Further Studies on Cytostatic Activity of Alkoxymethyl Purine and Pyrimidine Acyclonucleosides

Hanna Modrzejewska[a], Marcin Dramiński[b], Anna Zgit-Wróblewska[c] and Janusz Greger[a,*]

[a] Department of Biochemistry, Institute of Physiology and Biochemistry, Medical University of Łódź, Lindleya 6, 90–131 Łódź, Poland.
Fax: 048426782465. E-mail: tpietruc @ psk 2. am. lodz.pl

[b] Department of General Chemistry, the Ludwik Rydgier Medical University of Bydgoszcz, Karłowicza 24, 85–092 Bydgoszcz, Poland

[c] Department of General Chemistry, Institute of Basic Sciences, Military School of Medicine, pl.Hallera 1, 90–647 Łódź, Poland

* Author for correspondence and reprint requests

Z. Naturforsch. **54c,** 923–931 (1999); received March 3/June 25, 1999

Acyclonucleosides, Deoxynucleotide Kinases, Melanoma

The influence of 14 acyclonucleosides[1], derivatives of adenine, guanine, uracil and thymine on the phosphorylation of dAdo, dGuo, dCyd and dThd occurring in the cytosol of growing amelanotic melanoma transplanted to Syrian hamsters, as well as on inhibition of tumor growth were studied. From among the studied ACNs eight were tested earlier (Modrzejewska *et al.*, 1996, The influence of alkoxymethyl purine and pyrimidine acyclonucleosides on growth inhibition of Kirkman-Robbins hepatoma and possible mechanism of their cytostatic activity, Z. Naturforch. **51c**, 75–80); from among the newly synthesized ACNs, 1,3-*N,N*-diallyloxymethylthymine (AMT2), 1-*N*-allyloxymethyl-5,6-tetramethyleneuracil (AMUTM), and tested previously 1-*N*-allyloxymethylthymine (AMT1), administered i.p. in a dose of 0.2 mmol /kg body weight reduce the tumor mass from 0.98 g to 0.64 g ± 0.11 g (i.e. 35% ± 12%). 48 hours after i.p. administration of the mentioned ACNs in the same dose a reduction of tumor mass is accompanied by the inhibition of dAMP, dGMP and dTMP synthesis. AMT1 inhibits dThd phosphorylation from 6.2 to 4.22; AMT2 suppresses dAdo, dGuo and dThd phosphorylation by, correspondingly, from 2.8 to 1.7, from 10.8 to 7.5 and from 6.2 to 4.2; AMUTM depresses dAMP synthesis from 2.8 to 1.6 (all data: μmol of 2'dNMP formed per mg of protein per min. × 10^{-4}). None of the 14 studied acyclonucleosides influences dCMP synthesis. *In vivo*, after hydration of allyloxymethyl group to hydroxypropoxymethyl residue (having -CH_2OH group), AMT1, AMT2 and AMUTM undergo phosphorylation to corresponding triphosphates. Phosphorylated ACNs are not incorporated into tumor DNA, however they inhibit dAdo, dGuo and dThd incorporation into DNA. It is concluded that ACN triphosphates are not substrates for DNA polymerase but, competing with dATP dGTP and dTTP, inhibit incorporation of these 2'dNTP into DNA and, in consequence, reduce tumor growth, which is presumed to be the main mechanism of cytostatic activity of the studied ACNs.

[1] *Abbreviations:* ACN, acyclonucleoside; dAdo, 2'-deoxyadenosine; dGuo, 2'-deoxyguanosine; dCyd, 2'-deoxycytidine; dThd, 2'-deoxythymidine; 2'dN, 2'-deoxynucleoside; 2'dNMP, 2'-deoxynucleoside-5'-monophosphate; 2'dNTP, 2'-deoxynucleoside-5'-triphosphate; AMT1, 1-*N*-allyloxymethylthymine; AMT2, 1,3-*N,N*-diallyloxymethylthymine; DHPMT1, 1-*N*-[(2',3'-dihydroxy-1'-propoxy)methyl-]thymine; DHPMT2, 1-*N*-[(1',3'-dihydroxy-2'-propoxy)methyl]thymine; PMT, 1-*N*-propoxymethylthymine; AMU, 1-*N*-allyloxymethyluracil; DHPMU, 1-*N*-[(2',3'-dihydroxy-1'-propoxy)methyl]uracil; AMUTM, 1-*N*-allyloxymethyl-5,6-tetramethyleneuracil; DHPMUTM, 1-*N*-[(2',3'-dihydroxy-1'-propoxy)methyl]-5,6-tetramethyleneuracil; DHPMUDM, 1-*N*-[(2',3'-dihydroxy-1'-propoxy)methyl]-5,6-dekamethyleneuracil; AMUF, 1-*N*-allyloxymethyl-5-fluorouracil; AMA, 9-*N*-allyloxymethyladenine; AMG, 9-*N*-allyloxymethylguanine.

