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Antiviral activity of *O'*-methylated derivatives of adenine arabinoside

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Ara-A*, originally synthesized as a potential antitumor drug, has demonstrated significant *in vitro* and *in vivo* activity against a variety of DNA viruses [1], including herpes simplex, varicella-zoster and cytomegalovirus, i.e., viruses that are implicated in various often life-threatening or debilitating diseases in man. Preliminary clinical studies suggest that some of these virus infections may be amenable to ara-A therapy [2].

Ara-A is readily deaminated to ara-Hx (hypoxanthine arabinoside) by adenosine deaminase, ubiquitously present in tissues and cell culture systems. Only a minor part of ara-A is phosphorylated by cellular enzymes to yield ara-ATP [3, 4]. The primary site of action of ara-A appears to be the DNA-dependent DNA polymerase [5, 6]. In DNA synthesis catalyzed by this enzyme, ara-ATP would act as a competitive inhibitor of dATP. The major degradation product of ara-A, ara-Hx, is distinctly less effective than ara-A in inhibiting virus replication [7]. Thus, premature deamination may be expected to impair the antiviral potency of ara-A. Accordingly, addition of an adenosine deaminase inhibitor was found to increase the inhibitory effects of ara-A on virus replication in cell culture [8, 9]. The protective action of the adenosine deaminase inhibitor was particularly striking in LLC-MK_2 cells which are known to deaminate ara-A very rapidly [10].

Recently, we have observed that 2'-, 3'- and 5'-*O*-methylation markedly increases the resistance of ara-A to deamination by either rabbit kidney or calf intestine adenosine deaminase [11, 12]. It appeared interesting, therefore, to examine whether these *O'*-methylated derivatives of ara-A would also be endowed with enhanced biological activities (antiviral and/or antimetabolic).

Ara-A was obtained from Parke, Davis and Co. (Detroit, Michigan) through the courtesy of Dr. H. E. Machamer and Dr. R. A. Buchanan, and of Dr. R. Wolf (Parke-Davis Clinical Research Western Europe, München). The same batch (CI-673) was used to prepare the *O'*-methyl analogues: 2'-*O*-methyl ara-A, 3'-*O*-methyl ara-A, 2',3'-*O*-dimethyl ara-A and 5'-*O*-methyl ara-A. The synthesis and physico-chemical properties of the *O'*-methylated derivatives of ara-A have been described elsewhere [12].

The technique for evaluating the effects of the ara-A analogues on cytopathogenicity induced in PRK cells by vaccinia, herpes simplex (type 1) and vesicular stomatitis virus has been described previously [13]. The methodology for measuring vaccinia virus growth has also been described [13].

A microassay was developed to assess the effects of the ara-A analogues on [^{3}H]methyl-thymidine incorporation into PRK cell DNA. Serial dilutions of the compound stock solutions were prepared in MEM + 1% HCO_3^- + 5% foetal calf serum and 100 μl vol. of these dilutions were transferred into the wells of disposable Linbro plastic trays (model FB – 48 – TC; Linbro Chemical Co., New Haven, CT). To each well 50 μl of a PRK cell suspension, containing 10^5 cells per 50 μl, and 50 μl of a [^{3}H]methyl-thymidine (specific radioactivity: 12 Ci/m-mole) solution, containing 0.125 μCi per 50 μl, were added and the trays were incubated for 16 hr at 37° in a CO_2 controlled atmosphere. The cells were then treated with cold TCA (5%) for 30 min at 4°, washed 6 times with cold TCA (5%) and 4 times with cold ethanol, and, finally, allowed to dry for 1 hr at 37°. The cups were then cut out and assayed for radioactivity in a toluene based scintillant. DNA synthesis was also determined in PRK cells which had been infected with herpes simplex virus. To this end HSV (type 1) (10^5 $CCID_{50}$/ml) was included in the initial assay mixture. As compared to the uninfected PRK cells, HSV-infected cells showed a reduction of [^{3}H]methyl-thymidine incorporation by circa 75 per cent (measured after a 16 hr incubation period).

