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ANTIVIRAL ACTIVITY OF O'-ALKYLATED DERIVATIVES OF CYTOSINE ARABINOSIDE

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Abstract—The inhibitory effects of the 2'-O-methyl, 3'-O-methyl, 5'-O-methyl and 5'-O-ethyl derivatives of cytosine arabinoside (ara-C)* on (vaccinia) virus multiplication in primary rabbit kidney (PRK) cell cultures are negligible as compared to the unsubstituted parent compound. None of the analogues inhibited thymidine incorporation into PRK cell DNA. Nor did they suppress vaccinia virus-induced tail lesion formation in mice. The lack of antiviral activity of these analogues is consistent with, and supports, the proposal that the improved activity of 5'-O-acylated ara-C is due to the gradual release of free ara-C by tissue esterases.

Ara-C (1-β-D-arabinofuranosylcytosine, cytosine arabinoside, cytarabine, ara-cytidine) is a potent antimetabolite which exhibits antiviral, antitumor and immunosuppressive activities in animals and man. The influence of methods of administration of this drug, as well as treatment schedules, have been widely investigated and, e.g. treatment of L1210 leukemic mice is most effective with multiple closely spaced doses [1]. A variety of observations have demonstrated that intravenously administrated ara-C disappears from the serum of patients more rapidly than might be anticipated from its excretion in the urine, and considerable evidence now points to the fact that this is due to enzymatic deamination of the active ara-C to ara-U, which is metabolically inactive [2–4]. This would account, at least in part, for the lower effectiveness of ara-C as a therapeutic agent when administered as a single large dose.

The synthesis of a number of ara-C derivatives, in which one or more of the sugar hydroxyls were methylated or ethylated [5–8], was followed by the observation that such O'-alkyl analogues are appreciably or totally resistant to the action of cytidine deaminase [9]. The question could be raised, therefore, whether the potential therapeutic activity of such derivatives would be improved as compared to that of ara-C itself.

The foregoing appeared all the more interesting in the light of the observation of Gray *et al.* [10] that single doses of a variety of 5'-acyl derivatives of ara-C [11] were almost as effective as multiple closely spaced doses of ara-C, when tested for antitumor and antiviral activities in mice. One of the factors invoked to interpret this enhanced effectiveness was the presumed resistance to deamination conferred by the 5'-acyl substituents; subsequent slow hydrolysis of these acylated derivatives by tissue esterase(s) would then lead to a gradual release of active ara-C. Similar, although somewhat less effective, behavior was exhibited by some 2'- and 3'-acyl derivatives of ara-C [12].

Since O'-alkyl derivatives of ara-C are also resistant to dealkylation by tissue enzymes, an examination of the possible antiviral activity of these compounds should be of some interest in relation to the reported activities of the acylated ara-C derivatives.

MATERIALS AND METHODS

The *synthesis* and *physico-chemical characteristics* of the 2'-O-methyl, 3'-O-methyl, 5'-O-methyl and 5'-O-ethyl analogues of 1-β-(D-arabinofuranosyl) cytosine have been described previously [5–8]. The structure of these nucleosides is depicted in Fig. 1.

All *in vitro* experiments were carried out in PRK (primary rabbit kidney) cell cultures grown to confluency in Falcon plastic petri dishes or glass culture tubes. Eagle's minimal essential medium (MEM) was used as the cell culture medium (supplemented with 10% calf serum for growth of the cells or 3% calf serum for maintenance of the cells).

The explore the direct *effect of the O'-alkyl analogues of ara-C on viral cytopathogenicity*, PRK cells in tubes were inoculated with either vaccinia virus (500 $CCID_{50}$/tube), herpes simplex virus (50 $CCID_{50}$/tube) or vesicular stomatitis virus (500 $CCID_{50}$/tube) for 1 hr at 37°, and, immediately thereafter, exposed to different concentrations of the ara-C analogues (200, 40,

* Abbreviations used: ara-C—1-β-D-arabinofuranosylcytosine, cytosine arabinoside, cytarabine, ara-cytidine; $CCID_{50}$—cell culture infecting dose 50 (infecting 50% of the cell cultures); PFU—plaque forming units, both determined in primary rabbit kidney cell cultures; PRK—primary rabbit kidney; MEM—minimal essential medium; PBS—phosphate buffered saline.

