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# ANTIVIRAL ACTIVITY OF 5-ETHYL PYRIMIDINE DEOXYNUCLEOSIDES

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**Abstract**—The antiviral activity of the α- and β-anomers of 5-ethyl-2′-deoxyuridine (EtUdR) and 5-ethyl-2′-deoxycytidine (EtCdR) has been assessed in primary rabbit kidney (PRK) cell cultures. Cytosine arabinoside (ara-C) and 5-iodo-2′-deoxyuridine (IUdR) were included as reference materials. When inhibition of vaccinia virus multiplication was measured, the following order of (decreasing) activity was established: ara-C > IUdR > (β-)EtUdR > (β-)EtCdR. The α-anomers of EtUdR and EtCdR were completely inactive.

In marked contrast with ara-C and IUdR which were found to inhibit PRK cell growth and thymidine incorporation into cell DNA at relatively low doses (0·08–0·4 μg/ml and 8–40 μg/ml respectively), EtUdR did not inhibit cell growth or thymidine incorporation into DNA unless very high doses were used (200–500 μg/ml). At 0·4–200 μg/ml EtUdR had a stimulatory effect on the subsequent incorporation of thymidine and deoxycytidine into DNA, most probably due to a starvation of the cells for these precursors during their contact period with EtUdR. EtCdR did not markedly alter thymidine incorporation into DNA.

Antiviral indices were calculated for EtUdR, IUdR and ara-C. These were defined as the ratios of the minimum toxic doses (inhibiting cell growth or thymidine incorporation by 50 per cent) to the minimum effective doses (inhibiting vaccinia virus induced cytopathogenicity by 50 per cent). The antiviral indices of EtUdR and IUdR were almost identical but considerably greater than that of ara-C.

The antiviral and antimetabolic (antitumour, immunosuppressive, ...) activities of nucleoside analogues such as IUdR* (5-iodo-2′-deoxyuridine) and ara-C (cytosine arabinoside) depends, at least in part, on the intracellular phosphorylation to the triphosphate, followed by inhibition of DNA synthesis. The inhibition of DNA synthesis is apparently due to an inhibition of the activity of enzymes (e.g. DNA polymerase) involved in DNA synthesis and/or the incorporation of the nucleoside analogue into DNA. Prusoff [1] proposed that, in any event, the primary site of antiviral activity of IUdR is subsequent to its incorporation into viral DNA. However, IUdR and the related BrUdR (5-bromo-2′-deoxyuridine) are also incorporated into the DNA of bacterial and mammalian cells and the incorporation of compounds such as IUdR and BrUdR in host cell DNA is potentially hazardous since it may increase the rate of mutations [1], inhibit the formation of specific enzymes [2] and favour the expression of oncogenic virus genomes [3–5].

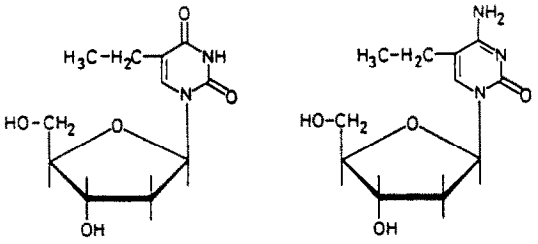
Unlike BrUdR and IUdR, 5-ethyl-2′-deoxyuridine (EtUdR) is devoid of mutagenic activity, at least in a phage system [6, 7], although 5-ethyluracil and its glycoside are readily incorporated into bacterial DNA [8] and bacteriophage DNA [6, 9]. Lack of mutagenicity of EtUdR has also been reported in a higher organism, *Drosophila melanogaster* [10], but this conclusion should be considered as tentative, because no evidence was provided that EtUdR was actually incorporated into the host cell DNA. More recently, Swierkowska *et al.* [11] reported that EtUdR restored amethopterine-suppressed DNA synthesis in human lymphocyte cultures; although EtUdR was incorporated into DNA of these cells, it did not increase the frequency of chromatid breaks as opposed to BrUdR [11].

