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The 5′-monophosphates of 5-propyl- and 5-ethyl-2′-deoxyuridine do not inhibit the replication of deoxythymidine kinase deficient (TK^-) mutants of herpes simplex virus

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5-Substituted 2′-deoxyuridines such as 5-ethyl-dUrd, 5-propyl-dUrd, 5-propynyloxy-dUrd, 5-vinyl-dUrd and 5-(2-halogenovinyl)-dUrd are potent and selective inhibitors of the replication of herpes simplex virus (HSV) [1–9]. The anti-herpes activity of these deoxythymidine (dThd) analogues depends on their phosphorylation by the virus-induced dThd kinase (TK), because HSV mutants which are unable to induce this TK activity are not inhibited by 5-ethyl-dUrd and its congeners [4, 10, 11]. However, one may expect the 5′-phosphorylated derivatives (5-ethyl-dUMP, 5-propyl-dUMP, . . .) to be effective against TK^- HSV, if at least two conditions are fulfilled: (i) that the genetic deficiency of the TK^- HSV mutant be restricted to the inability to induce TK activity, and (ii) that the 5′-phosphorylated derivatives are able to enter the cells. Thus, inhibition of TK^- HSV replication by the 5′-phosphorylated compounds might be considered as evidence for their uptake by the cells. We chose to evaluate this hypothesis using the deoxythymidylate analogues 5-ethyl-dUMP and 5-propyl-dUMP as probes.

The antiviral properties of 5-ethyl-dUMP, 5-propyl-dUMP and their free nucleosides, 5-ethyl-dUrd and 5-propyl-dUrd, were assessed in primary rabbit kidney cells, human skin fibroblasts (strain VGS), feline lung cells, HEp-2 cells (a continuous human cell line derived from an epidermoid carcinoma of the larynx) and BS-C-1 cells (a continuous cell kidney line derived from the African green monkey, *Cercopithecus aethiops*), which had been infected with either one of the following virus strains: HSV-1 (KOS), HSV-1 (McIntyre), HSV-1 (F), HSV-2 (LYONS), HSV-2 (G) or HSV-2 (196) (which are all TK^+ HSV strains), TK^- HSV-1 (B 2006) (obtained from Dr. Y. -C. Cheng, Chapel Hill, NC, U.S.A.), TK^- HSV-1 (C1 101) (obtained from Dr. H. J. Field, Cambridge, U.K.), or vaccinia virus. Vaccinia virus was included because it resembles TK^- HSV-1 in a certain aspect: indeed, the dThd kinase induced by vaccinia virus recognizes a much narrower spectrum of substrates than the HSV-induced TK [12], which implies that some nucleoside analogues which are effectively phosphorylated by HSV-induced TK may not be phosphorylated by vaccinia virus-induced TK. All antiviral assays were carried out in microtiter trays (Sterilin, Teddington, Middlesex, U.K.). When confluent, the cell cultures were inoculated with 100 $CCID_{50}$ of virus (1 $CCID_{50}$ being the virus dose infecting 50 per cent of the cell cultures) for 1 hr at 37°, and, immediately thereafter, exposed to varying concentrations of the test compounds in Eagle's minimal essential medium supplemented with 3 per cent fetal bovine serum. Viral cytopathogenicity was recorded as soon as it reached 100 per cent cell destruction in the control (virus-infected untreated) cell cultures. Antiviral activity is expressed as the minimum inhibitory concentration of compound required to reduce viral cytopathogenicity by 50 per cent.

Phosphorylation of 5-ethyl-dUrd and 5-propyl-dUrd was carried out enzymatically with the aid of wheat shoot phosphotransferase, as described elsewhere [13]. The resulting nucleotides were isolated by chromatography on Whatman 3MM paper with the solvent system isopropanol–conc. NH_4OH–H_2O (7:1:2, v/v), eluted with water and precipitated by addition of ethanol and acetone to give 5-ethyl-dUMP . $6H_2O$ and 5-propyl-dUMP . $6H_2O$, each in about 40 per cent yield. Both nucleotides were hydrolysed quantitatively to the parent nucleosides by purified 5′-nucleotidase.

The data obtained with 5-ethyl-dUMP and 5-propyl-dUMP in TK^- HSV-infected cells could only be interpreted correctly if the following requirements were met: (i) the 5′-monophosphates should not be cytotoxic, (ii) they should be effective against the TK^+ strains of HSV, and (iii) in their free nucleoside form they should not be active against TK^- HSV. This indeed proved to be the case: 5-ethyl-dUMP and 5-propyl-dUMP did not cause any toxic alteration of the cells (as detectable microsopically) at the highest doses tested (400 μg/ml) and they inhibited the replication of the TK^+ HSV strains at similar, if not identical, concentrations as 5-ethyl-dUrd and 5-propyl-dUrd (Table 1). Furthermore, 5-ethyl-dUrd and 5-propyl-dUrd were not inhibitory to the replication of TK^- HSV (Table 1), as has been reported previously [4, 10, 11].