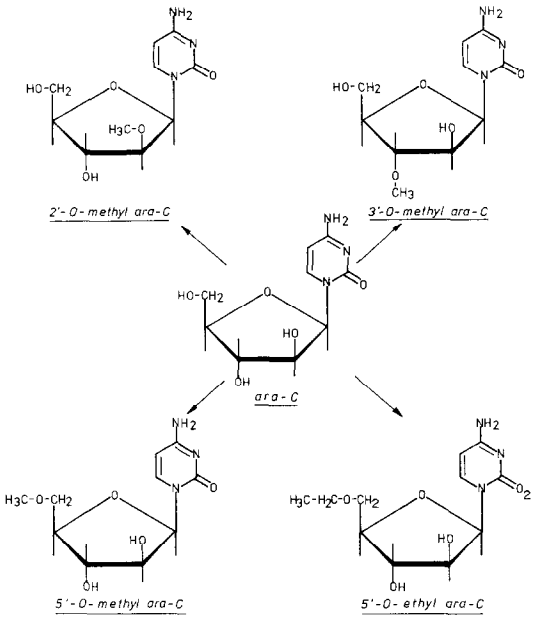

Fig. 1.

4, 0·4, ... μg/ml in MEM supplemented with 3% calf serum; 1 ml/tube). Viral cytopathogenicity was recorded 1 or 2 days later (vesicular stomatitis virus), 2 or 3 days later (vaccinia virus), and 3 or 4 days later (herpes simplex virus).

To evaluate the *effect of the O'-alkyl analogues of ara-C on vaccinia virus growth*, PRK cells in petri dishes were inoculated with vaccinia virus ($10^{4.5}$ PFU/petri dish) for 1 hr at 37°, and, immediately thereafter, exposed to different concentrations of the ara-C analogues (100 or 10 μg/ml in MEM supplemented with 3% calf serum; 4 ml/petri dish). The cell cultures were frozen (at −70°C) either immediately after addition of the compounds (that is 1 hr after virus infection) or 24, 48 or 72 hr after virus infection. Before virus titration, the cell cultures were thawed, frozen and thawed again to break up the cells and to release the virus. Cell debris was removed by centrifugation at 1500 rev/min for 15 min, and the supernatant titrated for virus plaque formation in PRK cell monolayers in petri dishes. Plaques were counted after 2 days.

To determine the *effect of the O'-alkyl analogues of ara-C on DNA synthesis*, PRK cells in petri dishes (approx. 10^6 cells/petri dish) were exposed to 40 μg/ml of either compound (in MEM supplemented with 3% calf serum; 2 ml/petri dish) for 24 hr at 37°. The cells were then washed with MEM and incubated with (^{3}H-methyl)-thymidine (specific radioactivity: 12 Ci/m-mole) at 2 μCi/ml MEM per petri dish for 30 min at 37°. After the cells had been washed again (3 times, with phosphate buffered saline) the petri dishes were emerged in a mixture of ethanol and acetic acid (2:1, v/v) for 15 min, washed with ethanol (70%), washed with distilled water and then emerged in perchloric acid (stock solution of 70%, diluted 1/32 in distilled water) for 30 min. The petri plates were allowed to dry for 2 hr at 37° (or overnight at room temperature) and broken up in pieces with a forceps. The pieces were then placed in scintillation vials, and readily dissolved in the scintillation fluid [2,5-diprenyloxazole (PPO)-toluene] added to the vials at 15 ml/vial. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. This procedure for measuring incorporation of (^{3}Hmethyl)-thymidine into DNA of cell monolayers has been proven more convenient and at least as reliable as the more routinely used procedure in which the acid-insoluble content of the cell (including DNA) precipitated by trichloroacetic acid is collected on glass fibre discs after the cells have been scraped off from the petri dishes and disrupted by ultrasonication.