Why would IUdR be mutagenic and EtUdR be nonmutagenic? Most probably because of their differences in $pK_a$ for dissociation of the N-3 hydrogen: 8·25 for IUdR as compared to 9·8 for EtUdR and thymidine [1, 12, 13]. This more acidic dissociation constant for the N-3 hydrogen of IUdR would increase the chances of faulty base pairing, e.g. with deoxyguanosine.

The ability of EtUdR to be incorporated into phage DNA, combined with its apparent lack of mutagenicity, prompted us to explore its antiviral activity in mammalian cell systems. The potential antiviral activity of EtUdR has been alluded to before, albeit without much experimental detail [7, 14]. It has also been reported that EtUdR does not suppress the primary immunoresponse of mice to sheep red blood cells [15] and that it inhibits the activity *in vitro* of thymidine kinase, a key enzyme in the multiplication of DNA viruses [16].

This report describes the antiviral activity of EtUdR and of the corresponding amino nucleoside, EtCdR (5-ethyl-2′-deoxycytidine), and their effects on

* Abbreviations used: EtUdR, β-anomer of 5-ethyl-2′-deoxyuridine; α-EtUdR, α-anomer of 5-ethyl-2′-deoxyuridine; EtCdR, β-anomer of 5-ethyl-2′-deoxycytidine; α-EtCdR, α-anomer of 5-ethyl-2′-deoxycytidine; CdR, 2′-deoxycytidine (β-anomer); IUdR, 5-iodo-2′-deoxyuridine (β-anomer) ara-C, 1-β-D-arabinofuranosylcytosine, cytosine arabinoside; TdR, 2′-deoxythymidine; $CCID_{50}$, cell culture infecting dose 50 (infecting 50 per cent of the cell cultures); PFU, plaque forming units; PRK, primary rabbit kidney; MEM, (Eagle's) minimal essential medium; PBS, (Dulbecco's) phosphate buffered saline.



EtUdR EtCdR

Fig. 1. Chemical structures of EtUdR and EtCdR.

DNA synthesis in primary rabbit kidney (PRK) cell cultures. The rationale behind the use of EtCdR was that, even if this analogue itself were to prove inactive, it might still undergo intracellular deamination to the more active EtUdR and serve as a depot form of EtUdR.

## MATERIALS AND METHODS

The *synthesis* and *physico-chemical characteristics* of EtUdR, α-EtUdR, EtCdR and α-EtCdR have been described previously [7, 17]. The structures of EtUdR and EtCdR are depicted in Fig. 1. IUdR and part of the ara-C employed were generously supplied by Cusi Laboratories (Brussels, Belgium) and Upjohn (Puurs, Belgium) respectively. CdR was purchased from Sigma Chemical Company (St. Louis, Missouri).

The techniques for evaluating the effects of EtUdR, EtCdR and the other compounds in PRK cells on (i) vaccinia virus, herpes simplex virus and vesicular stomatitis *virus-induced cytopathogenicity* (ii) *vaccinia virus growth*, and (iii) *DNA synthesis, as monitored by [$^3$H-methyl] thymidine incorporation* into host cell DNA, have been described before [18]. In addition, some compounds were tested for their effect on [$^3$H-5]deoxycytidine, [$^3$H-8]deoxyadenosine and [$^3$H-8]deoxyguanosine incorporation into cell DNA and [$^3$H-5]uridine incorporation into cell RNA. [$^3$H-methyl]thymidine, [$^3$H-5]deoxycytidine, [$^3$H-8]deoxyadenosine, [$^3$H-8]deoxyguanosine and [$^3$H-5]uridine were all purchased from the C.E.N. Radioisotopes Department (Mol, Belgium). Their specific radioactivities amounted to 12, 23, 12, 5 and 26 Ci/mmole respectively.

## RESULTS AND DISCUSSION

*Virus-inhibiting effects.* Previous studies, reviewed by Schabel and Montgomery [19], have clearly demonstrated that the antiviral activity of IUdR and ara-C is primarily confined to DNA viruses. Accordingly, IUdR and ara-C were found to inhibit vaccinia and herpes simplex virus-induced cytopathogenicity in PRK cells at concentrations as low as 0·1 and 0·04 μg/ml respectively (Table 1). EtUdR and EtCdR inhibited vaccinia and herpes simplex virus-induced cytopathogenicity at 1 and 40 μg/ml respectively, whereas CdR and the α-anomers of EtUdR and EtCdR failed to inhibit this cytopathogenicity even at 200 μg/ml, the highest concentration tested (Table 1). All compounds, except ara-C, were inactive against vesicular stomatitis virus, a representative of the rhabdo (RNA) virus group.

