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(E)-5-(2-bromovinyl)-2'-deoxyuridine-5'-triphosphate as a DNA polymerase substrate

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ABSTRACT

Time course of incorporation and the effect of 5'-triphosphate of the selective antiherpetic agent (E)-5-(2-bromovinyl)-2'-deoxyuridine (bv^5dUTP) on the incorporation of dTTP and dATP into template-primers of different structure were studied in E. coli DNA polymerase I Klenow fragment enzyme-catalyzed reactions. bv^5dUTP could substitute for dTTP depending on the structure of template-primer. E.g. into calf thymus DNA incorporation of bv^5dUMP was around 80 % of that of dTMP at 30 minutes of incubation. The analog has also inhibited dTMP incorporation, net DNA synthesis, however, was hardly affected. The substrate properties of the analog were studied with [2-^{14}C]-labelled bv^5dUTP.

INTRODUCTION

(E)-5-(2-bromovinyl)-2'-deoxyuridine (bv^5dU) is one of the most potent and selective antiviral agents so far found active against herpes simplex virus type 1 [1] and varicella zoster virus [2]. First step in selective inhibition of virus DNA replication is assumed to be the phosphorylation of this nucleoside by virus-induced thymidine kinase in virus-infected cells [3]. Second step would be the selective inhibition of target enzyme, the virus-induced DNA polymerase by the 5'-triphosphate derivative of bv^5dU, as it was proposed by De Clercq [4]. Allaudeen and coworkers have actually tested the effect of bv^5dUTP on isolated viral and cellular DNA polymerase activities [5]. They showed that K_i value for bv^5dUTP of alpha-polymerase was 14-fold of that of the virus-induced DNA polymerase. These results showed the selectivity, however, only in the inhibition of dTMP incorporation into calf thymus DNA ([^{3}H]dTTP was used as labelled substrate). In this way, nothing is known about the inhibition of net DNA synthesis and substrate properties of this

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compound. Here we wish to present results of experiments dealing with studies of substrate properties of bv^5dUTP. The enzyme system studied was Escherichia coli DNA polymerase I Klenow fragment enzyme (without 5'→3' exonuclease activity), with template-primers of different structure and $[2-^{14}C]bv^5dUTP$.

MATERIALS AND METHODS

Nucleotides dATP, dTTP, dGTP and dCTP, template-primer polynucleotides poly[d(A-T)], poly(dA).poly(dT), poly(dA).$(dT)_{10}$, poly(A).$(dT)_{10}$, poly[d(G-C)] and poly(dG).poly(dC), enzymes Escherichia coli MRE 600 DNA polymerase I Klenow fragment enzyme (6900 units/mg protein) and bovine pancreatic DNase were the products of Boehringer-Mannheim GmbH. Calf thymus DNA was purchased from Miles-Seravac, $[^3H]$dATP (17 Ci/mmol) and $[^3H]$-dGTP (15 Ci/mmol) were from New England Nuclear.

$[2-^{14}C]$dTTP (2 mCi/mmol) was prepared from $[^{14}C]$thiourea as described previously [6,7]. (E)-5-(2-bromovinyl)-2'-deoxyuridine (bv^5dU) was prepared from 5-ethyl-2'-deoxyuridine [8] by a bromination procedure of Bärwolff and Langen [9]: 3',5'-di-O-acetyl-5-ethyl-2'-deoxyuridine was brominated with 2 equivalent of bromine in refluxing CCl_4 by UV catalysis in argon atmosphere. Dehydrobromination with a tertier base and subsequent deacylation with sodium methylate yielded bv^5dU in 52 %. Structure of the nucleoside was confirmed by comparison of our NMR and UV data with the published values [10]. $[2-^{14}C]bv^5dU$ (20.3 mCi/mmol) was synthesized in the same way starting with $[2-^{14}C]$-labelled 5-ethyl-2'-deoxyuridine (21.9 mCi/mmol). The 5'-mono- and 5'-triphosphate derivatives of unlabelled and labelled bv^5-dU, bv^5dUMP and bv^5dUTP were prepared and characterized in a similar way as described for 5-alkyl-dUMPs and -dUTPs previously in reference [7]. Yields were 65 and 48 %, respectively. Specific activity of $[2-^{14}C]bv^5dUTP$ was 18.2 mCi/mmol. Thin-layer chromatography was performed on silica gel (Kieselgel 60 F_{254} DC-Alufolien, E. Merck) or cellulose (Pre-coated TLC-plates CEL 400-10 UV_{254}, Macherey-Nagel, Co.) in solvent system 2-propanol: cc.NH_4OH:water = 7:1:2. R_f data of the nucleoside and nucleotides were as follows: bv^5dU 0.73 (silica gel), 0.81 (cellulose); bv^5dUMP 0.28 or 0.17; bv^5dUTP 0.04 or 0.05.

