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ANTIVIRAL ACTIVITIES OF PYRIMIDINE NUCLEOSIDE ANALOGUES: SOME STRUCTURE - ACTIVITY RELATIONSHIPS

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Summary. — Seventeen nucleoside derivatives (derived from arabinosylcytosine, resp. cytidine, 5-fluorouracil and uracil) were tested by agar-diffusion plaque-inhibition test for their antiviral activity with herpes simplex, vaccinia, fowl plague, Newcastle disease and western equine encephalomyelitis viruses. The highest antiviral activity against DNA viruses exhibited arabinosylcytosine, N^4-acylarabinosylcytosines, arabinosylthiouracil, cyclocytidine and its 5′-chloroderivative. RNA viruses were inhibited by 5-fluorouridine only, whereas other tested compounds were ineffective or showing marginal activity only. By search for relationship between chemical structure and antiviral activity a tendency was found of higher antiviral activity at lower lipophilicity. This is probably due to better transport of the studied compounds into cell. The chemical structure, however, is the main reason of antiviral activity.

Key words: nucleoside derivatives; antiviral activity; structure-activity-relationship

Introduction

In a recent review on structure-activity relationships of nucleoside analogues (Beránek, 1986), the inhibition of DNA and RNA synthesis in L1210 cells was compared with the activity inhibiting growth of *E. coli* (Bártová *et al.*, 1983) and replication of herpes simplex virus type 1 (HSV). Attention was paid in particular to the derivatives of 5-fluorouracil and of arabinosylcytosine (araC), used in cancer chemotherapy (Krakoff, 1984; Preisler *et al.*, 1985). 5-Fluorouridine was found the most potent selective inhibitor of RNA synthesis. 2′-Deoxy-5-fluorouridine, as the only one from 5-fluorouracil derivatives, exhibited an inhibitory effect on DNA synthesis with a simultaneous strong inhibition of RNA synthesis (Beránek and Acton, 1984; Beránek, 1986); it exhibited an inhibition of HSV. AraC exhibited strong inhibition of DNA synthesis with a considerable selectivity (Beránek and

Acton, 1984; Beránek, 1986). Cyclocytidine (cCyd) exerted a very strong inhibition of RNA synthesis in addition to the effect on DNA synthesis. Thus it stands very close to the carcinostatic antibiotics, as the only one of the studied series. The N-acylderivatives of araC also inhibited both the DNA and RNA synthesis. It was proposed that the change of the C4-amino-group to an imino group (cCyd) or the substitution of the amino group (e.g. N-acylderivatives of araC) resulted in a change in the action of the parental araC (Beránek and Acton, 1984; Beránek, 1986).

The comparative study (Beránek, 1986) of araC and its derivatives in the treatment of acute myeloblastic leukaemia and the above mentioned studies were completed by studying the rate of their deamination (Kára *et al.*, 1982), their transport and metabolic conversion in the everted rat jejunum (Farghali *et al.*, 1984; Novotný *et al.*, 1984). Morevover, the rate of phosphorylation of araC to arabinosyl 5′-triphosphate, the final active form of araC *in vivo* (Chu and Fischer, 1962; Furth and Cohen, 1968), its protection against dephosphorylation and deactivation have been followed (Novotný and Plunkett, 1987). This effort aimed to find out new derivatives of araC possessing better therapeutic index, lower rate of deamination to arabinosyluracil

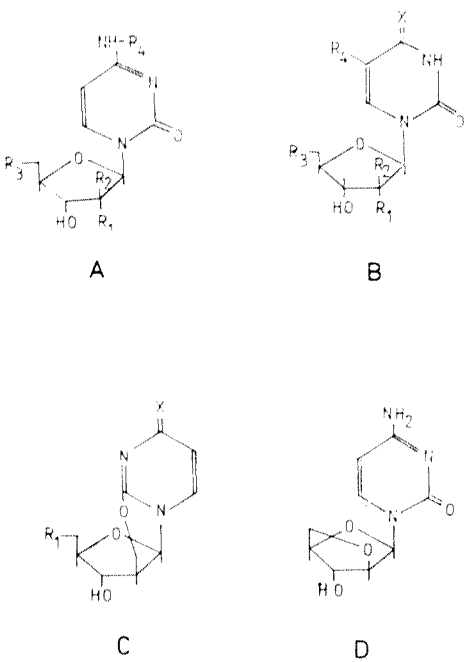

Fig. 1
Chemical structure of the tested compounds
For further data see Table I.