In PRK cell cultures ara-A inhibited vaccinia virus CPE at 0.3 μg/ml and herpes simplex virus CPE at 3 μg/ml (Table 1). These MICs (minimal inhibitory concentrations) compare favorably with those obtained before with ara-A in similar indicator systems (HEp-2 cells [7], human embryonic lung (WI-38) cells [14–16], rat brain cells [15], human foreskin fibroblasts [16] and mouse embryo fibroblasts [16]). The level of anti-vaccinia activity recorded with ara-A in PRK cells in the absence of adenosine deaminase inhibitor (Table 1) corresponds quite closely to the level of anti-vaccinia activity of ara-A in LLC-MK_2 cells in the presence of adenosine deaminase inhibitor [9]. A similar high level of activity was observed in chick embryo fibroblasts [17], also in the absence of adenosine deaminase inhibitor. Chick embryo cells are assumed to be poor deaminators of ara-A [10].

Ara-A proved also effective in curtailing the cytopathogenicity induced by vesicular stomatitis virus (Table 1), although the MIC at which ara-A did so was considerably higher than those required for inhibiting vaccinia and HSV CPE. The inhibitory effect of ara-A on VSV is not surprising in view of the previously reported inhibition of rhabdovirus multiplication by both ara-C [18] and ara-A [19]. Rhabdo- and leukoviruses are in fact the only RNA viruses that fall within the spectrum of activity of ara-C and ara-A.

As observed previously [13] with the 2'-, 3'- and 5'-*O*-alkyl analogues of ara-C, the 2'-, 3'- and 5'-*O*-methylated derivatives of ara-A were considerably less effective in inhibiting viral CPE than their parent compound (Table 1). 5'-*O*-methylation and 2',3'-*O*-dimethylation completely abolished the antiviral behaviour of ara-A. 2'-*O*-methylation or 3'-*O*-methylation reduced the anti-herpes and anti-VSV properties of ara-A beyond detectable levels but did not completely destroy its anti-vaccinia activity. The MICs of 2'-*O*-methyl ara-A and 3'-*O*-methyl ara-A in vaccinia

* *Abbreviations*: ara-A, adenine arabinoside, 9-β-D-arabinofuranosyl-adenine; ara-C, cytosine arabinoside, 1-β-D-arabinofuranosyl-cytosine; ara-Hx, 9-β-D-arabinofuranosyl-hypoxanthine; $CCID_{50}$, cell culture infecting dose 50 (infecting 50 per cent of the cell cultures); PFU, plaque forming units; MEM, minimal essential medium; PRK, primary rabbit kidney; HSV, herpes simplex virus; VSV, vesicular stomatitis virus; CPE, cytopathic effect, cytopathogenicity; MIC, minimal inhibitory concentration; ID_{50}, inhibitory dose-50.

Table 1. Effect of *O'*-methylated derivatives of ara-A on virus-induced cytopathogenicity in PRK cell cultures

Compounds†	Minimal inhibitory concentration* (μg/ml) Vaccinia virus‡	Herpes simplex virus (type 1)‡	Vesicular stomatitis virus‡
Ara-A	0.3 (0.1–1)	3 (1–10)	30
2'-*O*-methyl ara-A	30	> 100	> 100
3'-*O*-methyl ara-A	20 (3–30)	> 100	> 100
2',3'-*O*-dimethyl ara-A	> 100	> 100	> 100
5'-*O*-methyl ara-A	> 100	> 100	> 100

* Required to inhibit virus-induced cytopathogenicity by 50%, (mean values for 5 experiments). The data in parentheses refer to the range of minimal inhibitory concentrations obtained in the individual experiments.

† Compounds added immediately after virus adsorption.

‡ Virus input: 100 $CCID_{50}$ per tube.

virus-infected cells amounted to 30 and 20 μg/ml respectively (Table 1).

That the inhibitory effects of 2'-*O*-methyl ara-A and 3'-*O*-methyl ara-A on vaccinia virus CPE actually reflected an inhibition of virus multiplication, was ascertained by measuring virus growth in PRK cell cultures which had been inoculated with vaccinia virus and subsequently exposed to either 10 or 100 μg/ml of ara-A or the *O'*-methyl ethers thereof (Fig. 1). At 10 μg/ml, only ara-A caused an appreciable inhibition of vaccinia virus growth (Fig. 1a). At 100 μg/ml, ara-A completely arrested virus multiplication, 2'-*O*-methyl ara-A and 3'-*O*-methyl ara-A brought about a significant reduction in virus yield, and 5'-*O*-methyl ara-A and 2',3'-*O*-dimethyl ara-A were devoid of any inhibitory effect (Fig. 1b).