The *in vivo* experiments were carried out in female NMRI mice weighing 10 g (range from 9 to 11 g). To establish the *effect of the O'-alkyl analogues of ara-C on vaccinia virus-induced tail lesion formation*, the mice were given an intraperitoneal injection of either of the compounds (0·2 mg/0·2 ml per mouse), and immediately thereafter an intravenous injection of vaccinia virus (either $10^{3.6}$ or $10^{4.6}$ PFU/0·2 ml per mouse). The vaccinia virus stock used in these experiments was a calf lymph vaccine, kindly supplied by the "Rijksentstofinrichting" of Brussels. The stock itself titrated $10^{8.3}$ PFU/ml. It was stored at 4° and diluted to the appropriate concentration in PBS (phosphate buffered saline) before use. Tail lesions were counted 8 days after virus challenge. The statistical significance of the results was assessed by Student's *t*-test.

RESULTS AND DISCUSSION

The antiviral activity of the O'-alkylated derivatives of ara-C was assessed in PRK cell cultures infected with vaccinia, herpes simplex and vesicular stomatitis virus. It has been shown before that the activity of ara-C is confined to DNA (pox-, herpes viruses) [13] and rhabdo (rabies, vesicular stomatitis) viruses [14] (for a more complete review, see ref. 15). In accordance with these findings ara-C inhibited the cytopathogenicity of vaccinia and herpes simplex virus at concentrations as low as 0·04 μg/ml, and, at a significantly higher concentration, also the cytopathogenicity of vesicular stomatitis virus (Table 1). The 2'-O-, 3'-O- and 5'-O-methyl and 5'-O-ethyl analogues of ara-C were much less effective, and, with the exception of the 5'-O-methyl analogue, inactive at the highest concentration tested (200 μg/ml) (Table 1).

The multiplication of vaccinia virus was completely arrested in the presence of ara-C (at 10 or 100 μg/ml), added to the cell cultures immediately after virus adsorption (Table 2). No virus was detected in the cells treated with ara-C, whereas in the control group virus titers went up to 10^5 PFU/ml (after 24 hr) and 10^6 PFU/ml (after 48 and 72 hr). Addition of either 2'-O-, 3'-O- or 5'-O-methyl ara-C, or 5'-O-ethyl ara-C (at 10

Table 1. Effect of the O'-alkylated derivatives of ara-C on viral cytopathogenicity in PRK cells

Compounds†	Minimal inhibitory concn* (μg/ml)		
	Vaccinia virus‡	Herpes simplex virus	Vesicular stomatitis virus
Ara-C	0·04	0·04	40
2'-O-methyl ara-C	200	>200	>200
3'-O-methyl ara-C	>200	>200	>200
5'-O-methyl ara-C	10	40	>200
5'-O-ethyl ara-C	>200	>200	>200

* Required to inhibit viral cytopathogenicity by 50%.
† 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.

or 100 μg/ml) to the cells after virus adsorption did not appear to affect the virus yields (Table 2).

As shown in a variety of mammalian cells, ara-C specifically inhibits DNA synthesis [16–21]. Ben-Porat *et al.* [21] established that in rabbit kidney cells (herpes simplex or pseudorabies) virus multiplication was inhibited by ara-C to the same extent as DNA synthesis, and concluded from these data that the antiviral activity of ara-C was primarily due to its inhibitory effect on DNA synthesis and that ara-C was not a "selective" antiviral agent. The inhibitory effect of ara-C on DNA synthesis was also demonstrated in our experiments: (^{3}Hmethyl)-thymidine incorporation into DNA was reduced by about 95% in PRK cells which had been exposed to 40 μg/ml of ara-C for 24 hr (Table 3). This concentration corresponds to 100 ID_{50} (inhibitory dose, 50%) of ara-C: 50% inhibition of thymidine incorporation was recorded at a concentration of 0·4 μg/ml. At 40 μg/ml ara-C also inhibited the incorporation of ^{3}Hdeoxycytidine but not that of ^{3}Hdeoxyadenosine or ^{3}Hdeoxyguanosine (E. De Clercq, unpublished data). In marked contrast with ara-C itself, the O'-methyl (or -ethyl) analogues of ara-C failed to inhibit (^{3}Hmethyl)-thymidine incorporation into PRK cell DNA (Table 3); 2'-O-methyl ara-C slightly stimulated thymidine incorporation, even at concentrations as high as 200 μg/ml (thymidine incorporation 150 per cent as compared to the control group: data not shown).