Introduction

Results presented in this paper, being part of the current studies on acyclonucleosides (ElAshry and ElKiliany, 1997; Thormar *et al.*, 1998), resume our research on ACNs possessing potential cytostatic properties (Greger and Dramiński, 1989; Modrzejewska *et al.*, 1994; Modrzejewska *et al.*, 1996). The present results deal with eight previously tested and six newly synthesized alkoxymethylpyrimidine ACNs, derivatives of uracil and thymine, subjected to a study on a different biological model. ACNs tested previously (Fig. 1, compounds I,IV,V,VI,VII,XII,XIII,XIV) were in-

0939–5075/99/1100–0923 $ 06.00

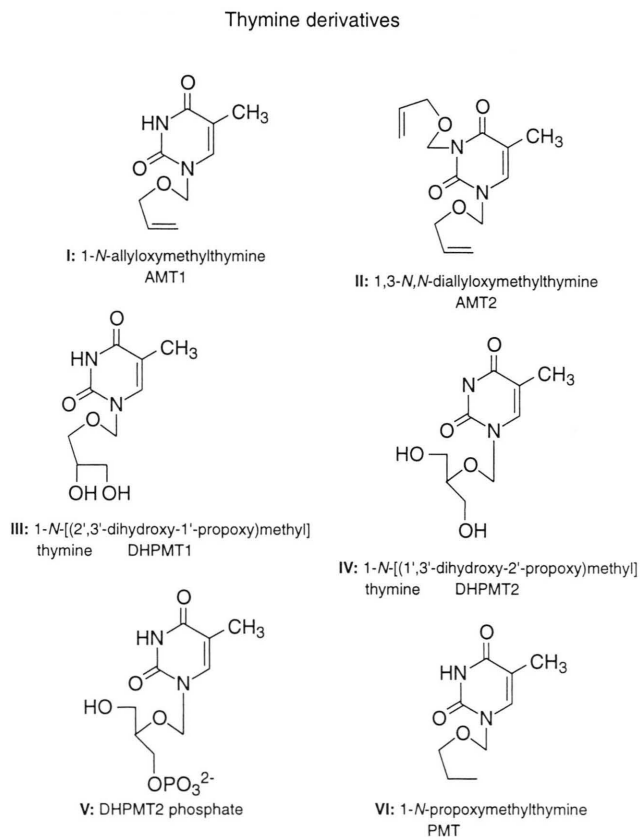
vestigated on the growing Kirkman-Robbins hepatoma. Now both groups of ACNs (Fig. 1, previously tested and newly synthesized ACNs, compounds: II,III,VIII,IX,X,XI) were subjected to tests on growing amelanotic melanoma transplanted , similarly as hepatoma, to Syrian hamsters.

A mechanism of cytostatic activity of allyloxymethyl derivatives of uracil and thymine has been proposed based on the results obtained in *in vivo* and *in vitro* experiments concerning 2'dN kinases activities present in the cytosol of a growing tumor subjected to biologically active ACNs (AMT1,AMT2, AMUTM), on phosphorylation of these ACNs and on incorporation of 2'dN into tumor DNA.

Materials and Methods

Chemicals

[^{14}C]dAdo,[^{14}C]dThd,[^{32}P]γ ATP were obtained from Amersham Corp. (Buckinghamshire, England); [^{14}C]dCyd from Sigma Chemical Company (St. Louis MO, USA) and [^{14}C]dGuo from Ustav pro Vyzkum Vyrobu a Vyuziti Radioactive (Prague, Czechoslovakia). Unlabeled 2'dNs, 2'dNMPs, and 2'dNTPs were purchased from Sigma Chemical Company (St. Louis, USA), Aldrich Chemical Company (Milwaukee, USA) and Boehringer (Mannheim, Germany). Other reagents used were highest quality commercially available products from Fluka AG, Loba-Chemie (Vienna, Austria), Koch-Light Lab. and Polskie Odczynniki Chemiczne (Gliwice, Poland). Allyloxymethyl purine and pyrimidine ACNs were obtained as described previously (Modrzejewska *et al.*, 1996; comp.: I,IV, V, VI, VII, XII, XIII, XIV; Fig. 1). Compounds III, VIII, IX, X ,XI (Fig. 1) were produced by oxidation of the appropriate 1-*N*-allyloxymethyl derivatives of heterocyclic bases. The oxidation agent was potassium chloride in the presence of osmium tetroxide. AMT2 (Fig. 1, comp. II) was obtained by condensation of 1,3-O(trimethylsilyl)thymine with excess of chloromethylacetyl ether. Purification was performed ac-



