7.9 μM) shows that 2-amino-dTTP is a very poor inhibitor of DNA polymerase.

higher pH, where protonation is less probable, was also performed. Data presented in Table 7 show a substitution assay carried out in 60 mM Tris.HCl, pH 8.8, instead of the usual pH 7.4.

Activity of the Klenow DNA polymerase enzyme decreased 7- to 8-fold at this higher pH, compared to that measured at pH 7.4 (Table 3). Percent incorporation of the test compounds was higher at pH 8.8 than at pH 7.4, reaching 5% and 4% for 2-amino-dTTP and 2-amino-EDUTP, respectively, calculated after subtracting background pmol incorporation (only [^{3}H]dATP present). The 5% and 4% may thus represent real incorporations. These values decrease with progression of the reaction, probably because of the proofreading activity of the Klenow enzyme.

In contrast to the 2-alkoxy-dTTPs, which are, albeit poor, substrates of DNA polymerases and are also incorporated into DNA,[8] the 2-amino-dTTP derivatives should not be sterically hindered during base-pairing with the complementary base. Substituents in C-2 of the pyrimidines are located in the narrow groove of the duplex DNA which can easily accommodate an amino group. This has been well established with the 2-amino-adenine/thymine base-pair in poly(amino^{2}dA-dT), synthesized with a DNA polymerase,[21] in which the exocyclic groups in the minor groove side are inverted relative to the 2-amino-thymine/adenine pair.

Finally, the type of inhibition of the enzymatic reaction by 2-amino-dTTP was also determined, with activated calf thymus DNA as the primer/template. Reciprocal plots are shown in Fig. 2. Inhibition of DNA synthesis by 2-amino-dTTP was found to be competitive with respect to dTTP. K_m of the Klenow DNA polymerase for dTTP was 7.9 μM, whereas the K_i for 2-amino-dTTP was 118.4 μM.

CONCLUSION

Annihilation of the marked anti-herpesvirus activity of EDU and BVDU following 2-amino substitution can be attributed to the inability of the 5'-triphosphate derivatives to inhibit HSV-1 DNA polymerase and to be incorporated in the HSV-1 DNA chain. This property is independent of the nature of the 5-substituent (i.e. methyl, ethyl or 2-bromovinyl). Bacterial and mammalian DNA polymerases behaved similarly as the viral DNA polymerase in that they did not accept the 2-amino-dUTP derivatives as substrates.

EXPERIMENTAL

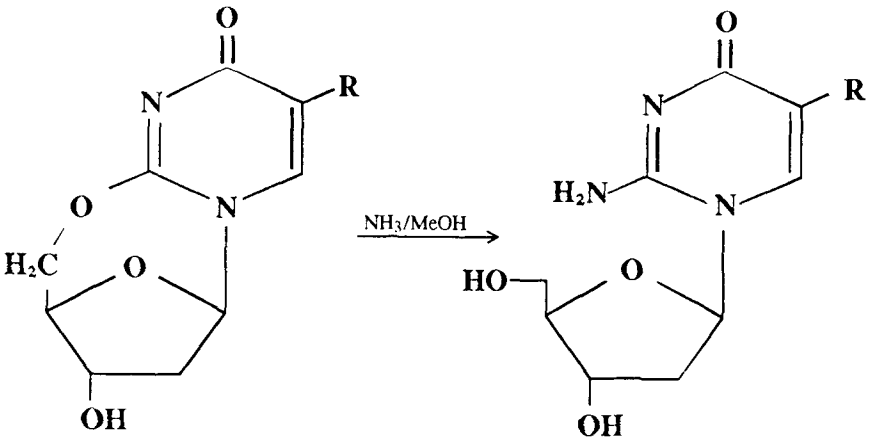
Chemistry

Synthesis of 2-amino derivatives of the 5-substituted 2'-deoxyuridines was accomplished *via* the 2,5'-anhydro derivatives (see Fig. 3).

2,5'-Anhydro-2'-deoxy-5-substituted uridines [I a-c]

Title compounds were prepared by slight modifications of the procedure reported by Watanabe *et al.*[22]

2,5'-Anhydrothymidine [I.a]. Mp. 214-6°C (lit. 216-8°C), anal. calcd. for $C_{10}H_{12}O_4N_2$: C, 53.56; H, 5.39; N, 12.49; found: C, 53.62; H, 5.52 and N, 12.47.



a. $R = -CH_3$

I. b. $R = -CH_2-CH_3$ II.

c. $R = -CH=\overset{Br}{\overset{|}{C}}H$

FIG. 3.