As shown in Table 1, none of the *O'*-methylated derivatives of ara-A proved particularly effective against herpes simplex virus. To ensure that this lack of activity was not related to some peculiarities of the HSV-PRK assay system employed, our investigations were extended to different herpes simplex strains in human diploid cells. Ara-A inhibited the CPE of all HSV strains tested: HSV (type 1) strain KOS, HSV (type 2) strain 333, HSV (type 1) strain LYONS and HSV (type 2) strain 196. In these tests, the MIC of ara-A ranged from 4 to 20 μg/ml, depending on the virus strain. The *O'*-methylated derivatives of ara-A, however, failed to inhibit the cytopathogenicity of any of the four HSV strains at concentrations equal to or below 100 μg/ml.

Is ara-A a selective antiviral agent or should its antiviral activity, like that of ara-C [13, 20], be regarded as the direct consequence of its inhibitory effect on cellular DNA synthesis? Shipman *et al.* [21] recently reported that ara-A specifically inhibited the synthesis of herpesvirus DNA at concentrations (3.2–32 μg/ml) which did not affect host cell DNA synthesis. In our assay system, ara-A effectively inhibited DNA synthesis, as monitored by [^{3}H]methyl-thymidine incorporation, of actively growing PRK cells (Fig. 2a). The ID_{50} (dose inhibiting DNA synthesis by 50 per cent after a 16 hr incubation period was approximately 25 μg/ml. Since the metabolism of ara-A in a virus-infected cell may differ drastically from its metabolism in a normal cell [20], ara-A was also tested for its inhibitory effect on DNA synthesis in HSV-infected PRK cells (Fig. 2b). The ID_{50} of ara-A in virus-infected cells was almost identical to the ID_{50} in noninfected cells. At lower concentrations

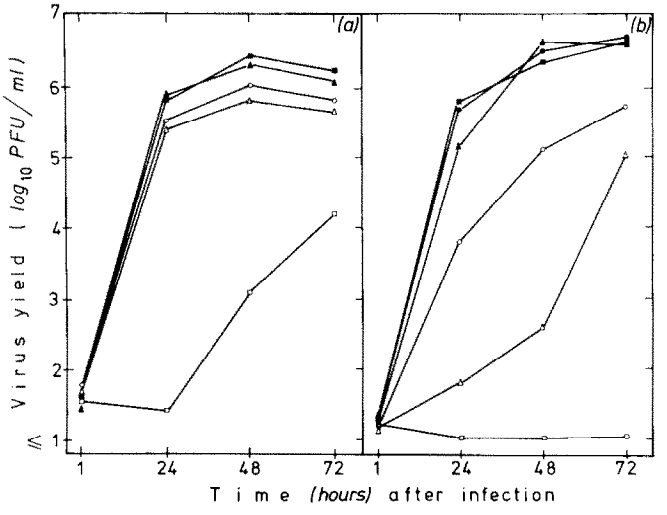

Fig. 1. Effect of the O'-methylated derivatives of ara-A on vaccinia virus growth in PRK cells. Compounds added at either 10 μg/ml (*a*) or 100 μg/ml (*b*), immediately after virus adsorption. Virus input: $10^{4.5}$ PFU (*a*) or $10^{4.5}$ $CCID_{50}$ (*b*) per petri dish. Control (■), Ara-A (□), 2'-O-methyl ara-A (○), 3'-O-methyl ara-A (△), 2',3'-O-dimethyl ara-A (●), 5'-O-methyl ara-A (▲).