Table 1. Effect of the 5-ethyl pyrimidine nucleosides on viral cytopathogenicity in PRK cell cultures

| Compounds† | Minimum inhibitory concentration* (μg/ml) | | |
|---|---|---|---|
| | Vaccinia virus‡ | Herpes simplex virus | Vesicular stomatitis virus |
| EtUdR | 1 | 1 | >200 |
| EtCdR | 40 | 40 | >200 |
| α-EtUdR | >200 | >200 | >200 |
| α-EtCdR | >200 | >200 | >200 |
| CdR | >200 | >200 | >200 |
| IUdR | 0·1 | 0·1 | >200 |
| Ara-C | 0·04 | 0·04 | 40 |

* Required to inhibit viral cytopathogenicity by 50 per cent.

† Compounds added immediately after virus adsorption.

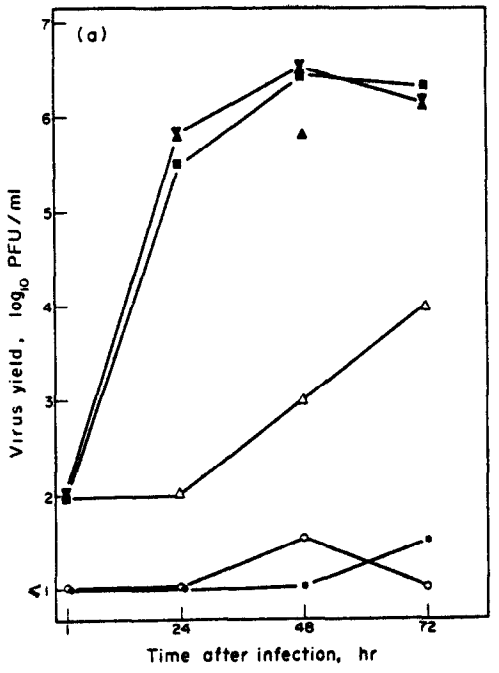
‡ Virus input: 500 $CCID_{50}$ (vaccinia and vesicular stomatitis virus) or 50 $CCID_{50}$ (herpes simplex virus) per tube.

Vaccinia virus growth in PRK cells was completely arrested by IUdR and ara-C, added at either 100 μg/ml (Fig. 2a) or 10 μg/ml (Fig. 2b) immediately after virus adsorption. A significant reduction in virus yield was also obtained if EtUdR (at 100 or 10 μg/ml) was added after virus adsorption. EtCdR (and CdR), however, did not appear to affect vaccinia virus growth (Fig. 2a and b).

The lack of antiviral activity of α-EtUdR and α-EtCdR (Table 1) is not entirely unexpected [7], although several examples have been recently reported of antibiotic and antiviral activities of the α-anomers of nucleosides. Somewhat more surprising, at first sight, is the relatively low activity of the β-anomer of EtCdR (Table 1, Fig. 2a and b). The latter was significantly less active than EtUdR, in marked contrast to the 5-halogeno derivatives of 2′-deoxycytidine, which proved to be more active than the parent 5-halogeno-2′-deoxyuridines in inhibiting the growth and cytopathogenicity of human cytomegalovirus in WI-38 cells [20]. There is little doubt that in this instance the 5-halogeno-2′-deoxycytidines are depot forms of the corresponding 5-halogeno-2′-deoxyuridines, to which they are converted by intracellular cytidine deaminase.

We have no direct data as to the susceptibility of EtCdR to intracellular deamination in PRK cells, and tests along these lines are planned. Meanwhile, *in vitro* tests conducted by Dr. E. Krajewska (personal communication) with a highly active extract of cytidine deaminase from *Salmonella typhimurium* LT2, demonstrated that the relative rates of deamination of deoxycytidine, 5-methyldeoxycytidine and 5-ethyldeoxycytidine were 1:0·70:0·23. The considerably reduced deamination rate obtained for EtCdR is most probably due to steric hindrance by the 5-ethyl side chain and may account for the low antiviral activity of this analogue.