2,5'-Anhydro-2'-deoxy-5-ethyluridine [I.b]. Mp. 180-183°C, anal. calcd. for $C_{11}H_{14}O_4N_2$: C, 55.45; H, 5.92 and N, 11.75; found: C, 55.7; H, 6.08 and N, 11.82.

2,5'-Anhydro-2'-deoxy-5-(*E*)-(2-bromovinyl)uridine [I.c]. Mp. 188-191°C, anal. calcd.for $C_{11}H_{10}O_4N_2Br$: C, 41.92; H, 3.51; N, 8.89 and Br, 25.35; found: C, 42.02; H, 3.68; N, 8.80 and Br, 25.45.

2-Amino-2'-deoxy-5-substituted uridines [II. a-c]

2-Aminothymidine [II.a]. 400 mg (1.78 mmol) of I.a dissolved in 30 ml methanol/NH_3 (30%) was kept at ambient temperature for four days. Solvent was removed in vacuum. Residue was purified by silica gel column chromatography (2x25 cm). The material adsorbed on silica gel was eluted by $CHCl_3$-MeOH (8:2) mixture and recrystallized from MeOH. Yield was 310 mg (71%); mp. 169°C; R_f = 0.35 with $CHCl_3$-MeOH (7:3) on silica gel tlc. Anal. Calcd. for $C_{10}H_{15}O_4N_3$: C, 49.79; H, 6.26 and N, 17.42; found: C, 49.71; H, 6.17 and N, 17.43. UV (H_2O, pH 5.5): λ_{max} = 261 nm; ε_{261} = 6340 $M^{-1}cm^{-1}$, λ_{min} = 251 nm; ε_{251} = 5610 $M^{-1}cm^{-1}$; $[\alpha]_D^{25}$ = +26.2 (c 1.99, 1N NaOH).

^{1}H-NMR (DMSO-d_6 + $CDCl_3$): δ 1.82 (3H, d, $J_{CH_3,6}$= 1.2 Hz; C5-CH_3), 2.2-2.4 (2H, m; C2'-H_2), 3.71 (2H, d, $J_{4',5'}$= 3.0 Hz; C5'-H_2), 3.88 (1H, td, $J_{3',4'}$= 3.0 Hz; C4'-H), 4.40 (1H, m; C3'-H), 5.91 (1H, t, $J_{1',2'}$= 6.8 Hz; C1'-H), 5.1 and 6.6 (2x1H, br s; -NH_2), 7.48 (1H, q; C6-H).

^{13}C-NMR (DMSO-d_6 + $CDCl_3$): δ 13.55 (C5-$\underline{C}H_3$), 39.30 (C2'), 60.79 (C5'), 69.99 (C3'), 87.49 (C1'), 88.09 (C4'), 114.25 (C5), 134.43 (C6), 154.10 (C2), 170.85 (C4).

2-Amino-2'-deoxy-5-ethyluridine (II.b). Starting with 647 mg (2.5) mmol of compound I.b, II.b was prepared by the method described above for the preparation of II.a. Yield was 454 mg (65%), mp. 147°C. R_f on silica gel was 0.42 with $CHCl_3$-MeOH (7:3). Anal. calcd. for $C_{11}H_{17}O_4N_3$: C, 51.75; H, 6.71 and N, 16.46; found: C, 51.33; H, 6.55 and N, 16.32. UV (H_2O, pH 5.5); λ_{max} = 261 nm, ε_{261} = 5950 $M^{-1}cm^{-1}$, λ_{min} = 251 nm, ε_{251} = 5290 $M^{-1}cm^{-1}$; $[\alpha]_D^{25}$ = +30.2 (c 1.0, 1N NaOH).

^{1}H NMR (DMSO-d_6 + $CDCl_3$): δ 1.08 (3H, t, J = 7.2 Hz; -$CH_2\underline{C}H_3$), 2.28 (2H, q; $\underline{C}H_2CH_3$), 2.2-2.4 (2H, m; C2'-H_2), 3.73 (2H, d, $J_{4',5'}$ = 3.0 Hz; C5'-H_2), 3.93 (1H, td, $J_{3',4'}$ = 3.6 Hz; C4'-H), 4.43 (1H, td, $J_{2',3'}$ = 4.7 Hz; C3'-H), 5.96 (1H, t, $J_{1',2'}$ = 6.8 Hz; C1'-H), 5.3 and 6.6 (2x1H; brs; -NH_2), 7.45 (1H, s; C6-H).

