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5-Substituted deoxyuridines – structural requirements for antiviral activity against herpes simplex virus types 1 and 2 and possible biochemical basis for relative potency*

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Summary

A number of structurally related 5-substituted pyrimidine 2'-deoxyribonucleosides were tested for antiviral activity against herpes simplex virus types 1 and 2 in cell culture. A minimum inhibitory concentration was determined for each compound and from a comparison of these values a number of conclusions were drawn with regard to those molecular features which enhance or reduce antiviral activity. Analogues in which the 5-substituent was unsaturated and conjugated with the pyrimidine ring were more potent antiviral drugs than the corresponding non-conjugated and alkyl-substituted analogues. The length of the 5-substituent and the nature of any heteroatoms contained within it also affected antiviral activity. When one pair of isomers was examined in more detail, differences in antiviral activity similar to those observed in cell culture occurred in virus-infected mice. The biochemical basis for the greater antiviral activity of the preferred isomer was related to affinity both for virus thymidine kinase and virus DNA polymerase.

5-substituted deoxyuridine; antiviral activity; herpes simplex virus

Introduction

In the 22 years that have elapsed since the first discovery [13] of the antiviral activity of 5-iodo-2'-deoxyuridine (IDU) against herpes simplex virus type 1 (HSV-1) a number of nucleoside analogues have been reported to exhibit significant antiviral

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activity against herpes viruses and a few are now in clinical use (for review, see Ref. 11). Because of the limitations in the use of IDU, much effort has been directed to the synthesis of other 5-substituted deoxyuridines (Fig. 1) which are more active but show less cellular toxicity. However, the development of such compounds has been largely empirical and only more recently have studies [6,7,10,12] on the in vitro antiviral properties of a number of these compounds led to a broader definition of the structural features required for optimum anti-HSV-1 activity. Although it is clear that herpes simplex virus type 2 (HSV-2) differs from HSV-1 in its susceptibility to inhibition by a wide range of nucleoside analogues, there are few detailed studies on the anti-HSV-2 activity of 5-substituted deoxyuridines [3,7,9].

The mechanism of action of the known pyrimidine nucleoside antivirals is, in many cases, not clearly defined. Some, as the nucleoside 5'-triphosphate, may exert an antiviral effect by interaction with the virus-coded DNA polymerase [1,21], either as substrates or as inhibitors, while inhibition of the putative virus-induced ribonucleotide reductase has also been proposed [19]. However, such interactions require that the compounds first be phosphorylated. This is best accomplished intracellularly as nucleotides are inefficiently taken up by cells and therefore have little potential for therapeutic use. HSV-1 and 2 each code for a unique thymidine kinase [14,17] which increases the ability of infected cells to phosphorylate thymidine compared with uninfected cells. Consequently, analogues of thymidine are potentially more active as antimetabolites in herpes virus-infected cells than in uninfected cells and those compounds that interact specifically with herpesvirus-induced, but not host cell, thymidine kinase would be expected to exhibit a high degree of selectivity of drug action.

This paper reports the anti-herpes virus activity of a series of pyrimidine-2'-deoxynucleosides with unsaturated substituents at the 5-position. Certain features in these substituents are identified as promoting antiviral activity against HSV-1 and HSV-2 both in vitro and in vivo. The possible biochemical basis for the greater antiviral activity of certain compounds is discussed.

Materials and Methods

Compounds

The synthesis of compounds *1–7* and *12–14* has been described elsewhere [12]. Compound *10* was prepared from 5-(4-carbomethoxy-1,3-butadienyl)-2'- deoxyuridine (see Ref. 12) by decarboxylation (R. Upton, unpublished observations). Com-

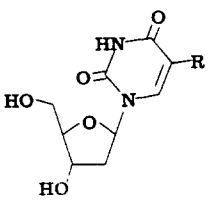

Fig. 1. Antiviral deoxyuridine nucleosides prepared by the introduction of various 5-substituents (R).

pound *8* [20] was a gift from Dr. R.L. Dyer and *9* was generously provided by Professor K.K. Gauri, Universitäts-Augenklinik, Hamburg. Compound *11* was synthesised as described previously [16] and *15* [15] was a gift from Professor A.S. Jones, University of Birmingham, Birmingham, U.K.