Reaction mixture for E. coli DNA polymerase I Klenow fragment enzyme consisted of 60 mM potassium phosphate buffer (pH 7.4), 6 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. Specific activity of the labelled nucleotides used in the reactions was as follows: [^{3}H]dATP, 19 dpm/pmol; [^{14}C]dTTP, 8 dpm/pmol; [^{14}C]bv^5-dUTP, 40 dpm/pmol; [^{3}H]dGTP, 12 dpm/pmol. Reactions were started with addition of the enzyme (0.25 μg/40 μl of mixture) to the preincubated mixture at 37°C. At definite times of incubation 25 μl samples were pipetted onto GF/C glass fibre filters (Whatman), precipitated in 5 % trichloroacetic acid, washed, dried and counted. Data given in the Figure and Tables are the mean values of two to four experiments.

Three types of DNA polymerase reactions were carried out: a./ study of the effect of bv^{5}dUTP on DNA replication, b./determination of K_m value for dTTP and bv^{5}dUTP in the presence of poly[d(A-T)], c./ study of substrate properties of [^{14}C]bv^{5}dUTP in comparison with [^{14}C]dTTP in the presence of natural and synthetic DNA template-primers.

a./ Reaction mixture in a final volume of 40 μl contained [^{3}H]-dATP (250 μM), [^{14}C]dTTP (100 μM) and bv^{5}dUTP in concentrations given in Figure 1 with poly[d(A-T)] (100 μM/P/) or poly(dA). poly(dT) (100 μM/P/). With pancreatic DNase-activated calf thymus DNA [11] (150 μg/ml) dGTP (250 μM) and dCTP (250 μM) were also applied in addition to the above substrates. The 25 μl samples were taken at 30 minutes of incubation.

b./ Reaction mixture of 40 μl contained poly[d(A-T)] (100 μM/P/), [^{3}H]dATP (250 μM) and [^{14}C]dTTP or [^{14}C]bv^{5}dUTP in eight different concentrations from 1 to 100 μM. Samples were taken at 5 min. of incubation. K_m values were obtained from 1/v - 1/s plotting of the data.

c./ Reaction mixture of 165 μl contained in 250-250 μM concentrations [^{3}H]dATP, dGTP, dCTP and [^{14}C]dTTP or [^{14}C]bv^{5}dUTP with activated calf thymus DNA (150 μg/ml) as a template-primer. Reaction mixture for the limited synthesis contained only three substrates: [^{3}H]dATP, dGTP and dCTP. Samples were taken at intervals indicated in Table 1.

Reaction mixtures of 165 μl with poly[d(A-T)] as a template-primer (100 μM/P/) contained 250-250 μM of [^{3}H]dATP and

$[^{14}C]$dTTP or $[^{14}C]$bv^{5}dUTP. Limited syntheses were presented with $[^3H]$dATP, $[^{14}C]$dTTP and $[^{14}C]$bv^{5}dUTP as well (Table 2).

With poly(dA).poly(dT) (100 μM/P/) as a template-primer reaction mixtures (165 μl) contained 250-250 μM of $[^3H]$dATP and $[^{14}C]$dTTP or $[^{14}C]$bv^{5}dUTP. One-substrate reactions were presented for $[^3H]$dATP, $[^{14}C]$dTTP and $[^{14}C]$bv^{5}dUTP as well. Samples were taken at the indicated times (Table 3).

Reaction mixtures of 82 μl with poly(dA).$(dT)_{10}$ (100 μM/P/, adenine:thymine = 20:1) or poly(A).$(dT)_{10}$ (100 μM/P/, adenine: thymine = 1:1) template-primer contained 250-250 μM of $[^3H]$dATP and $[^{14}C]$dTTP or $[^{14}C]$bv^{5}dUTP. The 25 μl samples were taken at 30 and 60 minutes of incubation (Table 4).