2-Amino-2'-deoxy-5-(*E*)-(2-bromovinyl)uridine (II.c). This compound was prepared from 600 mg (1.9 mmol) of compound I.c with the method described above for II.a, except that the adsorbed material was eluted with $CHCl_3$-MeOH (7:3) and that the eluted product was recrystallized from MeOH-EtAc (1:1). Yield was 255 mg (40%), mp. 170-171°C. R_f on silica gel with $CHCl_3$-MeOH (7:3) was 0.28. Anal. calcd. for $C_{11}H_{14}O_4N_3Br$: C, 39.74; H, 4.24; N, 12.65 and Br, 24.05; found: C, 39.92, H, 4.82, N, 12.58 and Br, 24.03. UV (20% MeOH/H_2O, pH 5.5); λ_{max} = 261 nm, ε_{261} = 11520 $M^{-1}cm^{-1}$, λ_{min} = 239 nm, ε_{239} = 8930 $M^{-1}cm^{-1}$; $[\alpha]_D^{25}$ = +38.6 (c 1.0, 1 N NaOH).

^{1}H NMR (DMSO-d_6 + $CDCl_3$): δ 2.25-2.45 (2H, m; C2'-H_2), 3.81 (2H, d, $J_{4',5'}$ = 3.0 Hz; C5'-H_2), 3.99 (1H, td, $J_{3',4'}$ = 2.8 Hz; C4'-H), 4.48 (1H, td, $J_{2',3'}$ = 4.8 Hz; 3'-H), 6.00 (1H, t, $J_{1',2'}$ = 6.5 Hz; C1'-H), 5.0 and 7.1 (2 x 1 H, brs; -NH_2), 6.81 (1H, d, J_{trans} = 13.2 Hz; Cα-H), 7.49 (1H, d; Cβ-H), and 7.94 (1H, s; C6-H).

2-Amino-5-substituted-dUTPs

5'-Triphosphate derivatives of the 2-amino nucleosides have been prepared by a modification of the standard $POCl_3$ method[23,24] on an 0.2 mmol scale. 2-Amino-thymidine, 2-amino-5-ethyl-2'-deoxyuridine or 2-

amino-(*E*)-5-(2-bromovinyl)-2'-deoxyuridine (0.2 mmol) were dissolved by stirring in 0.5 ml dry trimethylphosphate, under argon atmosphere. Solution was cooled down to -20°C and 20 µl (0.22 mmol) $POCl_3$ was added. Mixture was held at -20°C for 24-48 hr. One mmol of bis-tri-n-butylammonium pyrophosphate in 0.2 ml tributylamine was then added to the mixture at -20°C. After 24 hours, 8 ml $Et_3N.H_2CO_3$ (1 M) was added, the mixture was evaporated in vacuo, dissolved in n-PrOH-NH_4OH-H_2O (60-30-10) and separated by silica gel column chromatography (3 x 45 cm) with the n-PrOH eluent mixture. Pooled triphosphate fractions (determined by silica gel tlc) were evaporated in vacuo at 20°C, dissolved in deionized water, centrifuged to remove occasional silica gel contamination, and clear solutions of ammonium salts of the triphosphates were freeze-dried and stored at -25°C. Yields, calculated on starting nucleosides, were 35%, 51% and 20% for 2-amino-dTTP, 2-amino-EDUTP and 2-amino-BVDUTP, respectively. R_f values on silica gel tlc plates and with n-PrOH:NH_3:H_2O (5:4:1) were as follows: 2-amino-dTTP, 0.26; 2-amino-EDUTP, 0.3; 2-amino-BVDUTP, 0.35. HPLC retention times were 3.67 min for dTTP, 3.92 min 2-amino-dTTP, 4.30 min for 2-amino-EDUTP and 4.13 min for 2-amino-BVDUTP, respectively (ISCO, APS-Hypersil weak anion exchanger column [100 x 4.6 mm, particle size 3 microns]; buffer A: 0.1 M LiCl, 20% acetonitrile, 20% Tris solution; buffer B: 1 M LiCl, 20% acetonitrile, 20% Tris solution (Tris solution: 0.1 M Tris.HCl, pH 7.2, 0.01 M EDTA).

Confirmation of the structure was performed by NMR (400 MHz):

2-Amino-dTTP. ^1H NMR (D_2O): δ 1.92 (3H, s, CH_3), 2.4-2.6 (2H, m, C2'-H_2), 4.24 (2H, m, C5'-H_2), 4.71 (1 H, m, C3'-H), 4.89 (1 H, m, C4'-H), 5.93 (1H, t, J = 7 Hz, C1'-H), 7.65 (1 H, s, C6-H).

^{31}P NMR (D_2O): δ -5.6 (αP), -9.6 (γP), -20.4 (βP).