Antiviral assays

The antiviral activity of compounds against HSV-1, strain S3, in vitro was assessed using a micro-plaque reduction assay in BHK 21 cells as described elsewhere [22]. For anti-HSV-2 activity, a cytopathic effect (CPE) inhibition assay was employed as follows: monolayers of BHK 21 cells (4×10^4 cells/well) in 96-well microtitre plates (Falcon) were infected with HSV-2 strain 3345 [23], $10^{3.5}$ p.f.u. in Eagle's minimal essential medium, Dulbecco's modification, plus 10% calf serum (Flow Laboratories) (growth medium). After virus adsorption for 1 h at 37°C, monolayers were overlaid with growth medium containing 0.5% (w/v) carboxymethylcellulose and test compound at the required concentration. Infected untreated cultures were included as controls as were uninfected cultures treated with compound. Cultures were incubated until the virus induced-CPE had reached 90–100% in infected controls (usually 23–27 h) when plates were fixed (formal saline) and stained (carbol fuchsin). The extent of virus CPE in infected control and in drug-treated wells was estimated by examination of the stained cultures. Previous experiments had shown that there was a good (inverse) correlation between the extent of virus CPE (judged by examination under the light microscope) and the amount of stain retained by fixed cultures. Each compound was tested in a two-fold dilution series and the lowest concentration that caused a 50% inhibition of virus CPE (MIC) determined. In vivo assays for antiviral activity against HSV-1 and thymidine kinase inhibition assays were performed as described elsewhere [22].

Results and Discussion

The effect of conjugation and chain length on antiviral activity

The results of in vitro antiviral testing of a number of related alkene- and alkane-substituted deoxyuridines against HSV-1 in vitro are shown in Table 1. There was a

TABLE 1

Effect of conjugation on the antiviral activity of 5-substituted deoxyuridines against HSV-1 in vitro

Compound	Substituent	MIC[a] (μg/ml)
1	$CH_2CH{=}CH_2$	100
2	(E)-$CH{=}CHCH_3$	0.5
3	-$CH_2CH{=}CHCH_3$	10
4	(E)-$CH{=}CHCH_2CH_3$	0.5
5	-$CH_2CH_2CH_2CH_3$	10

[a] Minimum inhibitory concentration = lowest concentration required to give a 1 $\log_{10}$ reduction in the infectivity end point of HSV-1, strain S3, titrated on monolayers of BHK 21 cells.

considerable increase in antiviral activity against HSV-1 when the double bond in the side chain of *1* or *3* was brought into conjugation with the pyrimidine ring, as in *2* and *4* respectively. The saturated butyl derivative *5* had similar anti-HSV-1 activity to the non-conjugated alkene *3*. A similar effect has also been reported with the saturated propyl (not shown here) which was less active than the conjugated propene and the saturated ethyl which was less active than the vinyl [6]. In order to determine whether the differential antiviral activities of conjugated and non-conjugated isomers observed in vitro are also apparent in vivo, the efficacy of a pair of isomers, *1* and *2*, was examined in a disseminated HSV-1 infection of mice. C3H mice inoculated with HSV-1 by the intraperitoneal route became infected systemically and succumbed to a fatal encephalitis 5–12 days after infection. Lethally infected mice were treated with the conjugated alkene *2*, 500 mg/kg/day or 50 mg/kg/day, or the non-conjugated isomer *1*, 500 mg/kg/day, orally by gavage commencing 8 h post-infection. The results (Fig. 2) show that significant protection of mice was achieved when treated with *2* either 500 mg/kg/day ($P<0.001$) or 50 mg/kg/day ($P<0.05$). Compound *1* (500 mg/kg/day) also afforded significant protection compared with controls ($P<0.05$) but the effect was significantly less ($P<0.05$) than that achieved with the same dose of *2*.

