응용약물학회지, The Journal of Applied Pharmacology 2, 125-130(1993)

In vitro Evaluation of AraC Prodrugs for Their Antiviral Activity

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(Received August 23, 1993; accepted September 7, 1993)

Abstract—The araC prodrugs (**1**~**5**) carrying a special acyl group at 5′-*O*- or N^4-position were evaluated for *in vitro* antiviral activity against various human viruses. When tested against HSV-1 and HSV-2 cultured in the vero cells, the prodrugs exhibited slightly higher ED_{50} values compared with one of the parent araC but showed more increased CC_{50} values in all cases. Consequently the overall selectivity indexes of prodrugs were higher than that of araC. The prodrugs, except compound **5**, exhibited very potent activity similar to that of araC (ED_{50} about 0.12 μg/ml) when evaluated against another human DNA virus, cytomegarovirus. However, theses araC prodrugs were completely inactive against RNA viruses i.e. poliovirus and coxackie B3 virus at the concentration of 250 μg/ml.

Keywords □ araC prodrugs, 5′-*O*-alkyloxyacetylaraC, N^4-alkylthioacetylaraC, antiviral activity, *Herpes simplex* virus, cytomegarovirus.

The nucleoside analogue 1-β-D-arabinofuranosylcytosine (araC) has long been known as an antitumor and antiviral agent (Reynolds, 1982). It shows inhibitory activities after phosphrylated in the cells to actual cytotoxic agent, araCTP which is a potent inhibitor of DNA polymerase.

However there are several problems associated with its use as an antitumor and antiviral agent. Such problems include its rapid catabolism to the biologically ineffective 1-β-D-arabinofuranosyluracil (araU) by the cytidine deaminases which is mainly present in the human liver and particularly its excessive cytotoxicity aganinst the fast-growing cells (Montgomery *et al.*, 1979). Up to now many approches have been attempted to overcome these difficulties by preparing various types of prodrugs such as 5′-*O*-acylaraC (Ho and Neil, 1977), N^4-acylaraC (Hori *et al.*, 1984) and phospholipidoaraC (Matsushita, *et al.*, 1982). Most of these araC analogues were prepared in the prospect of better absorption into tissues due to increased lipophilicity and hydrolytic release of the parent araC in cells. These prodrugs were mainly evaluated for their antitumor activity and the ones commonly bearing long-chain fatty acyl group exhibited potent action. It was also proved that the 5′-*O*- or N^4-derivatives of araC are protected from enzymatic deamination. But they are presently not in clinical use due to such problems as poor water-solubility, poor absorption and other unexpected side effects.

Recently similar types of prodrugs with antiviral nucleosides such as AZT and ddC were synthesized and the increasing inhibitory activity of these prodrugs on the HIV cytopathocity were reported (Aggarwal *et al.*, 1990; Kerr and Kalman, 1992).

We previously have synthesized araC prodrugs **1**~**3** (Table I) which hold a special acyl ester group at the 5′-*O*-position and reported antitumor activity of these prodrugs evaluated with in vitro tumor cell cultures (Lee *et al.*, 1986; 1988). The acyl ester group on these prodrugs had an α-electron withdrawing group ($ROCH_2CO$- or $RSCH_2CO$-) and thus was expected to be more readily hydrolyzed to the parent than the esters with ordinary acyl groups. N^4-Derivatives **4**~**5** of araC were lately prepared by forming more stable amide bond with the same type of special acyl group ($ROCH_2CON^4$ and $RSCH_2CON^4$).