Table 1. Structure of the studied compounds

Structure	R_1	R_2	R_3	R_4	X	Derivative	Abbreviation	Reference
Fig. 1A	H	OH	OH	H		Arabinosylcytosine (cytarabinum)	AraC	(5)
	H	OH	Cl	H		5′-Chloroarabinosylcytosine	5′-Cl-araC	(19)
	H	OH	OH	$OCCH_3$		N-Acetylarabinosylcytosine	Ac-araC	(7)
	H	OH	OH	$OCCH_2NHCOCH_3$		N-Acetylglycylarabinosylcytosine	AcGly-araC	(17)
	H	OH	OH	$OC(CH_2)_5NHOCCH_3$		N-Acetylaminokaproylarabinosylcytosine	AcNHCao-araC	(18)
	H	OH	OH	CH_2COOH		N-Carboxymethylarabinosylcytosine	CM-araC	(18)
	OH	H	Cl	H		5′-Chlorocytidine	5′-Cl-Cyd	(19)
Fig. 1B	H	OH	OH	H	O	Arabinosyluracil	AraU	(8)
	H	OH	OH	H	S	Arabinosyl-4-thiouracil	AraSU	(8)
	OH	H	OH	F	O	5-Fluorouridine	FUrd	(6)
	H	H	OH	F	O	5-Fluoro-2′-deoxyuridine (floxuridine)	FdUrd	(9)
	OH	H	Cl	F	O	5′-Chloro-5-fluorouridine	5′-Cl-FUrd	(15)
	H	OH	Cl	F	O	5′-Chloroarabinosyl-5-fluorouracil	5′-Cl-araFU	(16)
Fig. 1C	OH				NH.HCl	O^2,2′-Cyclocytidine hydrochloride (anticabine)	cCyd	(4)
	Cl				NH.HCl	5′-Chloro-O^2,2′-cyclocytidine hydrochloride	5′-Cl-cCyd	(19)
	OH				O	O^2,2′-Cyclouridine	cUrd	(31)
Fig. 1D						2′,5′-Anhydroarabinosylcytosine	2′,5′-Anhydro-araC	(20)

(araU) (Ho and Frei, 1971), which is inactive, and particular derivatives suitable for peroral application.

There was also clearly shown that the derivatives of araC belong to the most potent antiviral analogues in the studied series (Beránek, 1986). In the present study a variety of araC derivatives was selected for determination of the antiviral activity using a spectrum of five viruses, in order to compare the derivatives inhibiting DNA synthesis with those inhibiting both DNA and RNA synthesis. Subsequently, the study was extented to derivatives of 5-fluorouracil, uracil, and cytidine altered in base, sugar or in both moieties of the molecule.

Materials and Methods

Compounds under investigation were synthesized in our laboratories according to the references indicated in Table 1. The series of studied analogues included derivatives of araC, 5-fluorouracil, uracil and the analogue 5'-chlorocytidine (Table 1).

Viruses. Herpes simplex virus (HSV), type 1, strain Kupka stock was prepared in Vero cells and stored in aliquots at −70 °C. Vaccinia virus, strain WR stock was prepared by infection of HeLa cell monolayers and stored in aliquots at −70 °C. Fowl plague virus (FPV), strain Dobson was inoculated into allantoic sacs of 10-days-old chick embryos. Twenty eight hours after inoculation the allantoic fluid was harvested and stored in aliquots at −70 °C. Newcastle disease virus (NDV), strain Hertfordshire stock was prepared by infection of the allantoic fluid of chick embryos, harvested two days after inoculation and stored in aliquots at −18 °C. Western equine encephalomyelitis (WEE) virus stock was prepared in chick embryo monolayer cells and stored in aliquots at −70 °C. Vaccinia virus, strain WR was obtained from Dr. N. P. Salzman, National Institute of Allergy and Infectious Diseases, Bethesda, U.S.A. All other viruses were obtained from the collection of the Institute of Virology, Bratislava.

Cells. Experiments with HSV were performed in mouse embryo cells. Primary cultures of mouse embryo cells were prepared from 18—20-days-old mouse embryos (SPF strain rom the breed Dobrá Voda) by a standard trypsinization procedure. Experiments with other viruses were performed in chick embryo cells. Primary cultures of chick embryo cells were prepared from 11-days-old chick embryos. Both cells were grown (usually two days) in monolayer cultures (in glass dishes of 100 mm diameter) in Eagle's basal medium containing 10% calf serum.

Plaque-inhibition test has been described earlier (Rada and Závada, 1962). Briefly, monolayers of mouse embryo cells (for HSV) or chick embryo cells in 100 mm Petri dishes were infected with a virus dose producing semiconfluent plaques. After adsorption of the virus inoculum, the monolayers were overlayed with Earle's medium containing 0.5% agarose (for HSV) or 1% agar (for other viruses). After the agarose (agar) overlay had solidified glass cylinders were placed in the centre of the overlay and solutions of substances (50 µl) were applied. A concentration gradient of the substance is formed by diffusion in agar overlay. The applied concentration is therefore approximately two logs higher than the actual inhibitory concentration. The cultures were stained with neutral red after 3 days cultivation. Zones of plaque inhibition and zones of toxicity (indicated by loss of neutral red uptake) were estimated. The diameter of the inhibitory zone is a semiquantitative measure of the efficacy of studied compound.

