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A study of the influence of newly synthesized acyclonucleosides and 1,2,3,4-tetrahydroisoquinoline derivatives on deoxythymidine and deoxycytidine kinase activities in human neurofibrosarcoma and ovarian cancer

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The influence of nine newly synthesized uracil acyclonucleosides, and 36 derivatives of 1,2,3,4-tetrahydroisoquinoline on the activity of enzymes catalysing dTMP and dGMP synthesis, on the content of dTTP and dGTP in acid soluble fraction and on the incorporation of [^{14}C]dThd and [^{14}C]dGuo into DNA in tumour homogenates was studied. The influence of the compounds was studied in the cytosol from intraoperatively excised human tumours — neurofibrosarcoma and ovarian cancer.

It was shown that dTMP and dGMP synthesis is inhibited competitively by 34.1±4.0% in both types of tumours by 0.2 mM 1-*N*-(3'-hydroxypropyl)-6-methyluracil (1) and 0.2 mM 1-*N*-(3'-hydroxypropyl)- 5,6- tetramethyleneuracil (2). The mentioned acyclonucleosides reduced the content of dTTP and dGTP in the acid soluble fraction of tumours (59.7±3.1% of control). 1-(4-chlorophenyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (3), 1-(2,3-dichlorophenyl)-6,7-dihydroxy 1,2,3,4-tetrahydroisoquinoline (4) and 1-(3-methoxyphenyl)-6,7-dihydroxy 1,2,3,4-tetrahydroisoquinoline (5) at 0.2 mM concentration caused a mixed type inhibition of the synthesis of dTMP and dGMP by, on average, 33.2±4.4%, and reduced the content of dTTP and dGTP in the acid soluble fraction (52.6±3.7% of control) but were active only in the cytosol of neurofibrosarcoma. While acyclonucleosides undergo

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Abbreviations: ACN, acyclonucleoside; HMDS, hexamethyldisilazane; THI, tetrahydroisoquinoline.

phosphorylation in the cytosol by cellular kinases, with their triphosphates being active acyclonucleoside metabolites, active 1,3,4,5-tetrahydroisoquinoline derivatives (compounds not containing a deoxyribose moiety), cannot be phosphorylated. ACN and THI derivatives which inhibit dThd and dCyd kinase activities, inhibit also the incorporation of [^{14}C]dThd and [^{14}C]dGuo (ACN — 50.2±2.7%, THI — 53.4±3.9% of incorporation inhibition) into tumour DNA. The obtained results point to the mechanism of uracil acyclonucleosides and 1,2,3,4-tetrahydroisoquinoline biological activity consisting in inhibiting the synthesis of DNA components.

In this paper we present a continuation of our study on compounds exhibiting cytostatic properties. Previously we have found that alkoxymethyl purine and pyrimidine acyclonucleosides tested on experimental tumours (Kirkman-Robbins hepatoma, amelanotic melanoma) inhibit tumour growth, the synthesis of dAMP, dGMP and dTMP, and the incorporation of dNTP into DNA (Greger & Dramiński, 1989; Modrzejewska *et al.*, 1996; 1999). The results presented herein concern 45 newly synthesized compounds. Nine of them are acyclonucleoside (ACN) uracil derivatives and 36 are tetrahydroisoquinoline (THI) derivatives. The inclusion of THI derivatives has resulted from the fact that these compounds, apart from other properties, exhibit also cytostatic activity (Lin M-C *et al.*, 1995; Goldbrunner *et al.*, 1997). Among the investigated compounds two ACN derivatives (Fig. 1, comp. 1, 2) and three THI derivatives (Fig. 1, comp. 3, 4 and 5) reveal moderate biological activity. Compounds 6 and 7 are hardly biologically active; the reason of their inclusion in the study will be explained in the Results and Discussion. The study on the biological properties of the ACN and THI derivatives were carried out on intraoperatively obtained human tumours — neurofibrosarcoma and ovarian cancer. Determination of dTMP synthesized by dThd kinase enabled the assessment of this enzyme activity. dGMP synthesis served to evaluate dCyd kinase activity, as dGuo and not dCyd is the preferred substrate of this enzyme (Usova & Eriksson, 1997). dGuo kinase occurs mainly in mitochondria (Wang *et al.,* 1993; Jullig & Eriksson, 2001) dCyd kinase is responsible for the synthesis of dGMP in the cytosol (Arner & Eriksson, 1995). Apart from the determination of the activities of both kinases which were subject to the effect of active forms of ACNs and THIs, we studied the content of dTTP and dGTP in the acid soluble fraction obtained from tumour homogenates, the phosphorylation of ACNs and THIs catalysed by kinases present in the cytosol of the tumours and also the incorporation of [^{14}C]dThd and [^{14}C]dGuo into DNA obtained fom both tumours in the presence of ACN and THI. The aim of the latter study was to explain what products of ACNs and THIs biotransformation are responsible for their biological activity.