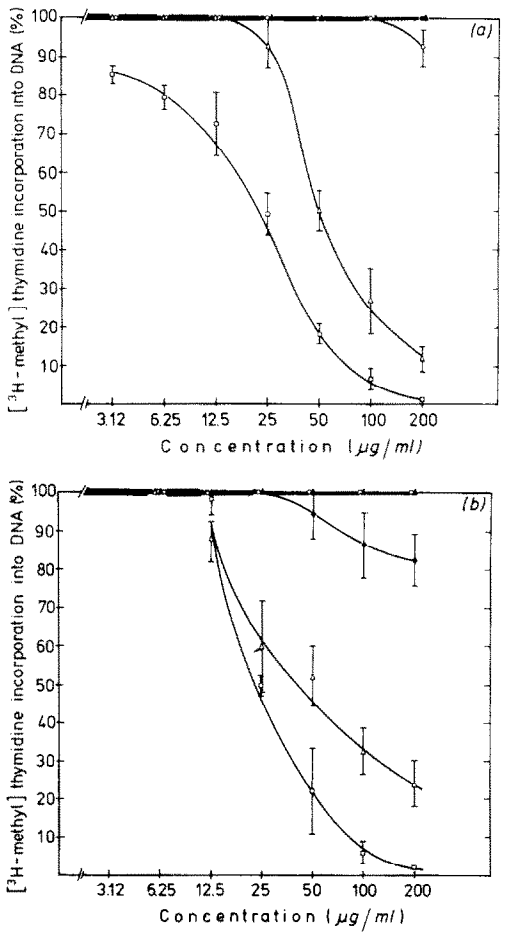

Fig. 2. Effect of the O'-methylated derivatives of ara-A on [^{3}H-methyl]-thymidine incorporation into DNA of normal PRK cells (*a*) and PRK cells infected with HSV (type 1) (10^5 $CCID_{50}$ per ml) (*b*). The cells were exposed to different concentrations of the compounds (indicated in the abscissa) for 24 hours. Data represent means ± S.D. Ara-A (□), 2'-O-methyl ara-A (○), 3'-O-methyl ara-A (△), 2',3'-O-dimethyl ara-A (●), 5'-O-methyl ara-A (▲).

(3.12–12.5 μg/ml), ara-A inhibited DNA synthesis in noninfected cells by 15 to 30 per cent (Fig. 2a). In HSV-infected cells, it failed to do so (Fig. 2b).

2'-*O*-methyl ara-A, 5'-*O*-methyl ara-A and 2',3'-*O*-dimethyl ara-A exerted little, if any, inhibitory effect on DNA synthesis in either virus-infected or noninfected PRK cells (Fig. 2). 3'-*O*-methyl ara-A, however, displayed a dose-dependent inhibitory effect which resembled that of ara-A. As compared to ara-A, the dose–response curve of 3'-*O*-methyl ara-A was shifted to a 2–4-fold higher concentration (Fig. 2a and b).

The results described herein with ara-A, as well as those reported previously with ara-C [13, 22] and formycin [23], support the general contention that 2'-, 3'- or 5'-*O*-alkylation renders nucleosides, whether ribosyl or arabinosyl, less active, if not inactive, as antiviral or antimetabolic agents.

The inactivity of 5'-*O*-methyl ara-A is understandable if it is assumed that no free ara-A is released, hence phosphorylation to the biologically active 5'-triphosphate form does not occur. The inactivity or low activity of 2'-*O*-methyl and 3'-*O*-methyl ara-A is more difficult to rationalize, unless one assumes steric hindrance in the interaction of these analogues with the cellular kinases, or the necessity of 2'- and 3'-OH in enzyme-substrate complex formation.

In fact, 3'-*O*-methyl ara-A retained partial activity which varied considerably from one assay system to another: 3'-*O*-methyl ara-A was about 60 times less effective than ara-A in inhibiting vaccinia virus CPE in PRK (Table 1), but only 2–4 times less effective in inhibiting DNA synthesis in PRK cells (Fig. 2a and b).

It is noteworthy that the order of antiviral activity of the *O'*-methyl analogues of ara-A reflects the degree of their susceptibility to deamination by calf intestinal adenosine deaminase [12]. The order of decreasing antiviral activity (or decreasing susceptibility to deamination) is: ara-A > 3'-*O*-methyl ara-A > 2'-*O*-methyl ara-A > 2',3'-*O*-dimethyl ara-A ≃ 5'-*O*-methyl ara-A. It remains to be established, however, whether this relationship is causal or fortuitous.

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Ascorbic acid and heme synthesis in deficient guinea pig liver

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Ascorbic acid deficiency in guinea pigs results in marked reduction in the activity of the hepatic microsomal drug metabolism system. Examples of the type of reactions affected include *O*-demethylation, *N*-demethylation, hydroxylation, nitroreduction and steroid hydroxylation [1–5]. Furthermore, there is a marked decrease in the quantity of the microsomal electron transport component, cytochrome P-450 [6–8]. Luft *et al.* [9] found that the quantity of the heme protein, cytochrome P-450, approached normal levels when ascorbic acid-deficient guinea pigs were injected with δ-aminolevulinic acid (ALA), a key substrate in heme synthesis. These authors suggested that the reduced quantity of cytochrome P-450 could be due to an impairment in heme synthesis [9, 10]. It was important, therefore, to determine the effect of ascorbic acid deficiency on the activities of the key enzymes involved in heme biosynthesis, namely ALA synthetase, ALA dehydratase and ferrochelatase.