Determination of the apparent partition coefficient P. The amount of 20 mg of the compound was dissolved in 10 ml of the aqueous or in 10 ml of octanol phase. When the compound was dissolved in one phase, an equal volume of the other phase was added. The mixture was shaken for 30 min in stoppered flask at room temperature. The separation was achieved by centrifugation at 3500 rev/min for 10 min. The concentration of the compound was measured both in the aqueous and the octanol phase by HPLC as described previously (Farghali *et al.*, 1984). P was calculated from the formula:

$$P = \frac{c_o}{c_a}$$

where c_o is the concentration of the test compound in the octanol phase and c_a is its concentration in the aqueous phase. Each determination was repeated at least twice.

Results and Discussion

The most active compound against HSV and vaccinia virus of the series of arabinosylcytosine derivatives was araC itself. AraC did not inhibit RNA viruses (FPV, NDV, WEE). Activity of cyclocytidine against HSV and vaccinia virus was found similar to that of araC. Even when cCyd belongs to the group of substances inhibiting both RNA and DNA synthesis, in our experiments the replication of RNA viruses was not inhibited.

Several derivatives were synthesized to elucidate the role of aminogroup in the position 4 of araC. Substitution by sulphur at this position (i.e. arabinosyl-4-thiouracil) resulted in increase of toxicity for mouse embryo cells (diameter of the toxic zone = 38 mm) and in loss of inhibitory activity against HSV. On the other hand, a marked inhibition by araSU was observed against vaccinia virus; however, araU itself was not effective against vaccinia virus. Change of aminogroup in araC for oxygen resulting in araU led to marked decrease of antiviral activity against HSV and complete loss of activity against vaccinia virus. The same results were found previously by Kulikowski *et al.* (1979). Suitable substitution at the 5 position yielding 5-ethyl-araU and araT exerted antiherpetic effect (Kulikowski *et al.*, 1979). This effect was increased in (E)-5-(bromovinyl)- arabinosyluracil and 5-vinyl-arabinosyluracil (Reefschläger *et al.*, 1984) or 2′-fluoro-5-methyl-arabinosyluracil (Watanabe *et al.*, 1979; Su *et al.*, 1986). In previous experiments Reefschläger *et al.* (1983) have shown that 5-fluoro-araU exerted moderate increase of HSV inhibition in comparison with parental araU; in our experiments further substitution of 5′-hydroxyl group by chlorine in 5-fluoro-araU resulted in complete loss of antiviral effect. The same change of aminogroup for oxygen in cCyd resulting in cUrd led to the loss of activity against HSV and a marginal effect was retained against vaccinia virus (Table 2).

In order to prevent deamination of araC, a group of araC derivatives was synthesized, in which hydrogen of the aminogroup in the 4 position was substituted by acetyl-, acetylglycyl-, acetylaminokaproyl-, and carboxymethyl-. These derivatives exerted against vaccinia virus an antiviral effect similar to parental araC and against HSV a lowered one. Carboxymethyl-araC exerted the lowest efficacy against both viruses. The lowered action of this group of derivatives can be caused by delayed formation of the active form — araC by hydrolysis of the respective acylderivative.

To study the role of 5′-hydroxyl group in antiviral activity, three pairs of 5′-chloroderivatives were compared. Generally, this substitution resulted in a partial or total loss of antiviral activity. In the pair — cCyd and 5′-Cl-cCyd no or marginal decrease of activity was observed with vaccinia virus and about 20 per cent decrease with HSV. It is known that active form of cCyd is araC and similarly 5′-Cl-cCyd is converted to 5′-Cl-araC.

More expressed decrease of antiviral activity was observed in the pair — araC and 5′-Cl-araC (40—50%) with both DNA viruses. This effect may be explained by the fact that the phosphates of araC appear in case of this chloroderivative (Beránek, 1986; Fang *et al.*, 1987). Their inhibitory effect could be related to the inhibition of viral DNA polymerase. Out of

Table 2. Inhibitory activity of nucleoside analogues against herpes simplex and vaccinia viruses