MATERIALS AND METHODS

Chemistry. [^{14}C]dThd and [γ-^{32}P]ATP were obtained from Amersham (Buckinghamshire, England); [^{14}C]dCyd, [^{14}C]dGuo, unlabeled dCyd, dGuo and ATP were purchased from Sigma Chemical Company (St. Louis Mo, U.S.A.). Other reagents used were products of highest quality commercially available from Aldrich Chemical Company (Milwaukee, U.S.A.) Boehringer (Mannheim, Germany) Fluka A.G., Loba Chemie (Vienna, Austria) and Polskie Odczynniki Chemiczne (Gliwice, Poland).

1-N-(3′-hydroxypropyl)-6-methyluracil (1): 6-methyluracil 2.52 g (20 mmol) in 25 cm^3 hexamethyldisilazane (HMDS) was refluxed for 3 h to dissolve uracil. The solvent was evaporated to viscous oil and equimolar amount of 1-benzyloxy-3-chloropropane was added. The mixture was kept overnight at room temperature. The reaction was monitored by TLC and the mixture was coevaporated with ethanol and with ammonia so-

lution in ethanol. The residue was crystallised twice from ethanol. The yield of 1-*N*-(3′-hydroxypropyl)-6-methyluracil was 1.5 g (45%).

1-N-(3′-hydroxypropyl)-5,6-tetramethyleneuracil (2): 5,6-tetramethyleneuracil (3.32 g, 20 mmol) in 25 cm^3 HMDS was refluxed for 3 h to dissolve uracil. In a similar manner as above the yield of 1-*N*-(3′-hydroxypropyl)-5,6-tetramethyleneuracil was 1.8 g (40%).

3-N-(3′-hydroxypropyl)-5,6-tetramethylene-2-thio uracil (6): a mixture of 10 mmoles sodium methanolate in methanol [prepared by dissolving 0.23 g (10 mmol) sodium in 25 cm^3 of dry methanol], 1.7 g (10 mmol) ethyl cyclohexanon-2-carboxylate and 2.4 g (10 mmol) *N*-(3′-benzyloxypropyl) thiourea (prepared according to Anders & Jensen, 1958) was refluxed for 10 h. The solvent was evaporated and residue was dissolved in 20 cm^3 of water and acidified with 30% H_2SO_4. The precipitate was crystallized twice from ethanol, to give 60% yield of 3-*N*-(3′-hydroxypropyl)-5,6-tetramethylene-2-thio uracil.

3-N-(3′-hydroxypropyl)-5,6-tetramethyleneuracil (7): 0.48 g (2 mmol) 3-*N*-(3′-hydroxypropyl)-5,6-tetramethylene-2-thio uracil in 5 cm^3 10% solution of chloroacetic acid was refluxed for 10 h to give 50% yield of 3-*N*-(3′-hydroxypropyl)-5,6-tetramethyleneuracil.

Analytical data of the synthesized acycyclonucleosides are shown in Table 1. Biologically active THI derivatives were synthesized by Brzezińska (1996) and analytical data of these compounds were described in the mentioned paper.

Biology

Chemical and enzyme assays. Tumours (5–6 g) obtained intraoperatively were immediately homogenized at 0°C in a Potter-Elvehjem apparatus in 20–24 ml of 25 mM Tris/HCl buffer (pH 7.4) containing 250 mM sucrose, 25 mM KCl and 5 mM $MgCl_2$ (medium A). Nuclei and mitochondria were spun down at 10 000 × ***g*** for 20 min and the resulting supernatant, after centrifugation at 105 000 × ***g*** for 60 min representing the cytosol was used for the experiments. dThd and dCyd kinase activities were assayed as described previously (Greger & Dramiński,