When a homologous series of side chains substituted into deoxyuridine were tested for antiviral activity against HSV-1 in vitro, it was observed that extending the side chain longer than four carbons caused a sharp cut-off in antiviral activity (Table 2). In vitro the propene (*2*) and butene (*4*) were equally effective against HSV-1 while the pentene (*6*) and hexene (*7*) were without antiviral activity at the maximum concentration tested. Analysis of the data of Machida et al. [18], who reported the anti-HSV-1 activity of a series of 5-substituted 1-β-D-arabinofuranosyluracils, reveals a similar

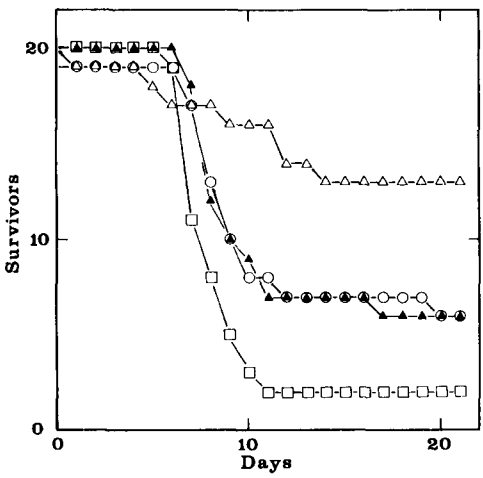

Fig. 2. Comparison of the antiviral effect of compounds *1* and *2* against intraperitoneal HSV-1 infection of mice. Compound *1* 250 mg/kg (○), *2*, 250 mg/kg (△) or 25 mg/kg (▲), was administered orally by gavage 8 h post-infection and twice daily for 5 days thereafter. □, Infected control.

TABLE 2

Effect of chain length on the antiviral activity of 5-substituted deoxyuridines against HSV-1 in vitro

Compound	Substituent	MIC[a] (μg/ml)
2	(E)-CH=CHCH$_3$	0.5
4	(E)-CH=CHCH$_2$CH$_3$	0.5
6	(E)-CH=CHCH$_2$CH$_2$CH$_3$	>100
7	(E)-CH=CHCH$_2$CH$_2$CH$_2$CH$_3$	>100

[a] Minimum inhibitory concentration = lowest concentration required to give a 1 $\log_{10}$ reduction in the infectivity end point of HSV-1, strain S3, titrated on monolayers of BHK 21 cells.

pattern of activity. The data show that the conjugated alkenes were more active than the corresponding alkanes and that within a series of homologues, antiviral activity decreased as chain length of the 5-substituent increased. De Clercq et al. [7] have observed a similar correlation between anti-HSV-1 activity and chain length with a series of 5-alkynyl-2'-deoxyuridines and arabinofuranosyluracils. However, the substituted deoxyuridines and, to a lesser extent, the ara-uracils also showed marked antimetabolite effects towards uninfected cells and cannot be considered to be selective antivirals.

A number of alkene and alkane-substituted deoxyuridines were also tested for antiviral activity against HSV-2 in vitro. The results (Table 3) show that the vinyl (*8*) was the most active and a spectrum of activity was observed for the remainder. Here also there are two structural features which appear to influence inhibitory activity: a) the presence of a vinylic group in the 5-substituent conjugated to the C–C double bond of the pyrimidine ring and, b) chain length. Compounds with a conjugated alkenyl group were more active than their non-conjugated isomers (compare *2* with *1* and *4* with *3*) whilst compounds with an alkane substituent were less effective than the

TABLE 3

Effect of conjugation and chain length on antiviral activity of 5-substituted deoxyuridines against HSV-2 in vitro

Compound	Substituent	MIC[a] (μg/ml)
8	-CH=CH$_2$	0.5
9	-CH$_2$CH$_3$	1.0
2	(E)-CH=CHCH$_3$	16
1	-CH$_2$CH=CH$_2$	32
10	(E)-CH=CHCH=CH$_2$	16
4	(E)-CH=CHCH$_2$CH$_3$	100
3	-CH$_2$CH=CHCH$_3$	>100
5	-CH$_2$CH$_2$CH$_2$CH$_3$	>100
6	(E)-CH=CHCH$_2$CH$_2$CH$_3$	>100

[a] Minimum inhibitory concentration = lowest concentration required to reduce virus-induced CPE by 50% in HSV-2, strain 3345, infected monolayers of BHK 21 cells.

conjugated compounds (compare *9* with *8* and *5* with *4*), and no more active than the non-conjugated alkenyl equivalent (see *5* and *3*). In compounds of longer chain length an extension of the conjugation by introduction of a second vinylic group resulted in a further increase in activity (compare *10* and *4*). The effect of chain length on antiviral activity is apparent from an examination of each of two series of related compounds. Amongst the conjugated alkenes, increase in chain length from 2 carbon atoms (*8*) to 3 and 4 carbons (*2* and *4*) resulted in decreased antiviral activity and the introduction of a further methylene group (*6*) yielded a compound which was inactive at the highest concentration tested. Comparison of the activities of the non-conjugated alkene and alkane-substituted deoxyuridines (*1, 3, 9* and *5*) similarly shows the adverse effect of chain length on antiviral activity. These effects are also shown in Fig. 3. A similar correlation between chain length and anti-HSV-2 activity has also been reported for 5-alkyne substituted deoxyuridines and ara-uracils [7].