The phosphorylated derivatives of 5-ethyl-dUrd and 5-propyl-dUrd did not confer any protection against TK^- HSV (Table 1), irrespective of the cell type in which they were assayed (primary rabbit kidney cells, human skin fibroblasts, feline lung cells, HEp-2 or BS-C-1 cells). In fact, there was no particular situation in which the 5′-monophosphates exhibited a significantly greater antiviral effect than their unphosphorylated counterparts. Although 5-ethyl-dUrd and 5-ethyl-dUMP were inhibitory to vaccinia, 5-proply-dUrd and 5-propyl-dUMP were not (Table 1). That 5-propyl-dUrd, unlike 5-ethyl-dUrd, lacks anti-vaccinia activity, has been pointed out previously [5]. According to the alternative substrate theory [14], one may assume that 5-ethyl-dUrd, but not 5-propyl-dUrd, is recognized as a substrate by the vaccinia virus-induced dThd kinase.

The salient feature emerging from the data presented in Table 1 is that, under conditions where the free nucleosides failed to demonstrate an antiviral activity, the nucleotides failed as well. Our findings may be interpreted to mean that the thymidylate analogues 5-ethyl-dUMP and 5-propyl-dUMP do not penetrate the cells, or, if they do, they may be dephosphorylated before they reach the site(s) of virus replication. Since 5-ethyl-dUMP and 5-propyl-dUMP are active against TK^+ HSV, one may postulate that, once dephosphorylated (either extra- or intracellularly), they are phosphorylated again intracellularly by the virus-encoded dThd kinase. Alternatively, the thymidylate analogues may enter cells and even reach the site(s) of virus replication, but due to the fact that TK^- HSV mutants suffer from additional genetic deficiencies other than lack of dThd kinase induction, viz. lack of thymidylate kinase induction, the thymidylate analogues may not be further processed to their active form (5′-triphosphate), hence failing to inhibit the synthesis of progeny TK^- HSV DNA. This alternative proposal is supported by the recent findings of

Table 1. Antiviral activity of the 5′-monophosphates of 5-ethyl-dUrd and 5-propyl-dUrd and their parent nucleosides in different cell cultures

Assay system	Minimum inhibitory concentration* (μmoles/ml $\times 10^{-3}$)			
	5-Ethyl-dUMP	5-Ethyl-dUrd	5-Propyl-dUMP	5-Propyl-dUrd
Primary rabbit kidney cells				
HSV-1 (KOS)	1.4	2.0	4.0	3.0
HSV-1 (Mc Intyre)	1.0	1.6	2.6	1.9
HSV-1 (F)	1.4	1.6	4.0	4.8
HSV-2 (LYONS)	0.4	0.4	6.0	4.8
HSV-2 (G)	0.4	0.4	5.0	9.3
HSV-2 (196)	0.2	0.4	5.0	5.6
HSV-1 TK$^-$ (B 2006)	>820	>1562	>797	>1481
HSV-1 TK$^-$ (Cl 101)	>820	⩾1562	>797	>1481
Vaccinia	174	20	>797	⩾1481
Human skin fibroblasts				
HSV-1 (KOS)	4.1	2.7	4.0	2.6
HSV-1 TK$^-$ (B 2006)	⩾820	⩾1562	>797	>1481
Vaccinia	2.0	2.7	>797	⩾1481
HEp-2 cells				
HSV-1 (KOS)	0.4	0.8	0.8	0.15
HSV-1 TK$^-$ (B 2006)	>820	>1562	>797	>1481
Vaccinia	8.2	16	>797	>1481
BS-C-1 cells				
HSV-1 (KOS)	4.1	3.9	20	15
HSV-1 TK$^-$ (B 2006)	>820	>1562	>797	>1481
Vaccinia	41	27	>797	>1481
Feline lung cells				
HSV-1 (KOS)	41	78	199	148
HSV-1 TK$^-$ (B 2006)	>820	>1562	>797	>1481
Vaccinia	410	586	>797	>1481

* Concentration required to inhibit virus-induced cytopathogenicity by 50 per cent. None of the compounds caused a microscopically detectable alteration of cell morphology at 400 μg/ml (~0.8 μmole/ml for 5-ethyl-dUMP and 5-propyl-dUMP, and ~1.5 μmole/ml for 5-ethyl-dUrd and 5-propyl-dUrd).

Chen *et al.* [15, 16], which indicate that TK$^-$ HSV mutants may also be deficient in inducing thymidylate kinase activity, and that both thymidylate kinase activity and TK activity may reside in the same virus-induced protein.

There is no doubt that nucleotides, viz. ara-AMP, can penetrate animal cells intact [17, 18]. If 5-ethyl-dUMP and 5-propyl-dUMP, like ara-AMP, do enter cells, one should expect some effect on TK$^-$ HSV replication. As even the slightest inhibition was absent, one may infer that additional deficiencies of the TK$^-$ HSV mutants, viz. lack of thymidylate kinase induction, contributed to their resistance to these thymidylate analogues.