Table 1. Analytical data of newly synthesized ACNs

	mp	TLC	UV λmax [nm] εx10³		H1-NMR						MS [m/e]%				
Comp.	[C°]	Rf/S_2	pH 7	pH 13	N-CH_2	N_3 - H	O-CH_2	C-CH_2-C	C-CH_3	∅-CH_3	M+	M-43	M-58	B + 1	43
1	165	0.49	262	262	5.31 s	8.94 bs	3.52 t	1.59 m	0.90 t	2.35	198	155	140	126	43
			8.5	6.5							20.07	55.55	62.48	70.11	100.0

TLC silica gel 60 F_{254}, S_2, $CHCl_3$/ETOH (92 : 8)

	mp	Rf TLC		UV λmax [nm] εx10³		H1-NMR					MS [m/e]%				
Comp.	[C°]	S_1	S_2	pH 7	pH 13	N-CH_2	O-CH_2	C-CH_2-C	N_3 - H	M+	M-18	M-44	M-59	M-88	M-102
2	152	0.61	0.34	271/10.50	270/9.30	3.94 m	3.67 m	1.65 m	-	224	206	180	165	136	122
										62.14	24.86	33.06	47.80	60.18	32.94

	mp	Rf TLC			UV λmax [nm] εx10³		H1-NMR				MS [m/e]%				
Comp.	[C°]	S_1	S_2	S_3	pH 7	pH 13	N-CH_2	O-CH_2	C-CH_2-C	N_1 - H	M+	M-18	M-44	M-59	M-88
6	191-3	0.60	0.50	0.67	270/8.92	282/11.70	4.10 t	3.52 t	1.75 m	10.25 bs	224	206	180	165	-
											20.97	32.92	100.0	57.43	
7	165-7	0.71	0.72	0.81	283/8.30	305/11.60	4.56 t	3.58 t	1.71 m	10.87 bs	240	222	196	181	207
											21.95	8.69	9.12	18.63	100.0

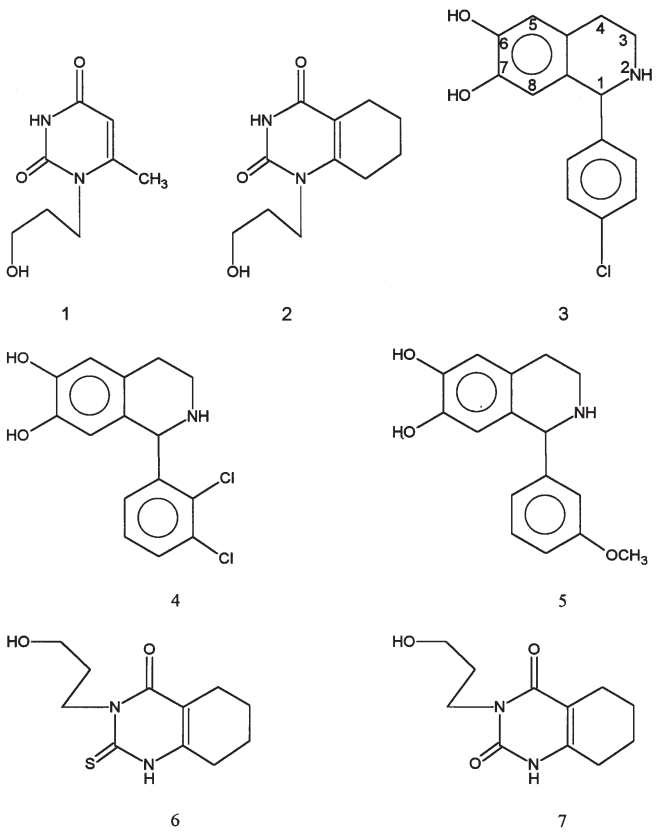
TLC, Kiesel gel 60 F_{254}, S_1, BuOH/H_2O (86 : 14); S_2, $CHCl_3$/EtOH, (92 : 8); S_3, CH_3-CO-CH_3; UV, Unicam SP 500; ^{1}H-NMR, Tesla 80 MHz, $CDCl_3$; MS-direct inlet, LKB 6000, 70 eV.