*Effects on DNA synthesis.* In marked contrast to IUdR and ara-C, which were found to reduce [$^3$H-methyl]thymidine incorporation into PRK cell DNA by 50 per cent at concentrations as low as 40 μg/ml and 0·4 μg/ml respectively, EtUdR enhanced thymidine incorporation even at concentrations up to 200



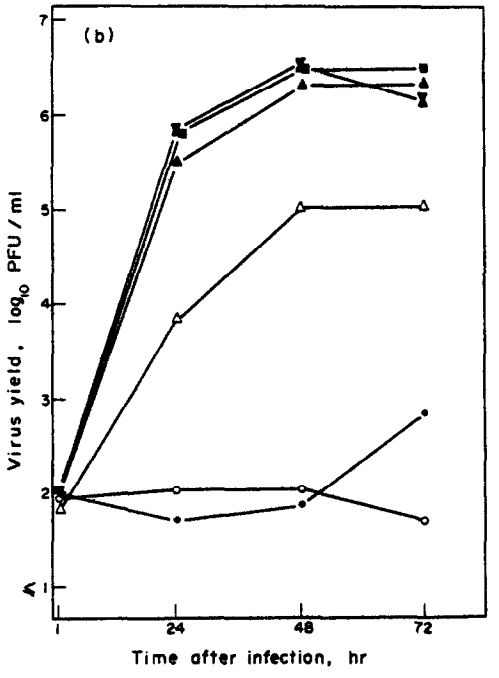




Fig. 2. Effect of the 5-ethyl pyrimidine nucleosides on vaccinia virus growth in PRK cells. (a) Virus input: 4·5 $\log_{10}$ PFU per petri dish. Compounds added at 100 μg/ml, immediately after virus adsorption: control (⧗), EtUdR (△), EtCdR (▲), CdR (■), Ara-C (○) and IUdR (●). (b) Virus input: 4·5 $\log_{10}$ PFU per petri dish. Compounds added at 10 μg/ml, immediately after virus adsorption: control (⧗), EtUdR (△), EtCdR (▲), CdR (■), Ara-C (○) and IUdR (●).

μg/ml (Fig. 3). The other compounds, EtCdR, CdR, α-EtUdR and α-EtCdR did not significantly alter [$^3$H-methyl]thymidine incorporation; at the highest concentration tested (200 μg/ml), EtCdR slightly enhanced, and CdR, α-EtUdR and α-EtCdR slightly depressed, thymidine incorporation (Fig. 3).

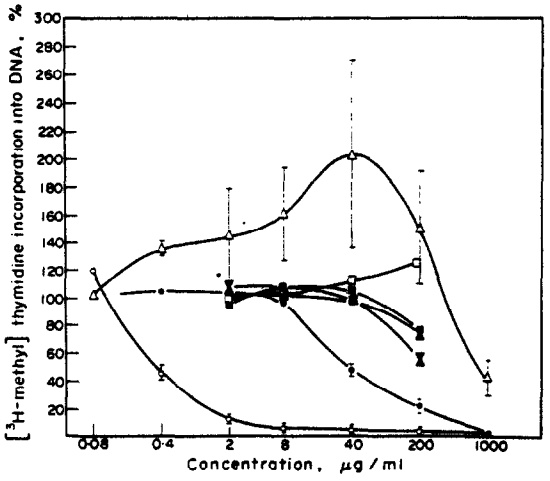



Fig. 3. Effect of the 5-ethyl pyrimidine nucleosides on [$^3$H-methyl]thymidine incorporation into DNA of PRK cells: EtUdR (△), α-EtUdR (▲), EtCdR (□), α-EtCdR (■), CdR (⧗), Ara-C (○) and IUdR (●). [$^3$H-methyl]thymidine incorporation into DNA was measured after the cells had been exposed to different concentrations of the compounds (as indicated in the abscissa) for 24 hr. Brackets indicate means ± S.D. (5–10 observations). Means are expressed as percent of controls.