The failure of the O'-alkylated derivatives of ara-C to inhibit DNA synthesis is most probably related to their lack of activity against DNA viruses. In this sense our results provide additional support for the contention [21] that the inhibitory effect of ara-C on the replication of DNA viruses is accounted for by inhibition of DNA synthesis.

The 5'-O-acyl derivatives of ara-C show an enhanced potency as immunosuppressive, antileukemic and antiviral agents as compared to the parent compound when administered as a single dose in the mouse [10, 11]. In marked contrast herewith, the O'-methyl analogues of ara-C did not achieve a significant

Table 2. Effect of the O'-alkylated derivatives of ara-C on vaccinia virus multiplication in PRK cells

	Vaccinia virus* titer (log_{10} PFU/ml) at ... hours after infection			
	1	24	48	72
A. Compounds added at 10 μg/ml immediately after virus adsorption				
Ara-C	<2·3	<2·3	<2·3	<2·3
2'-O-methyl ara-C	<2·3	4·8	5·9	5·8
3'-O-methyl ara-C	<2·3	5·2	6·1	5·9
5'-O-methyl ara-C	<2·3	4·9	6·1	5·8
5'-O-ethyl ara-C	<2·3	5·0	6·2	6·1
Control	<2·3	5·0	6·1	5·9
B. Compounds added at 100 μg/ml immediately after virus adsorption				
Ara-C	<1·3	<1·3	<1·3	<1·3
2'-O-methyl ara-C	<1·3	4·6	5·9	5·9
3'-O-methyl ara-C	<1·3	4·9	6·2	6·0
5'-O-methyl ara-C	<1·3	5·1	6·3	6·0
5'-O-ethyl ara-C	<1·3	5·0	6·2	6·1
Control	<1·3	4·9	6·2	6·1

* Virus input: 4·5 log_{10} PFU/petri dish.

Table 3. Effect of the O′-alkylated derivatives of ara-C on DNA synthesis in PRK cells

Compounds	(^{3}Hmethyl)-thymidine incorporated into DNA* (cpm/petri dish†) Average	S.D.
Ara-C	631	±95
2′-O-methyl ara-C	18577	±2599
3′-O-methyl ara-C	13968	±678
5′-O-methyl ara-C	11298	±470
5′-O-ethyl ara-C	11236	±48
Control	14723	±922

* Thymidine incorporation into DNA was measured after the cells had been exposed to the compounds (40 μg/ml) for 24 hr. Data represent average values for at least 4 observations (only 2 for 5′-O-ethyl ara-C).

† Average number of cells per petri dish: 1×10^6.

antiviral activity in mice infected with vaccinia virus (Table 4). Mice inoculated intravenously with vaccinia virus develop characteristic tail lesions which are readily suppressed by a variety of compounds such as methisazone [22, 23], 2′- and 3′-C-methyladenosine and 3′-C-methylcytidine [23], polyacrylic acid [24] and other interferon inducers [25]. Neither ara-C nor its O′-methyl analogues were significantly inhibitory in this system, although ara-C showed some activity in mice infected with the lower virus challenge dose (Table 4). In the same conditions (singe dose of 20 mg/kg of the compound injected intraperitoneally immediately before intravenous vaccinia virus inoculation), 2′-deoxyuridine analogues such as IUdR (5-iodo-2′-deoxyuridine) proved significantly more active than ara-C (E. De Clercq, unpublished data).

In vitro (Table 1), ara-C caused a 50 per cent reduction of vaccinia virus cytopathogenicity at 0·04 μg/ml. *In vivo*, however, a concentration of 20 μg/ml was required to inhibit vaccinia virus-induced lesions by 50 per cent (Table 4), at least if $10^{3.6}$ PFU/mouse was used as the virus challenge dose. It should be recognized that this challenge dose (400 PFU/g mouse) corresponded well to the virus challenge dose used *in vitro* (500 $CCID_{50}$/ml per tube). Thus, the minimal effective concentration of ara-C required to inhibit virus-induced cytotoxicity is considerably higher *in vivo* than *in vitro*. That ara-C is less effective *in vivo* than *in vitro* is not unexpected in view of its rapid deamination *in vivo* to the metabolically inactive ara-U [2–4].