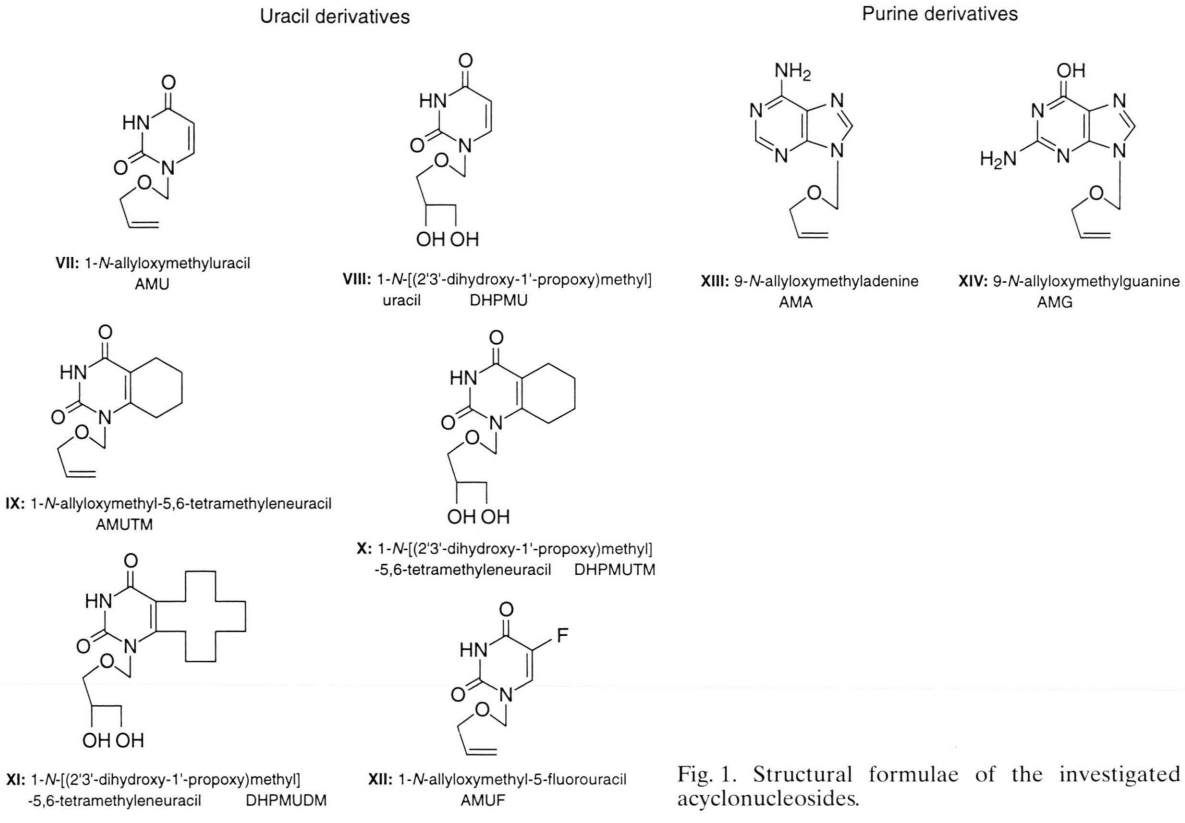


Fig. 1. Structural formulae of the investigated acyclonucleosides.

cording to a procedure of Dramiński and Frass, 1981. The ACNs were checked for chromatographic homogeneity with at least two solvent systems. Analytical data are given in Table I. [^{14}C]AMT1, [^{14}C]AMT2 and [^{14}C]AMUTM were obtained by the same methods as unlabeled ACNs using[^{14}C] thymine or [^{14}C] uracil instead of unlabeled thymine or uracil (Ozierov *et al.*, 1991; Drabikowska *et al.*, 1987). 1 cm zones were scraped from the TLC silica gel plates (Merck Kiselgel 60 F, 0.2 mm) and counted by a liquid scintillation counter (1600 TR Packard liquid scintillation analyzer). The ACN spots contained [^{14}C] label only.The rest of the chromatograms had background level activity.

Animals and preparation of subcellular fractions

A group of four female Syrian hamsters (weighing 80–90 g) was used for each experiment. Amelanotic melanoma, obtained from Department of Histology, Medical University of Gdańsk, belongs to a family of melanomas transplantable to hamsters (Słomiński and Paus, 1993; Bomirski *et al.*, 1998) and represents one of the fast growing tumors. The tumor was maintained as a subcutaneous transplant at 10 days' intervals. The most intensive growth of tumor occurred at 10–12 days after transplantation. From the 16–18 day regressive changes took place and within one week the tumor was eliminated. For the *in vivo* experiments, 10 days after tumor transplantation the animals were given i.p. respective ACNs (0.2 mmol per kg of body weight), 48 h later they were sacrificed by sectioning the cervical spinal chord, tumor was excised, homogenized at 0 °C in Potter-Elvehjem apparatus and nuclear and cytosol fractions obtained as described previously (Modrzejewska *et al.*, 1996) were used for the experiments. In *in vitro* experiments, 10 days after tumor transplantation the animals were sacrificed and the cytosol obtained as above was assayed for the enzyme activity.

Chemical and enzyme assays

2'dN kinase activities were assayed as described previously (Greger and Draminski, 1989). The phosphorylation of ACNs was performed under conditions matching all 2'dN kinases activities, using 0.2 mM ACN as a substrate and 10 mM[^{32}P] γ ATP of 0.5 μCi(0.0185 MBq) per sample as phosphate donor. Phosphorylation products were separated by TLC (Modrzejewska *et al.*, 1996) and spots corresponding to standards were counted as above (see Chemicals). DNA was isolated and [^{14}C]2'dN or [^{14}C] ACN were assayed according to methods described by Blin and Stafford (1976), and protein by Bradford (1976). 2'dN kinase activities and ACN phosphorylation products were expressed as μmol of 2'dNMP or ACN phosphates formed per mg of protein per min. $\times 10^{-4}$. Statistical calculations were made using Student's, Fisher-Snedecor tests and a Chem Windows 3 statistical computer program.

Results and Discussion

The influence of 14 ACNs on the growth of amelanotic melanoma and on 2'dN kinase activities present in the cytosol of the growing tumor were investigated. AMT1, AMT2 and AMUTM administered i.p. to animals 10 days after tumor transplantation in a dose of 0.2 mmol per kg of body weight reduced the tumor weight from 0.98 g to 0.64 g ± 0.11 g, i.e. 35±12% (mean ± SD for four separate experiments). Table II demonstrates the influence of AMT1, AMT2 and AMUTM on the activity of these enzymes. The above mentioned ACNs inhibit the synthesis of dTMP (AMT1, AMT2), dGMP(AMT2) and dAMP (AMT2, AMUTM) both *in vivo* and *in vitro*. This inhibition (expressed in μmol of 2'dNMP formed per mg. of protein per min. $\times 10^{-4}$) was found from 5.4 to 4.0 *in vitro* and from 6.2 to 4.2 *in vivo*, i.e. 26–34% for dThd kinase, from 11.2 to 8.1 *in vitro* and from 10.8 to 7.5 *in vivo*, i.e. 28–31% for dGuo kinase and from 2.3 to 1.5 *in vitro* and from 2.8 to 1.6.*in vivo*, i.e. 35–44% for dAdo kinase. None of the 14 mentioned ACNs influences the synthesis of dCMP. No significant differences have been found in results of experiments *in vivo* (average inhibition of 2'dN kinases activities in experiments *in vivo* was 35.8±5.1%, in experiments *in vitro:* 33.0±7.1%), whereas the K_i determined in experiments *in vitro* (Fig. 2) varied considerably (from 76 μM for AMUTM inhibiting the synthesis of dAMP to 227 μM for AMT2 inhibiting the synthesis of dGMP, see Table II). Attention should be drawn to the fact that ACNs which were the *in vivo* inhibitors of dGMP and dTMP synthesis in

Table I. Analytical data of newly synthesized ACN's.