As virus replication and DNA synthesis are reduced by ara-C to a comparable, if not identical degree, it is reasonable to ascribe the antiviral activity of ara-C to its inhibitory effect on DNA synthesis [18, 21]. However, this assumption apparently does not hold for IUdR and EtUdR, since both deoxyuridine analogues inhibited virus replication at concentrations far below the DNA synthesis-inhibiting concentrations. Over a wide range of concentrations, EtUdR even enhanced [$^3$H-methyl]thymidine incorporation into DNA. This latter effect may be due to a starvation of the PRK cells for thymidine during their 24-hr treatment with EtUdR, resulting in an overconsumption of [$^3$H-methyl]thymidine once this is substituted for EtUdR in the cell culture medium (rebound effect). If this interpretation is correct, one may expect that thymidine added to the cells together with EtUdR would prevent the stimulatory effect of EtUdR on subsequent [$^3$H-methyl]thymidine incorporation. As shown in Fig. 4a, thymidine added to the cell together with EtUdR did indeed counteract the stimulatory effect of EtUdR on [$^3$H-methyl]thymidine incorporation, even when thymidine was added at concentrations which were too low (0·08–0·4 μg/ml) to affect directly subsequent [$^3$H-methyl]thymidine incorporation.

EtUdR not only enhanced the incorporation of [$^3$H-methyl]thymidine into DNA but also that of [$^3$H-5]deoxycytidine (Table 2). Again, this stimulatory effect may be caused by a starvation of the cells for deoxycytidine during EtUdR treatment, followed by a rebound effect when EtUdR is withdrawn, resulting in an overconsumption of [$^3$H-5]deoxycytidine. The results shown in Fig. 4b support this hypothesis, since deoxycytidine added to the cells together with EtUdR markedly reduced the stimulatory effect of EtUdR on [$^3$H-5]deoxycytidine incorporation, even when deoxycytidine was added at concentrations (0·08–8 μg/ml) which did not directly inhibit subsequent [$^3$H-5]deoxycytidine incorporation.

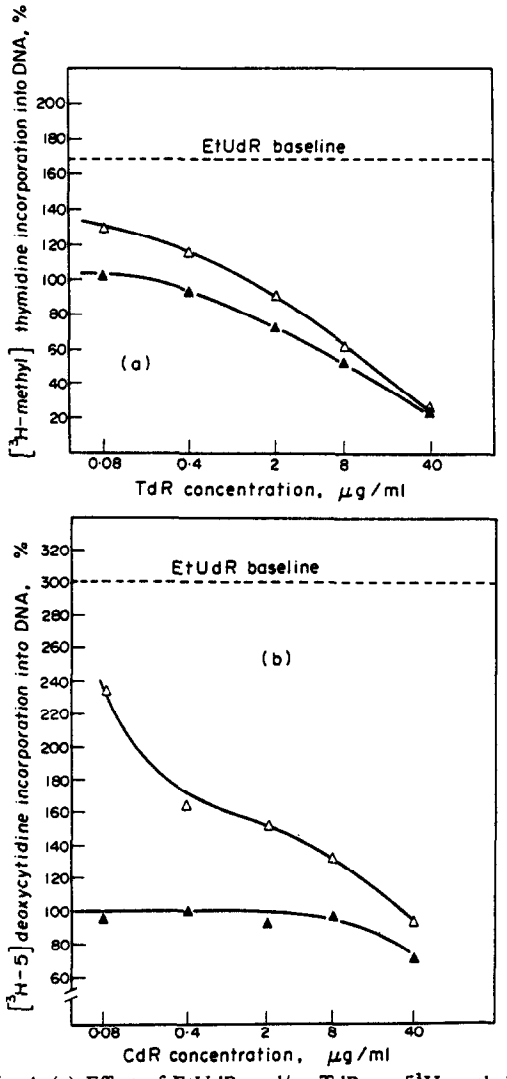



Fig. 4. (a) Effect of EtUdR and/or TdR on [$^3$H-methyl]-thymidine incorporation into DNA of PRK cells: EtUdR alone at 40 μg/ml (----), mixture of EtUdR (40 μg/ml) and TdR at the concentrations indicated in the abscissa (△), TdR alone at the concentrations indicated in the abscissa (▲). [$^3$H-methyl]thymidine incorporation into DNA was measured after the cells had been exposed to EtUdR and/or TdR for 24 hr. (b) Effect of EtUdR and/or CdR on [$^3$H-5]deoxycytidine incorporation into DNA of PRK cells: EtUdR alone at 40 μg/ml (---), mixture of EtUdR (40 μg/ml) and CdR at the concentrations indicated in the abscissa (△), CdR alone at the concentrations indicated in the abscissa (▲). [$^3$H-5]deoxycytidine incorporation into DNA was measured after the cells had been exposed to EtUdR and/or CdR for 24 hr.