Effect of bv^{5}dUTP on polymerization of dGTP and dCTP was studied in mixtures (82 μl) containing 250-250 μM of $[^3H]$dGTP, dCTP and $[^{14}C]$dTTP or $[^{14}C]$bv^{5}dUTP with poly[d(G-C)] (100 μM/P/) or poly(dG).poly(dC) (100 μM/P/). Samples were taken at 30 and 60 minutes of incubation.

RESULTS

Figure 1 shows the dependence on bv^{5}dUTP concentration of $[^{14}C]$dTMP and $[^3H]$dAMP incorporation (30 min.) into acid-insoluble polymer product catalyzed by E. coli DNA polymerase I Klenow fragment enzyme in the presence of different template-primer DNAs. With an increase in bv^{5}dUTP concentration a reduced rate of $[^{14}C]$dTMP incorporation into each template-primer studied was observed. Decreased rate of $[^3H]$dAMP incorporation was found, however, only in the presence of poly(dA).poly(dT). With calf thymus DNA or poly[d(A-T)] there was an increase of incorporation on an increase of bv^{5}dUTP concentration, i.e. net DNA synthesis increased. This would mean that bv^{5}dUTP is a substrate itself and incorporates into the DNA. To clarify whether this is the case, $[^{14}C]$-labelled bv^{5}dUTP was synthesized and time course of the analog incorporation was determined in comparison with dTTP into different template-primers.

Table 1 presents the results obtained in the presence of activated calf thymus DNA. bv^{5}dUTP was a good substrate of the enzyme. Compared to dTTP, replication rate with the analog was 79 % at 30 minutes, and at 5 hours of incubation the analog

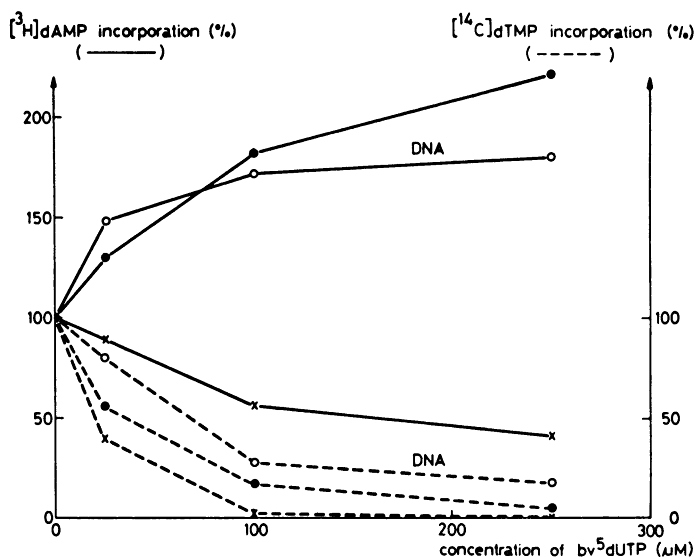

Figure 1. Effect of bv^5dUTP on the incorporation of $[^3H]dAMP$ and $[^{14}C]dTMP$ (100 µM) into template-primers of different structure: o - o, calf thymus DNA; • - •, poly[d(A-T)]; and x - x, poly(dA).poly(dT).

could completely substitute for dTTP.

Table 2 shows the results with poly[d(A-T)] copolymer as a template-primer. The analog was again a good substrate.

Table 1. Replication of activated calf thymus DNA

Triphosphates: dGTP + dCTP +	Incorporation of labelled substrates Incubation time					
	30 min.		60 min.		300 min.	
	nmol	%	nmol	%	nmol	%
$[^{14}C]dTTP$	1.14	100	1.35	100	2.20	100
$[^3H]dATP$	1.15		1.43		2.23	
$[^{14}C]bv^5dUTP$	0.90	79	1.08	80	2.41	110
$[^3H]dATP$	0.94		1.17		2.47	
$[^3H]dATP$	0.06		0.06		0.08	

Table 2. Incorporation of labelled substrates into poly[d(A-T)]