Although the O′-methyl analogues of ara-C are not deaminated [9], they were not more active than ara-C in inhibiting the development of pox tail lesion formation in mice infected with vaccinia virus (Table 4). It remains to be established whether these O′-alkyl analogues of ara-C are also ineffective as antileukemic [1] and immunosuppressive [26] agents.

The biological inactivity of the O′-alkylated derivatives of ara-C reported herein contrasts with the diverse (antiviral, immunosuppressive and antileukemic) activities attributed to the O′-acyl esters of ara-C [10–12]. The activity of the latter would ultimately reside in free ara-C, slowly released from its O′-acylates by enzymatic hydrolysis (in serum and/or tissues). The inactivity of the former may be ascribed to the lack of cleavage of free ara-C from its O′-alkyl analogues. The lack of activity of these analogues cannot be accounted for by modifications of the arabinose ring [27]. For 5′-O-methyl and 5′-O-ethyl ara-C the lack of activity is understandable, if it is assumed that ara-C must undergo intracellular phosphorylation by the appropriate kinases to become biologically active [28]. The inactivity of the 2′-O-methyl and 3′-O-methyl analogues of ara-C may also be due to the fact that they are not substrates for intracellular kinases, or, if they are, that their 5′-triphosphates are not inhibitors of DNA polymerase [29].

Table 4. Effect of the O′-alkylated derivatives of ara-C on tail lesions in mice infected with vaccinia virus

Compounds*	Number of lesions: Per individual mouse	Number of lesions: Average	Probability (compared to control group)
	A. Virus dose: 3·6 $\log_{10}$ PFU/mouse		
Ara-C	2, 3, 0, 5, 3, 5, 1, 0, 1, 5	2·5	<0·05
2′-O-methyl ara-C	0, 1, 4, 5, 6, 8, 3, 0, 1, 7	3·5	Non-significant
3′-O-methyl ara-C	2, 5, 5, 5, 1, 1, 5, 6, 7, 4	4·1	Non-significant
5′-O-methyl ara-C	5, 4, 7, 3, 3, 7, 1, 0, 0, 8	3·8	Non-significant
Control	3, 4, 5, 2, 9, 7, 2, 1, 9, 8	5·0	—
	B. Virus dose: 4·6 $\log_{10}$ PFU/mouse		
Ara-C	16, 9, 9, 20, 16, 14, 15, 20, 12, 13	14·4	Non-significant
2′-O-methyl ara-C	10, 13, 8, 13, 15, 5, 8, 12, 9, 22	11·5	<0·10
3′-O-methyl ara-C	3, 5, 17, 23, 6, 8, 16, 1, 19, 13	11·1	<0·20
5′-O-methyl ara-C	8, 8, 12, 2, 18, 12, 19, 7, 13, 9	10·8	<0·10
Control	17, 14, 7, 20, 18, 26, 11, 16, 12, 13	15·4	—

* Injected intraperitoneally (0·2 mg/mouse) immediately before intravenous vaccinia virus challenge.

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Note added in proof: Z. Darzynkiewicz (personal communication) has recently investigated the effects of the 2'-O-, 3'-O- and 5'-O-methyl analogues of ara-C on ^{3}Huridine incorporation into RNA of Sprague–Dawley rat thymus cell suspensions. At all concentrations tested (0·05–100 μg/ml) ara-C reduced ^{3}Huridine incorporation by more than 50 per cent, whereas the O'-methyl analogues of ara-C failed to do so: only a slight inhibition was noted at the higher concentrations (25 and 100 μg/ml): e.g. at 100 μg/ml 21·5% with 5'-O-methyl ara-C, 37·5% with 3'-O-methyl ara-C and 40·5% with 2'-O-methyl ara-C.

The 2'-O-methyl analogue of ara-C has also been synthesized by J. A. Montgomery and A. G. Laseter [*J. Med. Chem.* **17,** 360 (1974)]. As expected, 2'-O-methyl ara-C failed to inhibit L1210 leukemia *in vivo* and HEp2 cells *in vitro*.