Compound	Yield [%]	m.p.[°C]	TLC R_f S_1	TLC R_f S_2	UV λ_{max} [nm] $\epsilon \times 10^3$ pH=7 pH=13		MS (70ev) *m/z* [%] M	B	B+1	B+14	B-29	B+28	^{1}H-NMR ($CHCl_3$) 1-N-**CH_2**	1-N-**CH_2**
AMT2	28	oil	0.62	0.59	270	270	266	125	126	130	96	153	5.14	5.42 s
					7.6	5.8	1.3	1.1	2.9	11.1	28.6	1.4		
AMUTM	68	131–132	0.83	0.99	267	268	236	165	166	179	136			
					11.5	9.1	9.5	23.4	6.5	46.5	23.7		5.33 s	
DHPMT1	47	138–9	0.35	0.63	264	264	230	125	126	139	96		5.20 d	
					8.2	6.6	2.0	2.0	100	38.4	55.2			
DHPMU	60	132–4	0.26	0.46	259	259	216	111	112	125	82		5.22 d	
					10.2	9.4	0.8	1.3	51.9	57.2	100			
DHPMUDM	56	168–9	0.73	0.88	271	269	354	249	250	263	220		5.38 s	
					9.3	7.6	5.4	4.2	100	20.5	6.9			
DHPMUTM	65	146	0.48	0.78	268	269	270	165	166	179	136		5.35 s	
					12.5	11.0	3.3	18.9	100	10.5	13.9			

TLC: silica gel GF_{254}
S_1 – BuOH:H_2O = 86:14
S_2 – $CHCl_3$: EtOH = 7:3

Abbreviated compounds: AMT2, 1,3-*N,N*-diallyloxymethylthymine; AMUTM, 1-*N*-allyloxymethyl-5,6-tetramethyleneuracil; DHPMU, 1-*N*-[(2',3'-dihydroxy-1'-propoxy)methyl]uracil; DHPMT1, 1-*N*-[(2',3'-dihydroxy-1'-propoxy)-methyl]thymine; DHPMUDM, 1-*N*-[(2',3'-dixydroxy-1'-propoxy)methyl]-5,6-dekamethyleneuracil; DHPMUTM, 1-*N*-[(2',3'-dihydroxy-1'-propoxy)methyl]-5,6-tetramethyleneuracil.