Triphosphates	Time of incubation 30 min. nmol	%	60 min. nmol	%	120 min. nmol	%	300 min. nmol	%	24 h nmol
[^{14}C]dTTP	0.49	100	0.98	100	1.71	100	3.02	100	4.78
[^{3}H]dATP	0.50		0.96		1.75		3.10		4.80
[^{14}C]bv^{5}dUTP	0.36	73	0.72	73	1.37	80	2.58	85	4.75
[^{3}H]dATP	0.38		0.76		1.42		2.62		4.76
[^{3}H]dATP	<0.01		<0.01		<0.01		<0.01		0.01
[^{14}C]dTTP	<0.01		<0.01		<0.01		<0.01		<0.01
[^{14}C]bv^{5}dUTP	<0.01		<0.01		<0.01		<0.01		<0.01

Contrary to poly[d(A-T)], in the presence of the poly(dA). poly(dT) bv^{5}dUTP was a weak substrate of the enzyme and it inhibited dAMP incorporation as well (Table 3). With poly(dA).(dT)$_{10}$ and poly(A).(dT)$_{10}$ higher relative incorporation rates were measured at 30 minutes. The 60 min. values show, however, that

Table 3. Incorporation of labelled substrates into poly(dA). poly(dT) homopolynucleotide duplex template-primer

Triphosphates	Time of incubation 30 min. nmol	%	60 min. nmol	%	120 min. nmol	%	300 min. nmol	%
[^{14}C]dTTP	1.69	100	2.53	100	2.98	100	3.16	100
[^{3}H]dATP	1.65		2.55		3.05		3.10	
[^{14}C]bv^{5}dUTP	0.30	18	0.33	13	0.35	12	0.39	12
[^{3}H]dATP	0.27		0.29		0.29		0.31	
[^{3}H]dATP	0.20		0.27		0.27		0.29	
[^{14}C]dTTP	0.76		0.81		0.83		0.86	
[^{14}C]bv^{5}dUTP	0.25		0.28		0.26		0.26	

Table 4. Poly(dA).$(dT)_{10}$ and poly(A).$(dT)_{10}$ as template-primers

Triphosphates	Poly(dA).$(dT)_{10}$ Incubation time				Poly(A).$(dT)_{10}$ Incubation time			
	30 min.		60 min.		30 min.		60 min.	
	nmol	%	nmol	%	nmol	%	nmol	%
[^{14}C]dTTP	2.37	100	2.88	100	0.64	100	1.13	100
[^{3}H]dATP	0.73		0.92		0.42		0.89	
[^{14}C]bv^{5}dUTP	1.45	61	1.44	50	0.47	73	0.54	48
[^{3}H]dATP	0.16		0.16		0.07		0.11	

bv^{5}dUMP incorporation was limited also in these polymers (Table 4). Incorporation of dAMP was also inhibited.

bv^{5}dUTP interfered even dGMP and dCMP polymerization or copolymerization by E. coli DNA polymerase (Table 5). Misincorporation of the analog into these polymers could not be detected (specific activity was only 18.2 mCi/mmol).

DISCUSSION

Study of replication by E. coli DNA polymerase I Klenow fragment ("large proteolytic fragment") enzyme of different template-primers in the presence of bv^{5}dUTP, a dTTP analog, was carried out using double labelling ([^{14}C]dTTP and [^{3}H]dATP)

Table 5. Incorporation of [^{3}H]dGMP into poly[d(G-C)] and poly(dG).poly(dC) in the presence of dTTP or bv^{5}dUTP

Triphosphates: [^{3}H]dGTP + dCTP +	[^{3}H]dGMP incorporation into poly[d(G-C)] Time of incubation				poly(dG).poly(dC) Time of incubation			
	30 min.		60 min.		30 min.		60 min.	
	nmol	%	nmol	%	nmol	%	nmol	%
[^{14}C]dTTP	0.45	100	0.58	100	0.35	100	0.46	100
[^{14}C]bv^{5}dUTP	0.21	47	0.31	53	0.20	57	0.27	59