Of possible interest in relation to the foregoing is the recent demonstration that, in several normal and neoplastic mouse tissues, [$^3$H-5]deoxycytidine was a specific precursor of $^3$H-labeled DNA deoxycytidylate, and that the incorporation of [$^3$H-5]deoxycytidine into the DNA of three mouse neoplasms investigated was increased 2–3-fold on addition of tetrahydrouridine, a potent cytidine deaminase inhibitor [22]. Furthermore, lymphoid tissues of the rat and mouse exhibit quite high activities of deoxycytidine kinase activity, indicative of the utilization of exogenous deoxycytidine [23].

Table 2. Effect of EtUdR and ara-C on DNA synthesis* in PRK cells as monitored by [$^3$H-methyl]thymidine, [$^3$H-5]deoxycytidine, [$^3$H-8]deoxyadenosine† or [$^3$H-8]deoxyguanosine† incorporation into DNA

| Compounds | Gross counts (counts/min per petri dish) | Per cent (compared with control) |
|---|---|---|
| | [$^3$H-methyl]thymidine incorporation | |
| EtUdR | 24,681 | 327·6 |
| Ara-C | 900 | 11·9 |
| Control | 7534 | — |
| | [$^3$H-5]deoxycytidine incorporation | |
| EtUdR | 18,104 | 522·8 |
| Ara-C | 852 | 24·6 |
| Control | 3463 | — |
| | [$^3$H-8]deoxyadenosine incorporation | |
| EtUdR | 51,182 | 103·1 |
| Ara-C | 43,344 | 87·3 |
| Control | 49,648 | — |
| | [$^3$H-8]deoxyguanosine incorporation | |
| EtUdR | 38,761 | 113·0 |
| Ara-C | 34,329 | 100·1 |
| Control | 34,296 | — |

* Incorporation of the radiolabelled nucleosides into DNA was measured after the cells had been exposed to 40 μg/ml of either compound for 24 hr. Data represent mean values for 2 petri dishes.

† Note that, for the purine deoxynucleosides, the measurements may not necessarily represent direct incorporation into DNA (see Text).

EtUdR enhanced the incorporation into DNA only of the two pyrimidine deoxynucleosides [$^3$H-methyl]-thymidine and [$^3$H-5]deoxycytidine. It did not affect that of [$^3$H-8]deoxyadenosine or of [$^3$H-8]deoxyguanosine (Table 2). Neither did ara-C affect the incorporation of these labeled purine deoxyribonucleosides, although it suppressed the incorporation of the labeled pyrimidine deoxyribonucleosides (Table 2). However, interpretation of the results obtained with the use of purine deoxyribonucleoside "precursor" is likely to be somewhat more complex since, although both deoxyadenosine and deoxyguanosine undergo phosphorylation in animal cells, their incorporation into DNA may be limited as a result of extensive cleavage of the glycosidic linkages of the nucleosides [23].

It is, however, of considerable interest that EtUdR and ara-C exhibited opposing activities as regards incorporation into DNA of labeled thymidine and deoxycytidine. Neither compound affected RNA synthesis as monitored by [$^3$H-5]-uridine incorporation (Table 3). This is not surprising, since RNA synthesis has been shown to be relatively inert to the antimetabolic influence of ara-C [24–26].

