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In vivo phosphorylation of alloxymethyl purine and pyrimidine acyclonucleosides and the inhibitory effect of these compounds on thymidine and deoxyguanosine kinases

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High activity of dTK in human mammalian tumors [1] and cytostatic properties of pyrimidine acyclonucleotides, inhibiting the growth of experimental tumors by reducing the synthesis of dTMP and dGMP [2], were the reason for undertaking a study on the phosphorylation and inhibition of dTK and dGK activity of newly synthesized acyclonucleosides. The study comprised six alloxymethyl purine and pyrimidine acyclonucleosides obtained by Ozierow *et al.* [3] and 1*N*[(1',3'-dihydroxy)-2'-propoxymethyl]thymine synthesized by Draminski, as reported by Rutkowski & Korczak [4].

[^{14}C]dNs and [γ-^{32}P]ATP were obtained from Amersham (England). ACNs were prepared by Ozierow *et al.* [3] (Fig. 1, structures 1–6) and Draminski according to Rutkowski & Korczak [4] (Fig. 1, structure 7). All reagents used were the highest quality, commercially available products of Fluka AG, Loba Chemie (Vienna), Koch-Light Lab. and POCh (Poland). Syrian hamsters weighing 80–90 g were used for the experiments. Kirkman-Robbin's hepatoma (hepatoblastoma) was maintained as a subcutaneous transplant and transplanted at 10 days intervals. In the *in vivo* experiments, six days after heterotransplantation of Kirkman-Robbin's hepatoma, the animals were given ACN intraperitoneally at dosage of 80 mg per kg of body weight. After 48 hours the animals were sacrificed; the tumors were separated from the surrounding tissues, homogenized as described earlier [2] and the enzyme activities were assayed in cytosol obtained from the homogenate [2]. In the *in vitro* experiments, ACNs were incubated with cytosol obtained from the homogenate of six days growing tumors (as described above). Activities of dTK and dGK were assayed radiochemically under optimal conditions and under linear kinetics [2].

ACNs phosphorylations were performed in the same conditions as for the dTK activity determination, using [γ-^{32}P]ATP as a phosphate donor (10 mmol, 0.2 Ci per sample), and phosphorylation products were isolated by descending paper chromatography (Whatman 1) at room temperature in *n*-propanol:28% ammonia water:water (22:17:1) with R_F of ACN, ACN phosphate and ATP of 0.75, 0.38 and 0.2, respectively. The HeLa cells culture was grown in Eagle's medium enriched with bovine fetal serum containing gentamycin and Hepes. The growth of culture was estimated by calculating the number of cells before and after the addition of ACNs.

Among the *in vitro* tested ACNs, only AMT inhibits the dTK activity (Table 1A). The *in vivo* synthesis of dTMP and dGMP is reduced by AMT and Th5 (Table 1B). Similar effects are

[1] Abbreviations: ACN, acyclonucleoside; dN, 2'-deoxynucleoside; dTK, deoxythymidine kinase; dGK, deoxyguanosine kinase; dAK, deoxyadenosine kinase; dCK, deoxycytidine kinase.

Table 1

Activities of dTK and dGK (IU × 10^{-5} per mg of protein) in cytosol of six days growing hepatoma.

A: *In vitro* in the presence of 0.2 mmol of ACNs (for details see above). Each value = mean ± SEM for four experiments. The values of *P* were calculated using Student's *t* test. NS, mean non significant; control, dTK and dGK activity in cytosol of 6–8 days old hepatoma.