Effect of substitution in the vinyl group on antiviral activity

The antiviral activity of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) has been reported previously [8]. Against HSV-1 in vitro, BVDU is the most inhibitory compound of this type so far reported (see Ref. 6 and compound *11*) (Table 4). The replacement of Br in *11* with either nitrile (*12*) or methyl (*2*), both of which have a smaller molar refraction (an estimate of steric bulk) than bromine, failed to afford a compound more inhibitory to HSV-1 (Table 4). If, in this instance, size is not a constraint on antiviral activity then other properties of bromine, not shared with

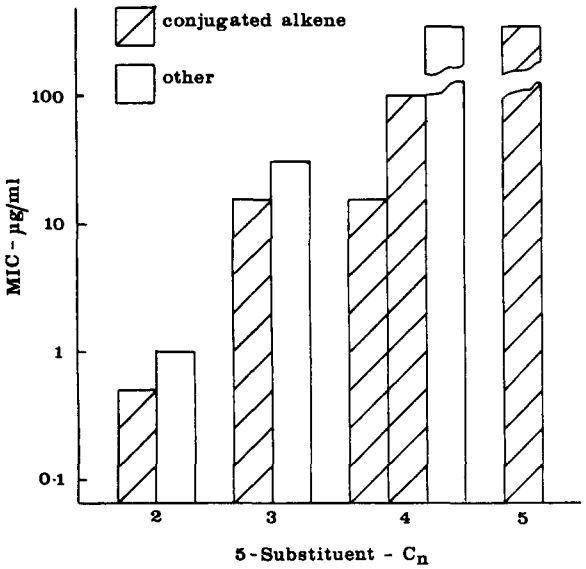

Fig. 3. In vitro anti-HSV-2 activity of 5-substituted deoxyuridines. BHK 21 cells were infected with HSV-2, strain 3345, and treated with compound to determine that concentration which caused a 50% inhibition of virus-induced CPE compared with infected, untreated controls after approximately 24 h growth at 37°C.

TABLE 4

Effect of various substitutions in the vinylic group on antiviral activity of 5-substituted deoxyuridines against HSV-1 in vitro

Compound	Substituent	MIC[a] (μg/ml)
11	(E)-CH=CHBr	0.005
12	(E)-CH=CHCN	>25
2	(E)-CH=CHCH$_3$	0.5
13	(E)-CH=CHSCH$_3$	0.5
14	CH=CBr$_2$	0.1
15	(Z)-CH=CHBr	0.1

[a] Minimum inhibitory concentration = lowest concentration required to give a 1 $\log_{10}$ reduction in infectivity end point of HSV-1, strain S3, titrated on monolayers of BHK 21 cells.

nitrile or methyl, may also be important in conferring antiviral properties on the substituted nucleoside. In particular, the hydrophobicity and inductive effects of bromine may be important. The reduced antiviral activity of *12* compared with *11* may be due to the hydrophilic nature of nitriles. The superior activity of *11* compared with *2* cannot be readily explained in terms of hydrophobic properties, methyl and bromine being very similar in this respect, but may be attributable to the inductive property of bromine not found with methyl. On the other hand, the greater size of thiomethyl (*13*) may be of prime importance in limiting its antiviral activity compared with *11*. However, there may be an optimum requirement for each of the properties – size, hydrophobicity and inductive effect – discussed here, or other properties not considered here may also be important. In this respect the lesser activity of the trifluoromethylvinyl analogue [(*E*)-5-(3,3,3-trifluoro-1-propenyl)-2′-deoxyuridine] compared with *11* [6] is of interest since trifluoromethyl is more hydrophobic and has a greater inductive effect than bromine while having a smaller molar refraction. The introduction of a second bromine atom (*14*) resulted in a compound that was less active than the monohalogenated *E* isomer; the monohalogenated *Z* isomer (*15*) was also less active than the *E* isomer (Table 4 and ref. 15). In 3 other cases where both *E* and *Z* isomers of a compound were tested, the *E* isomer was more active against HSV-1 than the *Z* isomer (data not shown). Studies with further compounds have also shown that branching of the 5-substituent, e.g., by methylation of either of the olefinic carbon atoms, substantially reduced antiviral activity (data not shown).