All these 5′-*O*- and N^4-acyl derivatives are expected to differ in the physicochemical properties from pre-

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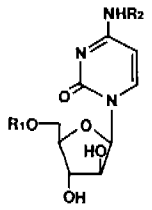
Table I. The prepared araC derivatives as prodrugs



Compound	R_1	R_2	mp (℃)	M.W.
araC	H	H		243
1 araC-MA[a)]	CH_3OCH_2C	H	155~157	315
2 araC-MTA[a)]	CH_3SCH_2C	H	188~190	331
3 araC-BTA[a)]	$CH_3CH_2CH_2CH_2SCH_2C$	H	78~80	373
4 araC-NH-MA	H	CH_3SCH_2C	156~158	315
5 araC-NH-BTA	H	$CH_3CH_2CH_2CH_2SCH_2C$	135~137	373

a) Reported (Lee *et al.*, 1984; 1988).

vious derivatives which carry a long-chain fatty acyl group: These derivatives would have different solubility character upon water and lipid, which is one of the important properties in the clinical use of a drug and would be protected from enzymatic deamination in the first-pass metabolism of liver as long as they stayed in the prodrug forms. However once they underwent into cells, the labile acyl group on these prodrugs would be more readily hydrolyzed to release the active parent araC than the long-chain acyl groups on previously reported prodrugs.

With these specific prodrugs, protected with hydrolytic-labile acyl group, of araC which is known as a broad-spectrum inhibitor on DNA synthesis, we were interested in the possibility of selective inhibitory activity of these prodrugs against the more fast replicating viruses in the living cells. The prepared araC prodrugs were evaluated for antiviral activity against various types of human viruses and the results are discussed in this report.

Materials and Methods

The whole screening was carried out with the method of Lee *et al.* (1992; Pauwels *et al.*, 1988) and here is only brief summary as follows.

Testing Chemicals

The prodrugs of araC (**1**~**3** on Table I) were synthesized from araC in this laboratory and the methods were reported previously (Lee *et al.*, 1984; 1988).

Synthesis of N^4-acyl-araC (4~5): AraC (4 mmol) and methoxyacetic acid or butylthioacetic acid (4 mmol) were dissolved in the mixed solvents of DMF (15 m*l*) and EtOH (15 m*l*). To this solution was added N-ethoxycarbonyl-2-ethoxy-1, 2-dihydro-quinoline (EEDQ, 4 mmol) and the reaction mixture was stirred at 55℃ for over-night. After removal of solvent, the corresponding product was separated by utilizing silica gel column eluted with the solvent of 10% MeOH-1% AcOH-$CHCl_3$ and crystallized from EtOH-EtOAc solvent.

N^4-Methoxyacetyl-araC: mp 156~158℃ ; R_f 0.37 (20% MeOH-1%AcOH-$CHCl_3$); UV (H_2O) λ_{max} 244, 299 nm (9800, 5700); IR (KBr) 3300 (br), 1640 cm^{-1}; NMR (DMSO-*d6*) δ3.48 (s, 3H, CH_3), 6.22 (d, J=3 Hz, 1H, $C_{1'}$-H), 7.39 (d, J=6 Hz, 1H, C_5-H), 8.29 (d, J=6 Hz, 1H, C_6 H).

N^4-Butylthioacetyl-araC: mp 135~137℃ ; R_f 0.49 (20% MeOH-1%AcOH-$CHCl_3$); UV (H_2O) λ_{max} 246, 298 nm (8800, 5900); IR (KBr) 3300 (br), 1630 cm^{-1}; NMR (DMSO-d_6) δ0.85 (t, J=6 Hz, 3H, CH_3), 1.45 (m, 4H, CH_2CH_2), 2.55 (t, J=8 Hz, 2H, SCH_2), 3.35 (s, 2H, $COCH_2S$), 6.0 (d, 5 Hz, 1H, $C_{1'}$-H), 7.13 (d, J=9 Hz, 1H, C_5-H), 8.04 (d, J=9 Hz, 1H, C_6-H).

The stock solutions of testing compounds were made

by dissolving each one in DMSO (20 mg/ml).

Hydrolytic Rates and Partition Coefficients of Prodrugs: Both experiments for N^4-amide prodrugs were carried out mainly according to the reported process (Lee *et al.*, 1988) and the final data were obtained by analyzing the UV absorption at 300 nm and collected on Table II.