ACN	dTK		dGK		% of inhibition	
	Control	In the presence of ACN	Control	In the presence of ACN	dTK	dGK
A						
AMU	92.0 ± 12.0	89.0 ± 12.1	31.0 ± 2.7	30.3 ± 3.0	3	2
AMT	91.0 ± 11.6	51.2 ± 6.1	34.2 ± 3.3	30.8 ± 2.2	43.8 (*P*=0.05)	10
AMFU	84.0 ± 10.0	92.0 ± 13.0	29.4 ± 2.3	32.1 ± 3.3	0	0
AMC	85.6 ± 10.4	93.0 ± 13.6	35.8 ± 4.1	31.3 ± 2.8	0	12.6 (NS)
AMA	96.0 ± 15.0	87.3 ± 11.6	28.6 ± 2.0	34.2 ± 3.8	9	0
AMG	94.7 ± 14.0	86.2 ± 11.2	27.9 ± 1.8	34.2 ± 4.0	9	0
Th5	93.0 ± 14.1	74.6 ± 9.2	30.8 ± 2.4	27.4 ± 2.1	19.8 (NS)	11 (NS)
B						
AMU	90.6 ± 11.2	57.9 ± 8.6	33.5 ± 3.2	22.5 ± 1.6	36 (NS)	33 (NS)
AMT	91.8 ± 12.6	37.5 ± 5.2	31.3 ± 3.0	14.6 ± 1.2	59.2 (*P*=0.01)	53.4 (*P*=0.005)
AMFU	87.4 ± 10.2	90.2 ± 12.1	34.6 ± 4.1	30.0 ± 2.6	0	13.3 (NS)
AMC	97.2 ± 15.1	90.3 ± 12.1	32.2 ± 3.0	35.4 ± 4.6	7	0
AMA	92.0 ± 13.0	89.4 ± 11.4	27.4 ± 2.1	33.5 ± 3.8	2.4	0
AMG	93.2 ± 13.1	84.3 ± 10.0	31.0 ± 3.2	34.8 ± 4.3	9.6	0
Th5	92.0 ± 13.6	38.6 ± 5.5	31.7 ± 3.0	14.2 ± 1.1	58 (*P*=0.02)	55.2 (*P*=0.002)

exhibited by the same compounds at the concentration of 1 μM/cm^3 of medium (i.e.: 200 μg of AMT and 230 μg of Th5) for the growth of HeLa cells of 54% and 56% ($P = 0.01$), respectively.

Since the biological activity of ACNs results from their *in vivo* phosphorylation [5] it should be assumed that alloxymethyl residue of AMT is subject to hydratation and that primary OH group formed similarly as the analogous OH group in Th5 is then phosphorylated by the kinases present in the growing tumor cytosol. Consequently, it should be expected that ACNs which do not reveal the biological activity are not hydrated and/or phosphorylated by the above mentioned enzymes. These suggestions are confirmed by the results presented in Table 2, since only AMT and Th5 are phosphorylated by the enzymes present in the growing hepatoma cytosol. The fact that AMT phosphorylation is catalysed with a 3-fold lower activity, as compared to Th5 phosphorylation (Table 2), evidences that AMT alloxymethyl residue hydratation may be the rate limiting reaction of AMT phosphorylation. The enzymatic hydratation of unsaturated binding of the ribosyl residue of *S*-adenosyl-L-homocysteine inhibitors was demonstrated by Jarvi *et al.* [6]. The

Table 2

The phosphorylation of ACN by kinases present in cytosol of six days growing hepatoma (IU × 10^{-5} per mg of protein).

Each datum: mean ± SEM for four experiments.

Substrate	[^{32}P] phosphate formed
AMU	0.12 ± 0.02
AMT	19.6 ± 2.5
AMFU	0.0
AMC	0.0
AMA	0.0
AMG	0.0
Th5	51.0 ± 7.2