Biochemical basis for relative antiviral potency

The requirement for herpes virus thymidine kinase in the antiviral action of the 5-substituted deoxyuridines of the general type discussed here is widely recognised. In order to determine whether the nature of the 5-substituent affected the interaction of nucleoside with thymidine kinase in a way that might account, at least in part, for the relative antiviral potency, we compared the ability of two isomers, *1* and *2* (Table 1), to inhibit the phosphorylation of [^{14}C]thymidine by HSV-1 thymidine kinase. In a simple competition experiment employing increasing ratios of analogue to substrate, the conjugated propene, *2*, was the more effective competitor, inhibiting the phospho-

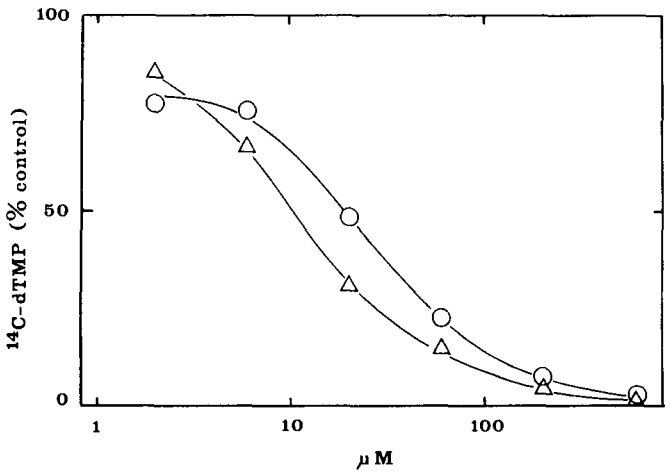

Fig. 4. Inhibition of thymidine phosphorylation by nucleoside analogues. [^{14}C]Thymidine was phosphorylated by HSV-1 thymidine kinase in the absence (control) or presence of increasing concentrations of: ○, compound *1*; △, compound *2*.

rylation of 20 μM thymidine by 50% at 10.5 μM analogue concentration (Fig. 4). By comparison, the non-conjugated isomer, *1*, had a corresponding 50% inhibitory dose of 20 μM (Fig. 4). Our data are in accord with K_i values of 0.2 μM and 0.7 μM for the conjugated and non-conjugated isomers respectively reported by Cheng and coworkers [2,5] who have further shown 5-propyl-2′-deoxyuridine to have a K_i for HSV-1 thymidine kinase of 0.6 μM [2] and to have lesser anti-HSV-1 activity in cell culture than the conjugated propene [5]. A correlation between antiviral activity and affinity for thymidine kinase is also apparent for the *E* and *Z* isomers of BVDU (compounds *11* and *15*, Table 4) which have inhibition constants of 0.24 μM and 2.47 μM respectively for HSV-1 thymidine kinase [4].

Thus the biochemical basis for the relative anti-HSV-1 activity of two types of isomeric 5-substituted 2′-deoxyuridines appears to reside, at least in part, in the higher affinity for virus thymidine kinase conferred on the nucleoside with the favoured isomeric form. Whether or not such interactions account for the differences in anti-HSV-1 activity amongst compounds where a different pattern of change in the 5-substituent pertains, e.g., chain length, remains to be seen. It is also not clear whether these arguments are applicable to HSV-2. In addition, it must be remembered that the mechanism of action of these 5-substituted deoxyuridines most probably requires the inhibition of other virus enzyme(s) by phosphorylated forms of the nucleoside. Differences in antiviral potency may also be accounted for by relative affinities of the phosphorylated nucleosides for their target enzymes, as appears to be the case for the 5′-triphosphates of *1* and *2* and their inhibitory effects on the HSV-1 DNA polymerase [22].

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