Male albino guinea pigs (Hartley strain) weighing 180–200 g were maintained on an ascorbic acid-deficient diet or on a normal chow diet for 18–21 days as previously described [11]. Animals were sacrificed by decapitation and their livers were perfused *in situ* with ice-cold physiological saline. Livers were homogenized with a Potter–Elvehjem glass homogenizer in 0.9% NaCl and 0.01 M Tris (pH 7.4) for the determination of ALA synthetase activity, in 0.25 M sucrose and 0.01 M Tris (pH 7.4) for ALA dehydratase activity, or in 0.25 M sucrose 0.05 M Tris (pH 8.0) and 0.001 M EDTA for ferrochelatase activity.

ALA synthetase activity was determined using a modification of the radiochemical assay of Ebert *et al.* [12] with [2,3-^{14}C]succinate as the substrate. One ml of whole liver homogenate (2.5 to 5%, w/v) was added to 1 ml of a solution containing 200 mM glycine, 150 mM Tris (pH 7.4), 20 mM EDTA, 0.4 mM pyridoxal phosphate and 1 μCi [^{14}C]succinate (22.5 mCi/m-mole). Incubations were carried out at 37° for 10 min and the reaction was terminated with 0.25 ml of 50% trichloroacetic acid. The trichloroacetic acid supernatants were prepared for chromatography according to Ebert *et al.* [12] and subsequently added to columns of Dowex 50-Na^+ resin (100–200 mesh). The columns were washed with 20 ml methanol—0.05 M acetate buffer (pH 4.0)(2:1, v/v), washed with 10 ml of 0.1 M HCl, and the [^{14}C]ALA eluted with 1 M NH_4OH. The initial 2 ml of 1 M NH_4OH eluate was discarded, since it did not contain appreciable radioactivity. [^{14}C]ALA was eluted in the subsequent 3 ml of 1 M NH_4OH. Aliquots (0.5 ml) were added to 10 ml naphthalene dioxane scintillation fluid and counted. The recovery of [^{14}C]ALA from Dowex columns was monitored using 0.01 μCi [^{14}C]ALA (25.4 mCi/m-mole) and was 81 ± 3 per cent. Enzyme activity was corrected for this recovery. ALA synthetase activity was also determined in sonicated liver mitochondria [13] to eliminate possible effects on activity arising from differences in mitochondrial integrity or amounts of endogenous cofactors present. Washed mitochondria were diluted to 1 mg protein/ml and sonicated using two 5-sec bursts at 4-8 A with a model S 125 Bronson sonifier. The incubation mixture (2 ml) contained 200 μg of mitochondrial protein, 75 mM Tris (pH 7.4), 10 mM EDTA, 125 mM sucrose, 10 mM $MgCl_2$, 100 mM glycine, 0.2 mM pyridoxal phosphate, 0.1 mM GTP, 0.1 mM CoA, 0.5 unit succinyl CoA synthetase (Succinic thiokinase from the Sigma Chemical Co.) and 1 μCi [^{14}C]succinate (22.5 mCi/m-mole). Incubations were carried out at 37° for periods up to 15 min and the [^{14}C]ALA formed was isolated and counted as described above. The modifications described were necessary to obtain guinea pig ALA synthetase activity which was proportional to enzyme concentration (up to 12 mg of whole homogenate protein or 200 μg of sonicated mitochondrial protein) and linear with time for at least 15 min.

ALA dehydratase activity was determined in 15,000 *g* supernatant fractions from guinea pig livers according to the method of Gibson *et al.* [14] except that the reaction was terminated with 1.0 ml of 10% trichloroacetic acid and 0.1 M $HgCl_2$. The amount of product, porphobilinogen, was measured directly in the deproteinized supernatants with Erlich's reagent using a molar extinction coefficient of 61,000 [15]; its recovery from liver supernatant fractions was greater than 94 per cent. In other experiments, porphobilinogen was isolated by column chromatography on Dowex 1-acetate [16].

Ferrochelatase activity was determined in liver mitochondria by the method of Wagner and Tephly [17], which measures the incorporation of ^{59}Fe into heme under anaerobic conditions. Ferrochelatase activity was proportional to enzyme concentration (1.5 to 4.0 protein/ml) and linear with time for at least 40 min. The labeled heme was extracted into cyclohexanone as described by Jones [18].