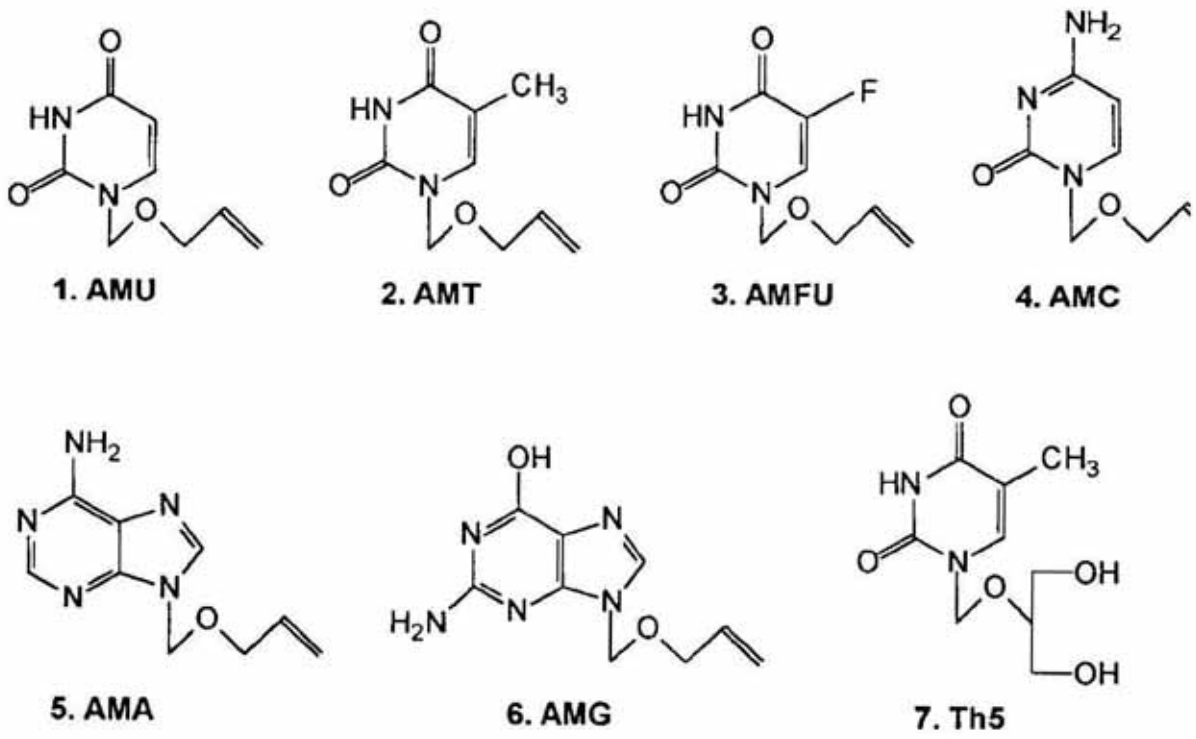

Fig. 1. Structure of the acyclonucleotides investigated.
Codes employed: AMU, 1*N*-alloxymethyluracil; AMT, 1*N*-allyloxymethylthymine; AMFU, 1*N*-alloxymethyl-5-fluorouracil; AMC, 1*N*-allyloxymethylcytosine; AMA, 1*N*-allyloxymethyladenine; AMG, 1*N*-allyloxymethylguanine; Th5, 1*N*[(1',3'-dihydroxy)-2'–propoxymethyl]thymine.

lack of this reaction *in vivo* in case of 1-(ethoxymethyl)-6-(phenylselenenyl) pyrimidines acyclonucleosides inhibiting the reverse transcriptase HIV-1 was revealed by Goudgaon *et al.* [7] who have also found these acyclonucleosides not to be phosphorylated *in vivo*. It confirms the suggestions presented in our paper that hydratation of the alloxymethyl residue in AMT in *in vivo* conditions precedes the phosphorylation of this compound. Hydratase present in the hepatoma cells catalyses the hydratation of the alloxymethyl residue only when this residue is thymine-bound (Tables 1B, 2).

It cannot be excluded that the lack of AMC and AMA biological activity results from 20–80-fold lower activity of dCK and dAK present in the growing hepatoma cytosol [2]. It is not clear, however, why AMU, a potential dTK substrate, and AMG, exhibiting similar properties against dGK, are not phosphorylated *in vivo* by appropriate tumor kinases.

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