^{13}C NMR (D_2O): δ 12.6 (CH_3), 37.6 (C2'), 64.8 (C5'), 69.8 (C3'), 85.5 (C4'), 88.6 (C1'), 115.0 (C5), 136.6 (C6), 154.9 (C2), 174.2 (C4).

2-Amino-EDUTP. ^1H NMR (D_2O): δ 1.11 (3H, t, J = 7 Hz, -CH_3), 2.29 (2H, q, J = 7 Hz, -CH_2), 2.4-2.6 (2 H, m, C2'-H_2), 4.25 (2 H, m, C5'-H_2), 4.70 (1 H, m, C3'-H), 4.90 (1 H, m, C4'-H), 5.98 (1 H, t, J = 7 Hz, C1'-H), 7.55 (1 H, s, C6-H).

^{31}P NMR (D_2O): δ -7.3 (αP), -9.68 (γP), -20.8 (βP).

Substrates and enzymes

Unlabelled natural triphosphates dTTP, dATP, dCTP and dGTP and the alternating copolymers poly(dA-dT) and poly(dI-dC) were purchased from

Boehringer Mannheim GmbH. [^{3}H]dTTP, [^{3}H]dATP, [^{3}H]dCTP and [^{3}H]dGTP were supplied by Amersham Int. Calf thymus DNA was from Sigma, activation was carried out by pancreatic DNase. *E.coli* Klenow DNA polymerase enzyme (2500 units/mg) was from Boehringer. Calf thymus DNA polymerase α was from P.L. Biochemicals (Milwaukee, Wisconsin, USA) and HSV-1 DNA polymerase was purified from HSV-1 (KOS)-infected HeLa cells as previously described.[25]

Methods

Incorporation substitution assays were carried out in 60 mM potassium phosphate buffer (pH 7.4) and 6 mM $MgCl_2$ solutions, except when indicated otherwise, in 50 µl final volumes. Concentration of the alternating sequence template-primers poly(dA-dT) or poly(dI-dC) was 100 µM (P) and that of the activated calf thymus DNA was 150 µg/ml. Triphosphates, either labelled or unlabelled, were applied at 200 µM. Specific activity of the labelled natural substrates used in the assays were as follows: [^{3}H]dTTP, 11.8 dpm/pmol; [^{3}H]dATP, 44.3 dpm/pmol; [^{3}H]dCTP, and [^{3}H]dGTP, 20.7 dpm/pmol. Reactions were started by addition of the Klenow DNA polymerase enzyme at a final concentration of 2.4 units/ml mix. Incubation was carried out at 37°C. Samples of 10 µl were withdrawn at times indicated in the Tables and then spotted onto GF/C filters, washed, dried and counted for acid-insoluble radioactivity. Data given in the Tables are mean values for two experiments. Kinetic parameters were calculated on an IBM compatible PC by using "Enzkin" program package (Compudrug, Budapest).

Inhibition experiments with HSV-1 DNA polymerase and calf thymus DNA polymerase α were carried out as follows. In the calf thymus DNA polymerase α assays, activated calf thymus DNA served as the template. The reaction mixture (40 µl) contained 20 mM Tris-HCl (pH 7.9), 200 µM dithiothreitol, 3 mM $MgCl_2$, 20 µg BSA, 2 µg activated DNA, 100 µM dGTP, dCTP and dATP, an appropriate concentration of [^{3}H]dTTP, 8 µl test compound solution (containing varying concentrations of the test compounds) and 12 µl of the DNA polymerase α preparation (diluted 40-fold). The reaction mixtures were incubated at 37°C for 30 min at which time 1 ml TCA 5% was added. After being kept on ice during 10 min, the TCA-insoluble material was washed with cold TCA 5%, dried with 95% ethanol, and analyzed for radioactivity.

In the HSV-1 DNA polymerase assays, the reaction mixture and test procedures were essentially the same as described for the DNA polymerase α assays, but 100 mM $(NH_4)_2SO_4$ was added to the reaction mixture and the enzyme was diluted 5-fold.

Determination of the antiviral activity of the nucleoside analogs were carried out in primary rabbit kidney (PRK) cell cultures. Briefly, confluent PRK cell cultures in 96-well microtiter trays were inoculated with 100 $CCID_{50}$ of virus (1 $CCID_{50}$ being the infective dose for 50% of the cell cultures). After 1 hr of virus adsorption, residual virus was removed and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Viral cytopathogenicity was recorded as soon as it reached completion in the control virus-infected cell cultures. The following virus strains were used in our studies: HSV-1 (KOS, F, and McIntyre), HSV-2 (G, 196 and Lyons), thymidine kinase-deficient (TK^-) HSV-1 (B2006 and VMW-1837), vaccinia virus (VV), and vesicular stomatitis virus (VSV).

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