1989), the phosphorylation of ACNs was performed under conditions optimal for dThd and dCyd kinase activities, using 0.2 mM ACN or THI derivatives as a substrate and 10 mM [γ-^{32}P]ATP (0.02 MBq per sample) as a phosphate donor. The phosphorylation products were separated by TLC ascending chromatography (silica gel plates, Merck Kiselgel 60 F, 0.2 mm) at room temperature in 1-propanol/conc. ammonia water (22:17, v/v), with R_f for ACN monophosphates 0.22–0.24, ACN diphosphates 0.18–0.20, ACN triphosphates 0.02–0.05 and spots corresponding to standards were counted in 5 ml toluene scintillator. ACN phosphates used as standards were obtained enzymatically using nucleoside phosphotransferase from *Enterobacter agglomerans* (Rutkowski & Korczak, 1992*)* As the enzyme from this bacterium does not catalyse THI phosphorylation, the radioactivity of chromatograms at 1 cm intervals (beginning with the starting position) was counted when investigating the THI phosphorylation catalyzed by the enzymes present in tumour cytosol. On the gel which was used for the separation of ACN phosphates, the accumulating ^{32}P of the lowest R_f corresponds to triphosphates. The isotope of higher R_f corresponds to diphosphates, and the one of the highest R_f — to monophosphates. Such a conclusion follows from the fact of chemical similarity of uracil ACN to THI. The enzyme activities and rates of ACN and THI phosphorylation were expressed as μmol of dNMP or dNTP formed per minute per milligram of protein $\times 10^{-5}$ (i.e. U per mg of protein $\times 10^{-5}$) [^{14}C]dTTP and [^{14}C]dGTP were assayed on acid soluble

Table 2. The influence of biologically active ACNs (Fig. 1 comp. 1, 2) and THIs derivatives (Fig. 1 comp. 3, 4 and 5) at 0.2 mM concentration alone and in the presence of EDTA (10 mM) on dThd and dCyd kinase acitivities (U per mg of protein $\times 10^{-5}$) in the cytosol of neurofibrosarcoma (A) and ovarian cancer (B).

Each value: mean ±S.D. for four separate experiments. *P* values, after checking variance conformability using Fisher-Snedecor test, were calculated using Student's *t*-test. In parentheses % of inhibition. NS, the difference non significant.

Compound tested	A dThd kinase	A dCyd kinase	B dThd kinase	B dCyd kinase
Control 11.2±1.7		13.2± 2.1	10.1± 1.6	13.2 ± 2.0
1	7.0 ± 1.0 (37) K_i 7.9×10^{-5} M *P*=0.002	8.8 ± 1.3 (33) K_i 7.6×10^{-5} M *P*=0.005	6.6 ± 1.0 (34) K_i 1.1×10^{-4} M *P*=0.005	9.5± 1.6 (28) K_i 1.6×10^{-5} M *P*=0.01
1 plus EDTA	10.8 ± 1.6 (4), NS	12.9± 2.0 (2), NS	9.8 ± 1.4 (3), NS	12.9± 1.9 (2), NS
2	7.7± 1.2 (31), K_i 1.0×10^{-4} M	7.8± 1.3 (41) K_i 5.6×10^{-5} M	6.4± 1.0 (36), K_i 1.0×10^{-4} M	8.8 ± 1.4 (33) K_i 9.6×10^{-5} M
2 plus EDTA	10.8 ± 1.5 (4), NS	12.8 ± 2.0 (3), NS	9.8 ± 1.5 (3), NS	12.9± 1.8 (2), NS
3	7.6± 1.2 (32), K_i 1.5×10^{-4} M *P*=0.005	9.6± 1.5 (27), K_i 1.2×10^{-4} M *P*=0.02	10.2 ± 1.6 (0), NS	13.0 ± 2.1 (1), NS
3 plus EDTA	7.3 ± 1.1 (35)	9.5 ± 1.5 (28), K_i 2.3×10^{-4} M	9.5±1.4 (5), NS	13.3±2.2 (0), NS
4	7.3± 1.2 (35), K_i 1.8×10^{-4} M *P*=0.005	12.4 ± 1.8 (6), NS	9.9 ± 1.6 (1), NS	12.1± 1.7 (8), NS
4 plus EDTA	7.2± 1.2 (36)	13.3±2.0 (0), NS	10.2±1.7 (0), NS	12.5±1.8 (5), NS
5	6.8 ± 1.0 (39), K_i 1.3×10^{-4} M *P*=0.002	8.9 ± 1.3 (33), K_i 1.5×10^{-4} M *P*=0.005	10.2± 1.7 (0), NS	12.1± 1.6 (8), NS
5 plus EDTA	7.0± 1.0 (38)	9.2 ± 1.4 (30), K_i 1.8×10^{-4} M	9.9±2.0 (1), NS	12.9±2.0 (2), NS
6	10.9±1.8 (3), NS	12.7±1.9 (4), NS	9.8±1.6 (2), NS	12.5±1.8 (5), NS
6 plus EDTA	10.7±1.6 (5), NS	12.5±1.8 (5), NS	9.5±1.4 (5), NS	12.5±1.9 (5), NS
7	10.6±1.6 (5), NS	12.5±1.9 (5), NS	9.5±1.4 (5), NS	12.6±1.7 (4), NS
7 plus EDTA	10.7±1.8 (5), NS	12.9±2.1 (2), NS	10.7±1.8 (4), NS	12.6±1.7 (4), NS