At least in the case of mouse fibroblasts (L cells), ara-AMP has been shown to be more cytotoxic than the parent ara-A, and it was reasonably well established that the nucleotide penetrates the cells intact, albeit at a rate of only 3–5 per cent of the rate of entry of the nucleoside [17, 18]. If 5-ethyl-dUMP and 5-propyl-dUMP do enter the cells embraced in the study of Plunkett *et al.* [17], one would expect some effect on TK$^-$ HSV replication. The absence of any such effect may, however, now be interpreted in terms of an additional deficiency of the TK$^-$ HSV mutants, viz. lack of thymidylate kinase activity, thus contributing to their resistance of the thymidylate analogues. It would clearly be desirable to seek physicochemical evidence regarding the ability of our thymidylate analogues to penetrate the cellular systems employed in the present and previous study [17].

Another point of interest emerges from an examination of the data in Table 1. If one were to examine the antiviral activity of 5-ethyl-dUrd, or its nucleotide, in infected feline lung cells, one would have to conclude that this well-known agent is only moderately active against HSV-1 (KOS) and virtually inactive against vaccinia. With the 5-propyl-dUrd analogue and its nucleotide, the activity against HSV-1 (KOS) is moderate in BS-C-1 cells and quite weak in the feline lung cells. This underlines the crucial significance of the choice of the cell system in evaluations of antiviral activity.

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Thiol *S*-methyltransferase: suggested role in detoxication of intestinal hydrogen sulfide

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Hydrogen sulfide is highly toxic to higher animals, causing death in the same range of atmospheric concentrations as does hydrogen cyanide [1]. Unlike cyanide, however, hydrogen sulfide is sufficiently common in biological systems to pose a threat to the organism. The anaerobic metabolism of many bacterial species common to the colon and periodontal spaces, acting on proteins and other sulfur-containing compounds, is known to liberate hydrogen sulfide [2–4]. Indeed, H_2S has been shown to form a small but offensive component in human flatus [5] and halitosis [6]. Direct ingestion of foods subjected to bacterial spoilage [7, 8] or of mineral sulfides dissolved in water offer other routes of exposure, as does dissolved H_2S that is found in fermented beverages [9].

Since the alimentary tract is the major portal of entry in each instance, it follows that the gut mucosa, in particular, may benefit from a mechanism for the detoxication of hydrogen sulfide. We propose this function for thiol *S*-methyltransferase, an enzyme present in the microsomes of many mammalian tissues [10]. The enzyme catalyzes

$$\text{RSH} + \textit{S}\text{-adenosyl-L-methionine} \rightarrow \text{RS–CH}_3 + \textit{S}\text{-adenosyl-L-homocysteine} \quad (1)$$

Reaction 1 in which R may be one of a large number of diverse groups as represented by thiophenols [11], aliphatic mercaptans [10–12], thiopyrimidines and thiopurines [13–16], dithiocarbamates [17, 18] and H_2S [10, 11]. We have described elsewhere the solubilization and purification to homogeneity of the enzyme from rat liver using 2-thioacetanilide as the assay substrate [11]. Here we examine the activity of the enzyme with specific attention to H_2S and methanethiol as potential physiologic substrates and to the tissue distribution of the enzyme in the rat. These data are in accord with a role for thiol *S*-methyltransferase in the detoxication of intestinal hydrogen sulfide.

Solutions of sodium sulfide and methanethiol were prepared in 20 mM KOH containing 10 μM EDTA; the concentration of each substrate was confirmed with *N,N*-dimethyl-*p*-phenylenediamine for H_2S [19] and by the method of Ellman [20] for methanethiol. *S*-Adenosyl-L-[methyl-^{3}H]methionine (9–15 Ci/mmole) was obtained from Amersham/Searle (Arlington Heights, IL) and diluted, when necessary, in 10 mM sulfuric acid.

The standard assay for thiol methyltransferase activity with 2-thioacetanalide and *S*-adenosylmethione has been described [11]. Because of the volatility of H_2S and its products at physiologic pH values, however, assays for these compounds required modification to minimize substrate or product losses. Small test tubes with an inside diameter of 4 mm (Kimax No. 45048) were cut to 30 mm in length so that they would hold slightly more than 0.4 ml. Hydrogen sulfide was added to each tube as an alkaline solution of sodium sulfide and was followed by a mixture containing enzyme and radiolabeled *S*-adenosylmethionine in a Triton X-100 containing buffer. The assay mixture contained, in a final volume of 0.4 ml, 0.25 μM *S*-adenosyl-[methyl-^{3}H]methionine, 0.2 mM dithiothreitol, 0.5% Triton X-100, 0.1 M potassium phosphate (pH 7.9), 1 mM EDTA and 10 mM sodium sulfide. The amount of enzyme was chosen to produce between 1 and 5 per cent conversion of added *S*-adenosylmethionine. Under these conditions, the reaction was a linear function of the amount of enzyme added.

After mixing the contents by suction and release with a 200 μl Eppendorf pipette, the tubes were sealed with Parafilm and incubated in a water bath at the desired temperature. Assays were terminated by transferring the