Virus and Host Cell Lines

For evaluation of antiviral activity of the prepared prodrugs, different types of viruses in various strains (Table III) were used: *Herpes simplex* virus type 1 and 2 (HSV-1 and HSV-2, enveloped DNA viruses); cytomegarovirus (CMV, nonenveloped DNA virus); poliovirus type 1 and coxsackie B virus type 3 (PV-1 and CoxB-3, enteric RNA virus). All these were obtained from authentic laboratories.

Vero cells (African green monkey kidney cells) and HeLa cells (human cervix epitheloid carcinoma cells) were obtained from American Type Culture Collection (ATCC) and cultured in the Dulbecco's modified Eagle (DME) media added with 4 μg/ml gentamycin and 5% fetal bovine serum (FBS). HEL 299 cells (human embryonic lung fibrioblast cells) were also purchased from ATCC and cultured in the same DME media except addition of 10% FBS and nonessential amino acids. These cell lines were used as host cells for propagation of each virus as shown on Table III.

Evaluation of Prodrugs against HSV-1 and HSV-2

The prodrugs were evaluated for their inhibitory effect on the herpesvirus-induced cytopathogenicity (CPE). In the 96-well plate the vero cells were cultured and then infected with the amount of 100 $CCID_{50}$ (50% cell-culture inhibitory dose) of HSV-1 and HSV-2. After 1 hr-adsorption at 37℃, the media was removed and

Table II. Relative hydrolytic rates and partition coefficients of prodrugs[a)]

	incubation (hr)	hydrolyzed (%)	$PC\left(\frac{[S]\ Hexanol}{[S]\ H_2O}\right)$
araC	–	–	0.0
2	1	all	0.125
3	1	all	0.838
4	24	50	0.04
5	24	60	15.8

a) The data for compound **2**~**3** were previously reported (Lee *et al.*, 1988).

Table III. The viral strains and test conditions used for evaluating the antiviral activity of prodrugs

Virus	Strain	Host cells	Inoculum size ($CCID_{50}$/well)	Incubation time (days)	Assay
HSV-1	F	Vero	100	3	CPE/MTT
	MacIntyre	Vero	100	3	CPE/MTT
	KOS	Vero	100	3	CPE/MTT
HSV-2	G	Vero	100	3	CPE/MTT
	MS	Vero	100	3	CPE/MTT
CMV	AD-169	HEL 299	2~4	7	CPE/Giemsa
	Davis	HEL 299	2~4	7	CPE/Giemsa
PV-1	Brunhilde	HeLa	100	2	CPE/MTT
CoxB-3	Nancy	HeLa	100	2	CPE/MTT

Table IV. *In vitro* antiviral activity of araC prodrugs against *Herpes simplex* viruses

Compound	Cytotoxicity (CC_{50}: μg/ml)[a)]		Antiviral Activity									
			EC_{50} (μg/ml)[b)]					SI (CC_{50}/EC_{50})[c)]				
			HSV-1			HSV-2		HSV-1			HSV-2	
	Cytostatic	Cytocidal	F	MI	KOS	G	MS	F	MI	KOS	G	MS
araC	2.4	10	0.4	2.3	1.3	1.6	3	25	4.3	7.7	6.3	3.3
1	14.78	65.24	2.7	3.8	2.7	5.6	9	24.2	17.2	24.2	11.6	7.2
2	18.88	95.63	5.2	6.8	4.5	4.1	9.5	18.3	14.1	21.2	23.2	10.0
3	14.09	69.85	2.5	5.5	3.2	4.2	10	27.9	12.7	21.8	16.6	7.0
4	15.37	64.14	2.3	8.8	6.7	3.9	10	27.9	7.2	9.6	16.4	6.4
5	23.74	91.38	4.9	10	9.2	3.8	10	18.6	9.1	9.9	24.0	9.1

a) The 50% cytotoxic concentration, based on the inhibition of the growth (cytostatic) and viability (cytocidal) of Vero cells
b) The 50% effective concentration, based on the inhibition of the cytopathicity of HSV-1 (F, MI and KOS) and HVS-2 (G and MS) to Vero cells
c) Selectivity index, calculated as the ratio of the CC_{50} (cytocidal) to EC_{50}.