*Antiviral indices.* Antiviral indices were calculated for the most active compounds (EtUdR, IUdR, ara-C) in both resting and growing PRK cell cultures (Table 4). The "antiviral index" was determined as the ratio of the minimum toxic dose (reducing thymidine incorporation or total cell count by 50 per cent in stationary and growing cell cultures respectively) to the minimum effective dose (inhibiting vaccinia virus-induced cytopathogenicity by 50 per cent). In both stationary and growing PRK cell cultures the antiviral index of EtUdR approximated that of IUdR, but significantly surpassed that of ara-C (Table 4). The relatively high antiviral activity and low toxicity of EtUdR, coupled with its apparent lack of mutagenicity [6, 7], would justify further experimental investigations with EtUdR in infections in animals and

Table 3. Effect of EtUdR, ara-C and IUdR on RNA synthesis in PRK cells as monitored by [$^3$H-5]uridine incorporation into RNA

| Compounds* | Dose (μg/ml) | [$^3$H-5]uridine incorporated into RNA: Gross counts (counts/min per petri dish) | [$^3$H-5]uridine incorporated into RNA: Per cent (compared with control) |
|---|---|---|---|
| EtUdR | 200 | 20,815 | 114·9 |
| | 40 | 19,943 | 110·1 |
| | 8 | 20,645 | 114·0 |
| | 2 | 20,452 | 112·9 |
| Ara-C | 200 | 18,472 | 102·0 |
| | 40 | 18,946 | 104·6 |
| | 8 | 17,733 | 97·9 |
| | 2 | 18,431 | 101·7 |
| IUdR | 200 | 16,073 | 88·7 |
| | 40 | 17,275 | 95·4 |
| | 8 | 19,514 | 107·7 |
| | 2 | 17,977 | 99·2 |
| Control | | 18,114 | — |

* Incorporation of [$^3$H-5]uridine into RNA was measured after the cells had been exposed to the compounds at the concentrations indicated for 24 hr. Data represent mean values for 2 petri dishes.

man in which IUdR would normally be indicated. On the basis of the antiviral indices recorded in cell culture, EtUdR and IUdR would appear equally promising, but EtUdR may eventually prove more useful because of its lack of mutagenicity.

It should be emphasized that EtUdR is a more "natural" thymidine analogue than IUdR. It has the same base-pairing properties as thymidine [12, 13]. Like thymidine, EtUdR may undergo photodimerization upon u.v.-irradiation; it may also undergo intramolecular photocyclization of the 5-ethyl group with the pyrimidine 5,6-bond, followed by photochemical dissociation of the resulting cyclobutane ring to yield deoxyuridine, a process which would be expected to be nonmutagenic [27]. It may be anticipated, therefore, that substitution of 5-ethyl for the 5-methyl in the thymidine of DNA would not lead to any marked changes in the normal conformation of DNA; and, in fact, phage T3 DNA in which about 65 per cent of the thymine residues have been replaced by 5-ethyluracil, renatures at virtually the same rate as normal phage DNA [6]. A DNA species has recently been isolated (from *Bacillus subtilis* phage SP-15) that contains a high percentage (12 moles per cent of all bases) of a thymine analogue with an even larger 5-side chain, 4',5'-dihydroxypentyl [28, 29]. An important characteristic of this unusual DNA was its low melting temperature ($T_m$). Such a destabilizing effect, albeit to a lesser extent, has also been noted upon introduction of a pyrimidine 5-ethyl substituent [6, 9, 12].

Table 4. Antiviral indices of EtUdR, IUdR and ara-C in stationary and growing PRK cell cultures

| Compounds | Minimum toxic dose* (μg/ml) | Minimum effective dose† (μg/ml) | Antiviral index |
|---|---|---|---|
| A. Stationary cell cultures | | | |
| EtUdR | 500 | 1 | 500 |
| IUdR | 40 | 0·1 | 400 |
| Ara-C | 0·4 | 0·04 | 10 |
| B. Growing cell cultures | | | |
| EtUdR | 200 | 20 | 10 |
| IUdR | 8 | 1 | 8 |
| Ara-C | 0·08 | 0·08 | 1 |

* Required to reduce [$^3$H-methyl]thymidine incorporation into host cell DNA by 50 per cent (stationary cell cultures) or to reduce total cell number by 50 per cent (growing cell cultures).

† Required to reduce vaccinia virus-induced cytopathogenicity by 50 per cent (both stationary and growing cell cultures).

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