1. 1-N-(3'hydroxypropyl)-6-methyluracil
2. 1-N-(3'hydroxypropyl)-5,6-tetramethyleneuracil
3. 1-(4-chlorophenyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline
4. 1-(2,3-dichlorophenyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline
5. 1-(3-methoxyphenyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline
6. 3-N-(3'hydroxypropyl)-2thio-5,6-tetramethyleneuracil
7. 3-N-(3'hydroxypropyl)-5,6-tetramethyleneuracil

Figure 1. Structural formulae of the active acyclonucleotides and 1,2,3,4-tetrahydroisoquinoline derivatives.

fraction obtained according to Letnansky (1964) from 20% (v/w) homogenates of neurofibrosarcoma and ovarian cancer in medium A enriched by 10 mM ATP. After 1 h incubation with [^{14}C]dThd or [^{14}C]dGuo (0.02 MBq per sample) the acid soluble fraction was passed through Dowex 1 × 8 (200–400 mesh, 0.8 × 10 cm column) with standards (dTTP or dGTP) of concentration determined spectrophotometrically. Two-milliliter fractions were eluted from column with formic acid (Akyoshi, 1984) and fractions in which spectrophotometrically identified and determined added standards were counted in toluene scintillator (as above) containing 30% (v/v) Triton X-100. DNA from both tumours was isolated and incorporation of [^{14}C]dThd and [^{14}C]dGuo into DNA was assayed according to the methods decribed by Blin & Stafford (1976). Protein was determined by the Bradford method (1976). Statistical calculations were made using Fisher-Snedecor, Student's *t*-tests and a Sigma Plot 2001 for Windows version 7 computer program.

Tumour specimens were obtained from the Surgical Clinic of Oncology (neurofibrosarcoma), and from the Dept. of Gynecology and Gynecological Oncology, Med. Univ. of Łódź (ovarian cancer).

RESULTS AND DISCUSSION

Biologically active 0.2 mM ACNs and THIs inhibit the synthesis of dTMP and dGMP to an identical level (ACN 34 ± 4%, THI 33 ±

4.4% inhibition, Table 2) in the cytosol of neurofibrosarcoma (ACN, THI) and ovarian cancer (ACN). Active derivatives of THI (3, 4, 5) inhibit the activity of dThd and dCyd kinases only in the cytosol of neurofibrosarcoma, being inactive in the presence of the same enzymes in the cytosol of ovarian cancer. EDTA diminishes 12-fold the percentage of the inhibition of dTMP and dGMP synthesis by active ACNs while the inhibitory properties of active THI in the presence of EDTA remain unchanged. The differences in K_i values for ACN and THI (ACN 79 ± 30 μM, THI 146 ± 23 μM) point to different types of inhibition of dThd and dCyd kinase activity by ACN and THI.