Table V. The antiviral effect of araC prodrugs on Cytomegarovirus (CMV, strain: AD-169 and Davis), Poliovirus (Brunhilde), Coxsackie B3 virus (Nancy)

Compound	EC_{50} ($\mu g/ml$)			
		CMV[a]	Polio[b]	Cox.B_3[b]
	AD-169	Davis	Brunhilde	Nancy
ara C	0.12	0.11	>250	>250
1	0.15	0.12	>250	>250
2	0.09	0.17	>250	>250
3	0.09	0.15	>250	>250
4	0.09	0.08	>250	>250
5	>10	>10	>250	>250

a) HEL 299 cells were used as host cells and the cytotoxicity of the drugs against the host cells was not tested.
b) HeLa cells were used as host cells and CC_{50} ($\mu g/ml$) of all drugs against this host cells was >250$\mu g/ml$.

the logalismic diluted drug solutions were treated twice into each well. After 3-day incubation at 37℃ in the CO_2-incubator, the number of living cells was measured with the MTT method. The antiviral effect was expressed as the concentration of each compound which increased the number of viable cells in infected cultures to 50% that of untreated controls (50% effective concentration, EC_{50}) and the results are shown on Table IV.

Evaluation of Prodrugs against CMV

The inhibitory effect of drugs on the CMV-induced cytopathogenicity was evaluated. As like above, in the 96-well plate the HEL 299 cells which stopped growing were added and the amount of 2~4 $CCID_{50}$ of CMV per well was inoculated. After virus adsorbed, the media was removed and each drug was treated twice in each corresponding well. After one-week incubation and removal of media, the cells were dyed with Giemsa solution and microscopically examined for the cytopathogenicity. The activity was determined as the concentration of each compound which decrease the number of cytopathic cells in infected cultures to 50% that of untreated controls (EC_{50}) and the results are summarized on Table V.

Evaluation of Prodrugs against PV-1 and CoxB-3

Whole procedure was the same one as in the test against HSV except that corresponding viruses were inoculated into HeLa cells and after drug treatment, the cells were cultured only for 2 days and evaluated. The results are on Table V.

Evaluation of Cytotoxicity on Vero Cells

For the evaluation of cytocidal effect of the compounds on stationary cells, 2×10^4 vero cells were put in 96 well plate and incubated for 3 days in order to form confluent monolayer. Each drug solution was added and the incubation was continued for 3 days. By utilizing MTT method, the drug concentration which made 50% of cells dead was obtained (50% cytotoxic concentraion). The test for the evaluation of cytostatic effect of drugs on growth cells was the same one as in the case for cytocidal test except that a drug was treated after 1-day incubation of vero cells in the plate. The results are shown on Table IV.

Results and Discussion

The synthetic methods for 5′-*O*-acyl derivatives **1~3** of araC on Table I were previously reported (Lee *et al.*, 1984; 1988) and the N^4-acyl amides 4~5 were prepared from the reaction of araC with a corresponding carboxylic acid in the mixed solvent of DMF-EtOH with the aid of dehydrating EEDQ and characterized as shown in the experiment. The acylation on N^4-position of araC was proved as maximum UV absorption at 245 and 299 nm which were quite distinguishable from the one, at 272 nm, of araC itself and 5′-*O*-acyl derivatives.

The initial purpose of araC modification was to protect it from metabolic deamination to inactive araU by cytidine deaminase present in the body. Therefore the relative hydrolytic rates of protecting groups on the prodrugs were examined in human plasma solution. As shown on Table III the 5′-*O*-acyl ester derivatives **2~3** were completely hydrolized to araC within 1 hr but, as expected, the N^4-acyl amides **4~5** remained 40~50% as itself after 24 hr incubation at 37℃. These results suggest that the amide derivatives *4~5* may have more increased plasma $t_{1/2}$ under the *in vivo* conditions when compared with the ones of ester analogues **2~3**. The partition coefficients of the prodrugs were determined using hexanol and water solvents and increased as shown on Table II. However, it also indicated that they, except compound **5**, are still quite water-soluble (PC (=[S] Hexanol/[S] H_2O)<1).