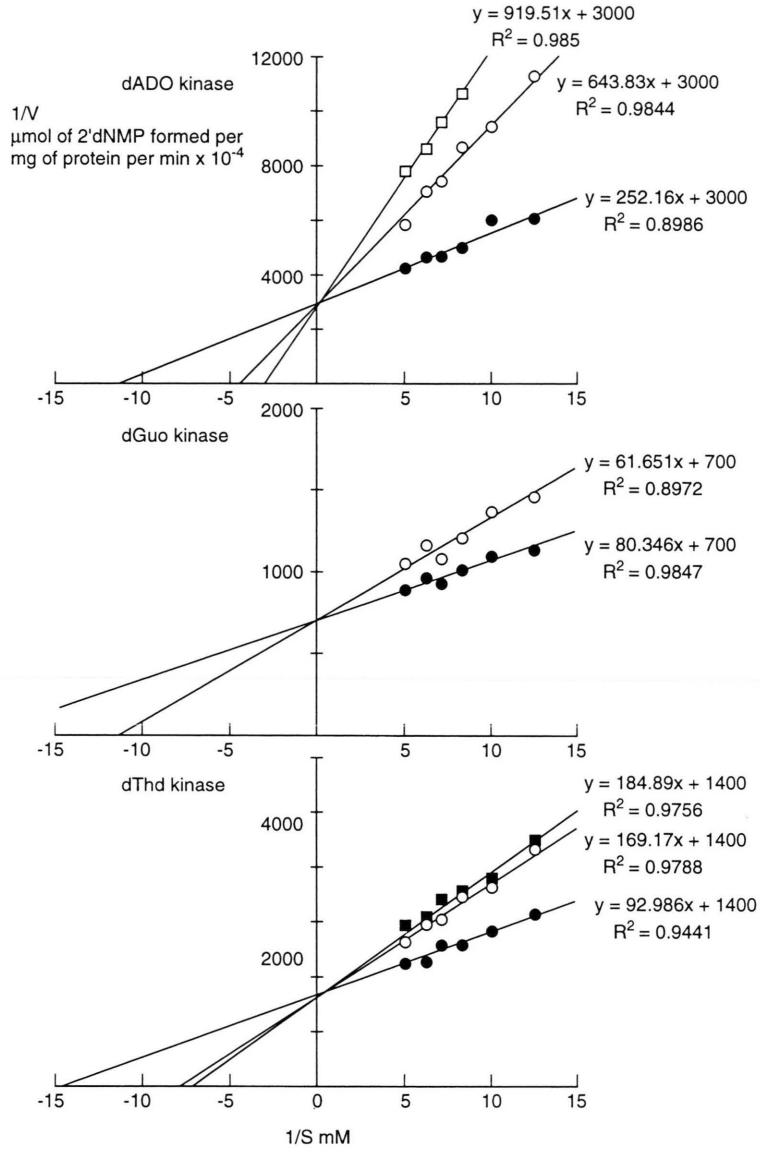


Fig. 2. Double reciprocal plots of the rate of dAdo, dGuo and dThd kinase activities in the cytosol of a 10-day growing melanoma (–•–•–•) in the presence of 0.2 mM AMT1 (–■–■–■–), AMT2 (–○–○–○–) and AMUTM (–□–□–□–). K_m calculated from these plots equal 84 µM for dAdo kinase, 50 µM for dGuo kinase and 66 µM for dThd kinase. K_i values presented in Table II calculated from K_{mi} values were obtained from these plots. y – regression equation; $R^2 > 0.5$ indicates that points belonging to the straight line are in the set of this line data (according to Chem.Windows 3 statistical computer program). K_{mi}: K_m constant in the presence of inhibitor.

Kirkman-Robbins hepatoma (AMU, DHPMT2, Modrzejewska *et al.*, 1996), were entirely inactive in case of melanoma, while AMT2, being an AMT1 derivative, inhibited the phosphorylation of as many as three 2'dNs (dAdo, dGuo, dThd) in melanoma.

Kinetics of 2'dN kinases inhibition (Fig. 2, double-reciprocal plots of dAdo, dGuo and dThd kinase activities in presence of AMT1, AMT2 and AMUTM) indicates that the mentioned ACNs are competitive inhibitors of dAMP, dGMP and dTMP synthesis. As it will be demonstrated later,

Table II. The influence of biologically active ACN's (AMT1, AMT2, AMUTM) on 2'dN kinase activities (μmol of 2'dNMP formed per mg of protein per min. $\times 10^{-4}$) in cytosol of growing melanoma. *In vitro* – 10 days after tumor transplantation in presence of respective ACN's (0,2 mM); *in vivo* – 10 days after tumor transplantation the animals were given ACN's (0.2 mmol per kg of body weight) and 48 h later the enzyme activities were assayed. Control: 2' dN kinase activities in cytosol of 10 (experiments *in vitro*) or 12 days (experiments *in vivo*) old melanoma. Each value: the mean ± SD for four separate experiments. NS-mean non significant (i.e. $p < 0.05$); p values, after checking variance conformability using Fisher-Snedecor test, were calculated using Student's t-test. In parentheses: % of inhibition. The remaining ACN's presented in Fig. 1 have no influence on 2'dNMP synthesis in melanoma cells.

	dAdo kinase	dGuo kinase	dCyd kinase	dThd kinase
in vitro				
Control	2.3±0.25	11.2±0.93	0.51±0.06	5.4±0.51
AMT1	2.2±0.22	9.4±0.80 (12%, NS)	0.48±0.05	4.0±0.31 (26%, p=0.005, K_i=188 μM)
AMT2	1.5±0.13 (35%, p=0.002, K_i=126 μM)	8.1±0.7 (28%, p=0.002, K_i=227 μM)	0.44±0.0.05 (14%, NS)	3.7±0.25 (32%, p=0.002, K_i=212 μM)
AMUTM	1.3±0.12 (44%, p=0.001, K_i=76 μM)	11.4±0.092	0.49±0.06	5.2±0.52
in vivo				
Control	2.8±0.38	10.8±0.86	0.46±0.05	6.2±0.62
AMT1	3.0±0.3	10.4±0.82	0.40±0.04 (14%, NS)	4.22±0.41 (32%, p=0.001)
AMT2	1.7±0.12 (39%, p=0.001)	7.5±0.71 (31%, p=0.002)	0.48±0.07	4.2±0.39 (34%, p=0.001)
AMUTM	1.6±0.11 (43%, p=0.001)	11.3±0.9	0.39±0.04 (16%, NS)	6.1±0.6

For abbreviations see Fig. 1.

the inhibitors of synthesis of the mentioned 2'd NMP are not AMT1, AMT2 and AMUTM but their triphosphates which are formed by ACN phosphorylation catalyzed by kinases present in the cytosol of the growing tumor. Table III demonstrates the phosphorylation of ACNs by kinases present in the cytosol of melanoma growing for 10 days. A 40 min. incubation with cytosol (i.e. during 2'dN incubation time sufficient to determine 2'dN kinases) indicates that besides AMA and AMG all the remaining 12 ACNs are phosphorylated to monophosphates; phosphorylation of AMU, AMUF, DHPMT2 and DHPMT2 phosphate is completed at the diphosphate stage, and only the biologically active ACNs (i.e. AMT1, AMT2 and AMUTM) are phosphorylated to triphosphates. Studying the kinetics of AMT1, AMT2 and AMUTM triphosphate formation we assume that the concentration of these phosphorylated derivatives increases until the 40th min. of incubation, then it does not change for the following 30–40 min. To confirm the fact that it is not AMT1, AMT2 and AMUTM but their triphosphates, which are the active species for inhibition of dAMP, dGMP and dTMP synthesis, an 80 min. incubation time was applied in experiments to study the *in vitro* inhibition of 2'dNMP synthesis.