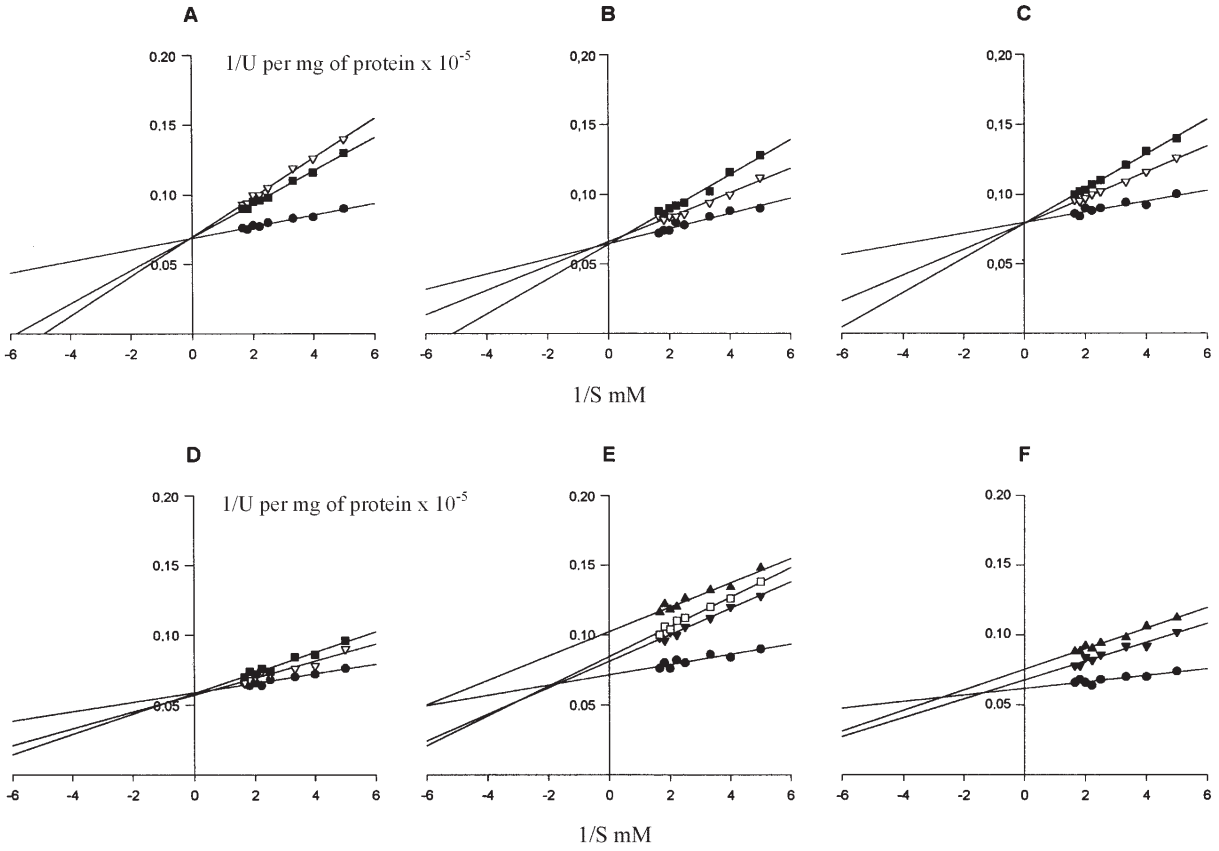


Figure 2. Lineweaver-Burk plots of dThd and dCyd kinase activities in cytosol of neurofibrosarcoma and ovarian cancer in the presence of 0.2 ACN mM (Fig. 1, comp. 1, 2) and THI (Fig. 1, comp. 3, 4 and 5) derivatives.

A, neurofibrosarcoma dThd kinase, (●, r^2 = 0.92), comp. 1 (▽, r^2 = 0.99), and 2 (■, r^2 = 0.98); **B**, neurofibrosarcoma dCyd kinase, (●, r^2 = 0.92), comp. 1 (▽, r^2 = 0.96), and 2 (■, r^2 = 0.97); **C**, ovarian cancer dThd kinase, (●, r^2 = 0.84), comp. 1 (▽, r^2 = 0.98), and 2 (■, r^2 = 0.99); **D**, ovarian cancer dCyd kinase, (●, r^2 = 0.92), comp. 1 (▽, r^2 = 0.91), and 2 (■, r^2 = 0.96); **E**, neurofibrosarcoma dThd kinase, (●, r^2 = 0.81), comp. 3 (▼, r^2 = 0.97), comp. 4 (❑, r^2 = 0.97) and 5 (▲, r^2 = 0.98); **F**, neurofibrosarcoma dCyd kinase, (●, r^2 = 0.78), comp. 3 (▼, r^2 = 0.94), and 5 (▲, r^2 = 0.94). K_m calculated from these plots equals: neurofibrosarcoma dThd kinase 51.2 μM, dCyd kinase 41.6 μM. Ovarian cancer: dThd kinase 53,7 μM, dCyd kinase 71,4 μM. K_{mi} values calculated from these plots are: neurofibrosarcoma dThd kinase comp. 1, 2, 3, 4 and 5: 1.8, 1.5, 1.2, 1.07, 1.3 × 10^{-4} M, respectively; dCyd kinase comp. 1, 2, 3 and 5: 1.5, 1.9, 1.1 × 10^{-4} M, 9.6 × 10^{-5} M, respectively. Ovarian cancer dThd kinase, comp. 1 and 2: 1.5, 1.6 × 10^{-4} M respectively; dCyd kinase, comp. 1 and 2: 9.8, 1.2 × 10^{-4} M, respectively. K_i values included in Table 2 were obtained from these K_{mi} values; $r^2 > 0.5$ indicates a high positive linear correlation coefficient (according to Sigma Plot 2001 for Windows version 7 statistical computer program).