Compound	HSV			Vaccinia			log P
	Concentration (mg/ml)	Zone of toxicity (mm)	Zone of inhibition (mm)	Concentration (mg/ml)	Zone of toxicity (mm)	Zone of inhibition (mm)	
AraC	50	8	85	50	0	100	−2.05
5′-Cl-araC	50	8	44	50	10	60	−0.71
Ac-araC	100	15	63	100	0−8	84	−1.35
AcGly-araC	100	8	56	100	8	100	
AcNHCao-araC	100	0	50	100	10	90	
CM-araC	100	0	13	100	10	23	
5′-Cl-Cyd	200	0	0	100	0−8	22	−1.05
AraU	100	8	37	100	0−9	0	−1.71
AraSU	100	38	0	100	14	100	
FUrd	100	0	57	100	0−9	20	−1.70
FdUrd	100	8	44	100	0	18	−1.21
5′-Cl-FUrd	100	8	0	100	0−9	0−10	−0.54
5′-Cl-araFU	100	0	0	100	0−10	0	
eCyd	50	0	80	50	0	100	−4.03
5′-Cl-eCyd	50	10	62	50	0	83−100	−3.10
eUrd	100	0−8	0	100	0−10	15	−2.85
2′,5′-Anhydro-araC	200	8	0	100	0	32	−1.64

Table 3. Inhibitory activity of nucleoside analogues against fowl plague, Newcastle disease and western equine encephalomyelitis viruses

Compound	Zone of toxicity (mm)	Zone of inhibition (mm)		
		FPV	NDV	WEE
5′-Cl-araC	0–10	0	0	17[a+]
5′-Cl-Cyd	0–8	0	0	14[a+]
AraU	0–9	0	14	0
FUrd	0–9	56	72	21[+]
5′-Cl-FUrd	0–9	0	0	15[+]
5′-Cl-cCyd	0	0	0	18[a]
cUrd	0–10	0	12	0

[a] at a concentration of 200 mg/ml (all other compounds tested at concentration of 100 mg/ml)
[+] small plaques are present
Other derivatives not given in Table 3 were without any inhibitory activity against RNA viruses.

derivatives prepared by us, 5′-Cl-cCyd belonged to the most active. Comparing the inhibitory activity of these 5′-chloroderivatives it is worthwhile to underline that the 5'-Cl-cCyd is more active than 5′-Cl-araC. This could be conditioned by the activity of the nonconverted compound itself. Both, 5′-Cl-araC and 5′-Cl-cCyd can be converted under mild alkaline conditions to 2′, 5′-anhydro-araC. However, since this 2′, 5′-anhydroderivative expressed substantially lower activity against vaccinia virus and no activity against HSV, possibility of this conversion should be excluded in our experimental conditions. The stability of this bond preventing the phosphorylation at 5′-position was determined (Hřebabecký *et al.*, 1982). Another anhydroderivative — cCyd, in which ether binding between 2′-hydroxyl and oxygen in the 2 position of araC was formed, retained the same activity against both DNA viruses as araC.

The broad-spectrum antiviral effect of 5-fluorouridine against RNA and also DNA viruses was completely lost in its 5′-chloro-congener due to the stability of nucleoside bond, i.e. between the sugar moiety and base in contrary to that in 5-FUrd (Bártová *et al.*, 1983). It is to be mentioned that the 5′-chloroderivatives (5′-Cl-araC, 5′-Cl-cCyd, 5′-Cl-5-FUrd, and 5′-Cl-Cyd) expressed some activity against WEE virus (Table 3).

Remarkable is the inhibitory activity of 5-FUrd against the whole spectrum of viruses tested. In contrast to preliminary results (Beránek, 1986), the inhibitory effect of 5-fluoro-2′-deoxyuridine was somewhat lower against HSV. The FPV, NDV, and WEE viruses were not inhibited in comparison to 5-FUrd. Thus it seems that the 2′-hydroxyl is responsible for the activity against RNA viruses.

The relation between antiviral activity and the lipophilicity has shown a linear course in the series of three araC derivatives (5′-Cl-araC, 5′-Cl-cCyd, cCyd) with the regression coefficient $r = 0.997$. If all derivatives studied (with determined log P) have been included (altogether 12), the regression

coefficient was reduced to r = 0.638. It seems that this finding supports the tendency the lower lipophilicity the higher antiviral activity. But the change in antiviral activity cannot be merely explained by the alteration in lipophilicity. A minute change in lipophilicity for N-acetyl-araC and 2′, 5′-anhydro-araC (log P = −1.35 and −1.64, respectively) was accompanied by the drop of the size of inhibitory zone with HSV from 63 mm to zero and with vaccinia virus from 84 mm to 32 mm.

The studied series of compounds was small to predict the antiviral activity from chemical structure. Nevertheless, the decrease of lipophilicity leads to the increased transport of the compound into the cell and consequently, to the increase of antiviral activity. The molecules of nucleosides possess many modification possibilities and for further experiments a series of compounds should be structurally more closely related and more numerous. Many of DNA viruses and retroviruses as well are sensitive to arabinosylnucleosides, while the majority of RNA viruses is not inhibited. The sensitivity of reverse transcriptase of retroviruses to araC seems to be a good supposition that some of our derivatives studied would be able to exert similar effect and that it would be reasonable to test the sensitivity of human immunodeficiency virus to these derivatives.

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