As it has been mentioned earlier (Modrzejewska *et al.*, 1996) phosphorylation of the allyloxymethyl group in AMT1, AMT2 and AMUTM must be preceded by an enzymatic hydration of unsaturated bonds *in vivo*. Hydration of such bonds described by Jarvi *et al.* (1991) transforms the allyloxymethyl group into a 1-hydroxypropoxymethyl residue ($-CH_2-O-CH_2-CH_2-CH_2OH$) or 2-hydroxypropoxymethyl residue ($-CH_2-O-CH_2-CHOH-CH_3$). Hydratases present in hepatoma (Modrzejewska *et al.*, 1996) prefer the allyloxymethyl group bound to pyrimidines (thymine, uracil). This is similar to melanoma where neither AMA nor AMG underwent phosphorylation (Table III). It should be expected that phosphorylation of the appropriate ACNs relates to a $-CH_2OH$ group and not a -CHOH group as cellular kinases responsible for ACN phosphorylation prefer first order alcoholic groups (Bouffard *et al.*, 1993; Salomon *et al.*, 1995). It is suggested that the effect of melanoma hydratases on allyloxymethylopyrimidines leads to the formation of $-CH_2-O-CH_2-CH_2OH$ groups in these ACNs.

Table III. The phosphorylation of ACN's (0.2 mM) during a 40-min incubation by kinases present in the cytosol of a 10-day growing melanoma (μmol of [^{32}P] ACN mono, di and triphosphate formed per mg of protein per min. × 10^{-4}). In parentheses: % of phosphorylation.

ACN	[^{32}P] ACN monophosphate	[^{32}P] ACN diphosphate	[^{32}P] ACN triphosphate
		Thymine derivatives	
AMT1	1.4±0.2	0.11+0.02 (6.8%)	0.088±0.011 (5.5%)
AMT2	1.2±0.16	0.1+0.05 (7.2%)	0.09±0.013 (6.8%)
DHPMT1	2.3±0.3	0.0	0.0
DHPMT2	3.6±0.4	0.87±0.13 (19.5%)	0.0
DHPMT2 phosphate	0.0	3.2±0.4 (99.5%)	0.017±0.002 (0.5%)
PMT	0.0	0.0	0.0
		Uracil derivatives	
AMU	0.95±0.12	0.88±0.1 (48%)	0.005±0.0007 (0.3%)
DHPMU	0.3±0.16	0.1±0.012 (7.2%)	0.0
AMUTM	1.6±0.2	0.14±0.018 (7.7%)	0.12±0.015 (6.6%)
DHPMUTM	1.2±0.15	0.09±0.013 (7.5%)	0.0
AMUF	0.9±0.13	0.04±0.006 (4.3%)	
DHPMUDM	0.0	0.0	0.0
		Purine derivatives	
AMA	0.0	0.0	0.0
AMG	0.0	0.0	0.0

Abbreviated compounds: AMT1, 1-*N*-allyloxymethylthymine; AMT2, 1,3-*N,N*-diallyloxymethylthymine; DHPMT1, 1-*N*-[(2',3'-dihydroxy-1'-propoxy)methyl]thymine; DHPMT2, 1-*N*-[(1',3'-dihydroxy-2'-propoxy)methyl]thymine; PMT, 1-*N*-propoxymethylthymine; AMU, 1-*N*-allyloxymethyluracil; DHPMU, 1-*N*-[(2',3'-dihydroxy-1'-propoxy)-methyl]uracil; AMUTM, 1-*N*-allyloxymethyl-5,6-tetramethyleneuracil; DHPMUTM, 1-*N*-[(2',3'-dihydroxy-1'-propoxy)methyl]-5,6-tetramethyleneuracil; DHPMUDM, 1-*N*-[(2',3'-dihydroxy-1'-propoxy)methyl]-5,6-dekamethyleneuracil; AMUF, 1-*N*-allyloxymethyl-5-fluorouracil; AMA, 9-*N*-allyloxymethyladenine; AMG, 9-*N*-allyloxymethylguanine.

A common feature of ACNs which are not phosphorylated to triphosphates *in vivo* is the presence of a chiral carbon atom in the synthon (Compounds: III,IV, V, VIII, X, XI – Table III)). Lack of PMT phosphorylation follows from the fact that the hydroxypropoxymethyl group constituting the synthon in PMT does not undergo hydration (Modrzejewska *et al.*, 1996). Lack of AMU, DHPMT2 and DHPMT2 phosphate triphosphates (and in consequence lack of their biological activity) results from a high concentration of these ACNs diphosphates (Table III) which are inhibitors of triphosphates synthesis *in vivo* (Krenitsky *et al.*, 1990).

AMUF is the only ACN whose lack of phosphorylation to a triphosphate is difficult to interpret. The presence of fluorine atom at position 5 of the pyrimidine chain does not seem to be decisive about the absence of phosphorylation as there are very many 5-fluoronucleosides and nucleotides which are phosphorylated *in vivo* to corresponding triphosphates (Sato *et al.*, 1993; Willmore and Durkacz, 1993).

Table IV shows the distribution of [^{14}C]2'dN, [^{14}C]ACN and [^{14}C]2'dN in the presence of ACN between DNA and cytosol in 10 days growing melanoma. Incorporation of [^{14}C] 2'dN, [^{14}C] ACN and [^{14}C] 2'dN with ACN present into tumor DNA indicates that all [^{14}C] 2'dNs are incorporated into DNA (average incorporation of all [^{14}C] 2'dNs : 88.7±1.7%), whereas practically none of [^{14}C] ACNs is incorporated into DNA (incorporation mean of [^{14}C] ACN: 0.66±0.0015%, 2'dN/ACN incorporation ratio: 1344). Biologically active ACNs significantly diminish incorporation of [^{14}C] 2'dN into DNA. AMT1 reduces incorporation of dThd from 32.2 nmol to 8.73 nmol per mg of DNA (i.e. reduction by 73%), AMT2 inhibits incorporation of dAdo from 21 nmol to 6.2 nmol per mg of DNA (i.e. reduction by 70%), dGuo from 34.2 nmol to 15.4 nmol per mg of DNA (i.e. reduction by 53%) and dThd from 32.2 nmol to 11 nM per mg of DNA