Figure 2 demonstrates the course of activity of dThd kinase from neurofibrosarcoma at increasing substrate concentrations without and in the presence of 0.2 mM active ACNs 1 and 2 (Table 2) as well as active THIs 3, 4 and 5. Changes in the enzyme activity in the presence of ACN derivatives point to a competitive type of inhibition of the enzyme by these compounds, while THI derivatives inhibit the enzyme in a mixed way. The inhibition of dGMP synthesis by active THI derivatives 3 and 5 in neurofibrosarcoma (Table 2) is also of a mixed character. This fact may be explained by a greater K_i value for THI where, unlike in competitive inhibition, the inhibitor binds both the free enzyme as well as the enzyme–substrate complex. Table 2 comprises aditionally a set of characteristics of two ACN derivatives 6 and 7. They exhibit only 5% of the activity of compounds 1 and 2, but they were subsequently used for the explanation of active ACNs biotransformation.

A loss of the inhibitory properties of ACN derivatives 1 and 2 in the presence of EDTA and a complete lack of this effect in the presence of ACN derivatives 6 and 7 and THI derivatives 3, 4 and 5 suggest a possibly different ACN and THI metabolism. This suggestion is confirmed by the results of ACN and THI phosphorylation presented in Table 3. Biologically active ACNs undergo phosphorylation to corresponding triphosphates by deoxynucleoside 5'-mono and 5'-diphosphate kinases present in the cytosol of both tumours. The extent of phosphorylation to the corresponding triphosphates of derivatives 1, 2, 6 and 7 is the same but the resulting amounts of ACN triphosphates of derivatives 6 and 7 is 20-fold lower than triphosphate derivatives 1 and 2. Both the above mentioned facts and the almost complete lack of inhibitory activity of derivatives 6 and 7 (Table 2) allow one to presume that ACN triphosphates are the inhibitors of dThd and dCyd kinases in neurofibrosarcoma and ovarian cancer. Another confirmation of this inference is the behaviour of active ACNs in the presence of EDTA. While inhibiting the activity of deoxynucleoside 5′-diphosphates kinase (Buczyński & Potter, 1990) EDTA, at the same time, inhibits the synthesis of ACN triphosphates which explains the almost complete loss of inhibitory properties of active ACNs (Table 2) 1 and 2. Active THI derivatives 3, 4 and 5 are not phosphorylated by the enzyme present in the tumour cytosol since they are not substrates for kinases. The OH groups present in the aromatic structure of THI are of acidic character, therefore, they cannot form esters with HPO_4^{2-}. For this reason EDTA exerts no influence on the inhibitory properties of THI derivatives.

Although the biological activity of modified nucleosides and nucleotides usually results from *in vivo* phosphorylation of these compounds (Parker *et al.,* 1995; Ilsley *et al.,* 1995; Modrzejewska *et al.,* 1999) some biologically active nucleosides inhibiting HIV-1 are not phosphorylated *in vivo* (Fossey *et al.,* 1994). Similarly, alkenophosphonic acid purine and pyrimidine derivatives acting as ACN triphosphate equivalents do not themselves undergo phosphorylation (Harnden *et al.,* 1993). Active THI derivatives, therefore, should be included among the compounds whose biological activity does not result from phosphorylation.

While commenting on the results included in Table 3 it should be noticed that the CH_2OH group, preferred by cellular kinases (Daher *et al.,* 1990) which is a component of the 3-hydroxypropyl group of the studied ACNs 1, 2, 6 and 7 (Fig. 1) is a substrate for these enzymes only when it is bound to the position N-1 of the modified pyrimidine base 1 and 2 (Fig. 1). The above mentioned group present in the 3-hydroxypropyl group bound to N-3 position of modified pyrimidine bases 6 and 7 (Fig. 1) is not recognized by cellular kinases. This explains the very low phosphorylation of ACN derivatives 6 and 7 and, as a consequence, the almost complete lack of inhibitory activity of these compounds (Table 2 and 3).