(Figure 1). The effect of bv^5dUTP on replication depended on the structure of template-primer. Considering the mechanism of action of antiherpetic (E)-5-(2-bromovinyl)-2'-deoxyuridine (bv^5dU) the activated DNA was of interest. Although bv^5dUTP inhibited $[^{14}]$dTMP incorporation in a concentration-dependent manner, net DNA synthesis was not inhibited in a similar way as it was indicated by $[^3H]$dAMP incorporation into acid-insoluble product. In the presence of 250 μM bv^5dUTP, $[^{14}C]$dTMP (100 μM) incorporation was under 20 % of the original value (without bv^5dUTP). (K_m value for dTTP of this enzyme was 24 μM with activated DNA [12].) At the same time $[^3H]$dAMP incorporation was 180 % of the original value. This indicates that bv^5dUTP is a competitive substrate. This was proved with $[^{14}C]bv^5dUTP$ using template-primers of different structures (Tables 1-4).

$[^{14}C]bv^5dUTP$ was a very good substrate of the enzyme with thymus DNA and the strictly alternating synthetic template-primer poly[d(A-T)]. (K_m for dTTP was 6.4 μM, K_m for bv^5dUTP was 6.6 μM with poly[d(A-T)], see Mat. and Meth.) Rate of incorporation of bv^5dUMP into these template-primers was 75-80 % of that of the dTTP at 30 minutes of incubation. After longer times of incubation (5-24 hours) equimolar quantities of $[^{14}C]$-dTMP or $[^{14}C]bv^5dUMP$ was found in the acid-insoluble material (Tables 1 and 2).

Interestingly enough, with the homopolymer duplex template-primer poly(dA).poly(dT) bv^5dUTP was a weak substrate of the enzyme (Table 3): low incorporation rate, and only a limited amount of $[^{14}C]bv^5dUMP$ (12 %) was found incorporated at 5 hours of incubation. $[^3H]$dAMP incorporation was also strongly inhibited, even in the presence of dTTP (Figure 1). Incorporation of bv^5dUMP into poly(dA).$(dT)_{10}$ or poly(A).$(dT)_{10}$ was limited as well, however, the relative amounts were higher. $[^3H]$dAMP incorporation was again strongly inhibited (Table 4).

The significant differences observed between incorporation of $[^{14}C]bv^5dUMP$ into template-primers containing nucleotides of adenine and thymine in different sequences may originate both from the known conformational differences between poly[d(A-T)] and poly(dA).poly(dT) [13] and from the effect of incorporated substrate analog on the secondary structure (template-primer

properties) of the polydeoxynucleotides. In the case of poly-[d(A-T)] the effect of incorporated analog is "diluted" as a consequence of the alternating sequence in the templating chain. Similar effect can be assumed with calf thymus DNA which has a heterogeneous nucleotide sequence. According to the results a nucleotide analog, like bv^5dUTP, can distinguish between templates of homopolymeric and copolymeric (heteropolymeric) DNA sequences in DNA polymerase reactions.

Incorporation of dGMP into poly[d(G-C)] or poly(dG).poly-(dC) was also affected by bv^5dUTP although misincorporation of the analog was not detected (low specific activity) (Table 5).

Compared to dTMP, incorporation of bv^5dUMP was not limited by the structure of calf thymus DNA in the E. coli DNA polymerase-catalyzed reactions (Table 1). In other words bv^5-dUTP could substitute for dTTP, and even the rate of replication of thymus DNA was hardly affected. Therefore, inhibition by bv^5dUTP of [^{3}H]dTMP incorporation into calf thymus DNA by other isolated enzymes, like herpes simplex virus type 1-induced DNA polymerase, does not mean also the inhibition of DNA synthesis as it was concluded [5].

Based on our results bv^5dUTP hardly inhibits replication of DNAs with "random" nucleotide sequence, like natural DNAs, and according to Allaudeen et al [5] K_m values for dTTP and K_i values for bv^5dUTP of viral and cellular DNA polymerases were similar. Therefore, antiherpetic (E)-5-(2-bromovinyl)-2'-deoxyuridine does not seem to exert essential inhibitory activity on virus DNA replication at the triphosphate level. On the other hand, the analog can be incorporated into DNA by DNA polymerase enzyme according to our results. In this way bv^5dUTP can be incorporated also into virus DNA at its replication in the virus-infected cell. Replacement of dTMP by bv^5dUMP in high percentage in the newly replicated DNA may change different properties of the DNA. These changes may affect replication of the virus.

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