Table IV. Distribution of [^{14}C] 2'dNs, [^{14}C] AMT1, [^{14}C] AMT2 and [^{14}C] AMUTM (A) and [^{14}C] 2'dNs in the presence of AMT1, AMT2 and AMUTM (B) between DNA and cytosol in a 10-day growing melanoma 48 h after administration of the compounds. Animals were given 0.2 mmol [^{14}C] 2'dNs, 4×10^6 cpm (0.066 MBq) or 0.2 mmol [^{14}C] ACN, 3×10^5 cpm (0.005 MBq) per kg of body weight (A) or [^{14}C] 2'dNs (labeled as above) with biologically active ACN (in doses as in A). The incorporation was expressed in nmol of [^{14}C] compounds per mg of DNA or nmol per mg of protein (cytosol) Each value: the mean ± SD for four separate experiments. In parentheses: % of incorporation.

	A				B			
			AMT1		AMT2		AMUTM	
	DNA	Cytosol	DNA	Cytosol	DNA	Cytosol	DNA	Cytosol
dAdo	21±1.8 (86)	3.24±0.3 (14)	17.8±1.6 (85)	3.1±0.3 (15)	6.21±0.7 (31)	13.7±0.9 (69)	7.3±0.8 (28)	19±2.6 (72)
dGuo	34.2±4.9 (85)	5.94±0.7 (15)	40±5.4 (93)	3±0.3 (7)	15.4±1.6 (38)	25±2.6 (62)	35.5±6.5 (90)	3.6±0.5 (10)
dCyd	12.6±0.9 (89)	1.62±0.17 (11)	11.7±1.6 (87)	1.8±0.2 (13)	11±0.9 (89)	1.26±0.17 (11)	12.6±1.5 (86)	2±0.3 (14)
dThd	32.2±5.3 (90)	3.28±0.4 (10)	8.73±0.9 (29)	21±2.8 (71)	12±1.5 (34)	23.4±2.7 (66)	28.8±4.5 (94)	1.8±0.2 (6)
AMT1	1.2±0.3 (3)	38.4±3.4 (97)	-	-	-	-	-	-
AMT2	1.4±0.4 (3)	43.2±4.7 (97)	-	-	-	-	-	-
AMUTM	1.7±0.4 (4)	41±4.2 (96)	-	-	-	-	-	-

Abbreviated ACNs: AMT1, 1-*N*-allyloxymethylthymine; AMT2, 1,3-*N,N*-diallyloxymethylthymine; AMUTM, 1-*N*-allyloxymethyl-5,6-tetramethyleneuracil.

(i.e. reduction by 64%). AMUTM, similarly, reduces dAdo incorporation from 21 nmol to 7.3 nmol (i.e. reduction by 66%). Quantitatively, the results do not correlate with the decrease of activities of 2'dN kinases by the mentioned ACNs. AMT1 reduces (*in vivo*) the activity of dThd kinase by 32% (Table II), but the same ACN inhibits dThd incorporation (in fact dTTP) into DNA by 70%, as this amount of dThd remains in cytosol in experiments with the use of [^{14}C] dThd with AMT1 (Table IV). Similarly, in case of [^{14}C] dAdo, [^{14}C] dGuo and [^{14}C] dThd, inhibition of incorporation of these [^{14}C] 2'dNs (phosphorylated *in vivo* to dATP, dGTP, and dTTP) by AMT2 and AMUTM, is always twice as high as the inhibition of activity of corresponding 2' dN kinases (Table IV). It is concluded that AMT1, AMT2 and AMUTM, beside inhibiting 2' dN kinases activities, are also inhibitors of DNA polymerase. Such inhibition of acyclonucleosides triphosphates, modified nucleosides and nucleotides has been reported (Derse *et al.*, 1981; Martin *et al.*, 1994; Ilsley *et al.*, 1995). Apparently the cytostatic activity of AMT1, AMT2 and AMUTM is of synergistic character: inhibiting the activity of 2' dN kinases it causes a reduction of substrates concentration for DNA polymerase, and, concurrently, inhibits DNA polymerase activity. Presumably, the latter inhibition occurs via a competition of AMT1, AMT2 and AMUTM triphosphates with natural substrates of DNA polymerase (dATP, dGTP, dTTP). The expected inhibition of pyrimidine nucleoside phosphorylase activity by biologically active ACNs (Modrzejewska *et al.*, 1996) has been confirmed for thymidine phosphorylase which is inhibited by AMT1 in human neoplasm (Miszczak-Zaborska *et al.*, 1997).

Because phosphorylases present in the tumor exhibit preferences to modify ribo- and 2'deoxynucleosides (Savarese *et al.*, 1981) this additional effect of allyloxymethylopyrimidine, by augmenting ACN concentration in tissues concurrently intensifies the cytostatic effect of ACN. The results on allyloxymethyl derivatives of thymine and uracil, confirming their cytostatic properties encourage further studies on these acyclonucleosides.

Blin N. and Stafford D. W. (1976), General methods for isolation of high molecular weight DNA from eukaryotes. Nucl. Acid Res. **3**, 2303–2308.

Bouffard D. Y., Laliberte J. and Momparler R. L. (1993), Kinetic studies on 2,2'difluorodeoxycytidine (gencitabine) with purified human deoxycytidine kinase and cytidine deaminase. Biochem. Pharmacol. **45**, 1857–1861.