The prodrugs were evaluated for their activity against various human DNA and RNA viruses under the in vitro conditions shown on Table III and their antiviral effects were compared with that of the parent araC.

Initially the compounds were tested for their inhibitory effect on the cytopathogenicity of herpes simplex viruses in vero cells and the antiviral activity was determined as the concentration of each drug which increased the number of viable cells in infected cultures to 50% that of untreated controls (50% effective concentration, EC_{50}). The cytotoxicity of compounds was

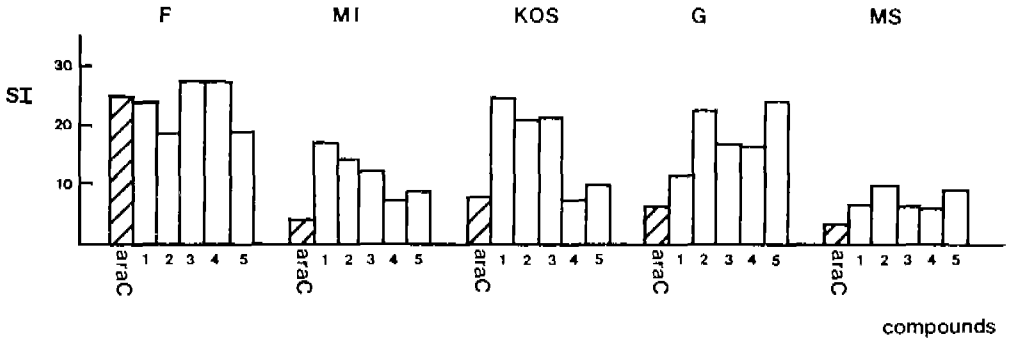


Fig. 1. The comparison of selectivity index (SI) of araC prodrugs calculated as the ratio of the toxic (cytocidal) concentraion against host cell, vero, to the effective concentration on various strains of HSV-1 and HSV-2.

also measured as 50% cytotoxic concentration (CC_{50}) based on the inhibition of the growth (cytostatic effect) or viability (cytocidal effect) of vero cells.

As shown on Table IV, all the prodrugs exhibited slightly higher ED_{50} values compared with one of the parent araC but showed more increased CC_{50} values in all cases. There was suprisingly not shown much activity difference between hydrolytic labile 5′-*O*-derivatives and more stable N^4-ones. To see the selective possibility of prodrugs as an antiherpesvirus agent, the selectivity index (SI) for prodrugs along with araC was calculated as the ratio of the toxic (cytocidal) concentration to the effective concentration(CC_{50}/EC_{50}, on Table IV). As illustrated on Fig. 1 overall SIs for prodrugs were increased compared with those of araC in almost all strains.

The prodrugs were evaluated against another herpesvirus cytomegarovirus with the method discribed in experimental section and except compound 5 exhibited very potent activity similar to that of araC (ED_{50} about 0.12 $\mu g/ml$) as shown on Table V. Finally they were tested against RNA viruses that are poliovirus and coxackie B_3 virus and almost no antiviral activity was noted at the concentration of 250 $\mu g/ml$ in all prodrugs including even the parent araC. At this concentration of each prodrugs, the growth rate of host HeLa cells was decreased about 10 to 15%.

From all these in vitro results it could be only concluded that without showing much differences among prodrugs, they were able to be hydrolyzed and converted to active araC phosphate in such cell lines as vero and HEL 299 cells (not much in HeLa cells). And then the active form might have more inhibitory effect against the fast replicating viral DNA than the one of host cells as demonstrated with increased selectivity index of prodrugs. It is noticeable that the prodrugs have the strong inhibitory potency against cytomegarovirus which is presently known as one of the most problem-pathogens in AIDS patients (Lee *et al.*, 1992).

However, a more miningful prodrug/drug comparison of biological activities could be further discussed after proper in vivo evaluation especially in these serial prodrugs which were originally designed for metabolic protection of araC known as a metabolically labile but a broad-spectrum inhibitor on various DNA synthesis.

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