Bradford M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254.

Bomirski A., Słomiński A. and Bigda J. (1998), The natural history of a family of transplantable melanomas in hamsters. Canc. Metastasis Rev. **7**, 95–118.

Derse D., Cheng Y-C, Furman P. A., St. Clair M. H. and Elion G. B. (1981), Inhibition of purified human and herpes simplex virus induced DNA polymerase by a 9(2-hydroxyethoxymethyl) guanine triphosphate. Effect on primer template function. J. Biol. Chem. **256**, 11447–11451.

Drabikowska A. K., Lissowska L., Dramiński M., Zgit-Wróblewska A. and Shugar D. (1987), Acyclonucleoside analogues consisting of 5- and 5,6- substituted uracil and different acyclic chains: inhibitory properties versus *E.coli* uridine phosphorylase. Z. Naturforsch. **42c**, 288–296.

Dramiński M. and Frass E. (1981), Alkylated derivatives of uracil. Part IX. Synthesis of N-(2, 3'-dihydroxypropyl) derivatives of 5,6-tetramethyleneuracil. Pol. J. Chem. **55**, 1547–1552.

ElAshry E. S. H. and ElKilany Y. (1997), Acyclonucleosides. Part 2: diseconucleosides. Adv. Heterocyclic Chem. **68**, 1–88.

Greger J. and Dramiński M. (1989), Growth inhibition of Kirkman-Robbins hepatoma by 1(1',3'-dihydroxy-2'-propoxymethyl) 5,6 tetramethylene uracil and possible mechanism of its biological activity. Z. Naturforsch. **44c**, 985–991.

Ilsley D. D., Lee S-H., Miller W. H. and Kuchta R. P. (1995), Acyclic guanosine analogs inhibit DNA polymerase α, β and ɜ with very different potentation and have unique mechanism of action. Biochemistry 34, 2504–2510.

Jarvi E. T., Mc Carthy J. R., Mehdi S., Matthews D. P., Edwards M. L., Prakash N. J., Bowlin T. L., Sunkara P. S. and Bey P. (1991), 4'5'unsaturated 5 halogenated nucleosides. Mechanism based and competitive inhibitors of S-adenosyl-L-homocysteine hydrolase. J. Med. Chem. **34**, 647–656.

Krenitsky T. A., Tuttle J. V., Miller W. H., Moorman A. R., Orr G. F. and Beauchamp L. (1990), Nucleotide analogue inhibitors of purine nucleoside phosphorylase. J. Biol. Chem. **265**, 3066–3069.

Martin J. L., Brown C. E., Matthews-Davis N. and Reardon J. E. (1994), Effect of antiviral nucleoside analogs on human DNA polymerase and mitochondrial DNA synthesis. Antimicrob. Agents Chemother. **38**, 2743–2749.

Miszczak-Zaborska E., Greger J., Woźniak K., Kowalska Koprek U. and Pajszczyk-Kieszkiewicz T. (1997), The activity of thymidine phosphorylase in the uterine myomas and the myometrium in perimenopausal women. Z.Naturforsch. **52c**, 850–854.

Modrzejewska H. Greger J. Lewandowska U, Fidek W. (1994), *In vivo* phosphorylation of allyloxymethyl purine and pyrimidine acyclonucleosides and the inhibitory effect of these compounds on thymidine and deoxyguanosine kinases. Acta Biochim. Pol. **41**, 185–187.

Modrzejewska H., Greger J., Dramiński M., Rutkowski M. (1996), The influence of alkoxymethyl purine and pyrimidine acyclonucleosides on growth inhibition of Kirkman-Robbins hepatoma and possible mechanism of their cytostatic activity Z. Naturforsch. **51c**, 75–80.

Ozierov A. A., Novkov M. C., Brel A. K., Andrejeva O. T., Vladyko G. V., Boreko E. I., Korbachenko L. Y. and Vervetchenko C. G. (1991), The synthesis and antiviral activity of newly synthesized unsaturated pyrimidine acyclonucleosides Chim. Pharm. **25**, 44–47.

Salomon B., Maury S., Loubiére L., Caruso M., Onclerq R. and Klatzman D. (1995), A truncated herpes simplex virus thymidine kinase phosphorylates thymidine and nucleoside analogs and does not cause sterility in transgenic mice. Mol. Cell. Biol. **15**, 5322–5328.

Sato S., Kohno K., Hidaka K., Hisatsungu T., Kuwano M. and Komiyama S. (1993), Differentially potentiating effects by dipyridamole on cytotoxicity of 5-fluorouracil against the human maxillary cancer cell lines derived from a single tumor. Anti Canc. Drug Design **8**, 289–297.

Savarese G. W., Crabtree G. M. and Parks Jr R. E. (1981), 5'-methylthioadenosine phosphorylase. Substrate activity of 5'-deoxyadenosine with the enzyme from Sarcoma 180 cells. Biochem. Pharmacol. **30**, 189–199.

Słomiński A. and Paus R. (1993), Bomirski melanomas: a versatile and powerful model for pigment cells and melanoma research. Int. J. Oncol. **2**, 221–228.

Thormar H., Georgsson G., Gunnarssson E., Naesens L., Torsteins S., Balzarini J. and De Clerg E. (1998), Treatment of visna virus infection in lambs with acyclic nucleoside phosphonate analogue 9-(2-phoshonylmethoxyethyl)adenine. Antiviral Chem. Chemother. **9**, 245–252.

Willmore E. and Durkacz B. (1993), Cytotoxic mechanism of 5'-fluoropyrimidines. Biochem. Pharmacol. **46**, 205–211.