Table 3. Phosphorylation of ACNs and THIs (0.2 mM) catalysed by kinases present in the cytosol of neurofibrosarcoma (A) and ovarian cancer (B) (μmol of [^{32} P]ACN or [^{32} P]THI mono-, di-, and triphosphate formed per min per mg of protein $\times$ 10^{-5}).

The active ACNs (alone or in presence of 10 mM EDTA) and THIs were incubated per 1 h with the cytosol obtained from tumours in the presence of 10 mM [γ-^{32}P]ATP (0.02 MBq per sample) and counted as described in Materials and Methods. ACNs phosphorylation proceeds to function y = f(x), were x is a straight line following y = ax + b equetion and the increase ACN phosphates concentration is a linear time function. In parentheses: % of phosphorylation. Each value: the mean ±S.D. for five separate experiments. Compound number according to Fig.1.

	A				B			
Compound tested	[^{32}P]mono-phosphate	[^{32}P]diphosphate	[^{32}P]triphosphate	sum of phosphorylation	[^{32}P]mono-phosphate	[^{32}P]diphosphate	[^{32}P]triphosphate	sum of phosphorylation
1	4.1±0.8 (84)	0.41±0.08 (8.4)	0.34±0.07 (7)	4.85	3.9±0.7 (85)	0.36± 0.08 (7.9)	0.3±0.05 (6.6)	4.56
1 plus EDTA	0.23±0.03 (91)	0.022±0.003 (8.7)	0.00	0.252	0.24±0.04 (91)	0.022±0.004 (8.4)	0.00	0.29
2	4.4±0.8 (87)	0.36±0.06 (7.1)	0.31±0.05 (6)	5.07	3.8±0.7 (86)	0.32±0.05 (7.2)	0.29±0.05 (6.6)	4.41
2 plus EDTA	0.28±0.04 (92)	0.023±0.003 (7.5)	0.00	0.303	0.25±0.04 (93)	0.018±0.002 (6.9)	0.00	0.268
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	0.3±0.05 (86)	0.028±0.004 (8.0)	0.02±0.003 (5.7)	0.348	0.27±0.04 (85)	0.028±0.004 (8.8)	0.02±0.003 (6.3)	0.318
7	0.27±0.05 (88)	0.02±0.003 (6.5)	0.016±0.003 (5.2)	0.306	0.24±0.04 (84)	0.03±0.004 (10)	0.016±0.002 (5.6)	0.286

Active ACNs and THIs decrease the synthesis of dTTP and dGTP in the acid soluble fraction of both tumours (Table 3, ACN derivatives: 41.5 ± 5.2%, THI derivatives: 47.2 ± 3.7% of synthesis inhibition, respectively). In the case of THI this concerns only these derivatives which inhibit the activity of dThd and dCyd kinases in the cytosol of neurofibrosarcoma. The THI derivatives without such activity have no influence on the content of dTTP and dGTP in the acid soluble fraction of both tumours. Incubation of [^{14}C]dN in the presence of ACN decrease dTTP and dGTP synthesis by, on average, 42%, as a consequence of inhibition by ACN triphosphates formed. THI derivatives which inhibit dTMP

Table 4. Synthesis of [^{14}C]dTTP and [^{14}C]dGTP in neurofibrosarcoma (A) and ovarian cancer (B) as a result of 1 h incubation of [^{14}C]dThd or [^{14}C]dGuo with 20% (v/w) tumor homogenates in medium A (containing additionally 10 mM ATP) with active ACNs (Fig. 1 comp. 1, 2) and THIs (Fig. 1 comp. 3, 4 and 5) alone or in the presence of 10 mM EDTA.

[^{14}C]dTTP or [^{14}C]dGTP were isolated from acid soluble fraction and counted (expressed in nmol per mg of tumour wet weight, for the detail see Materials and Methods). Each value: mean ±S.D. for five separate experiments. p value was counted as in Table 2. In parentheses: % of synthesis inhibition, NS, the difference non significant.

	A		B	
Compound tested	[^{14}C]dTTP	[^{14}C]dGTP	[^{14}C]dTTP	[^{14}C]dGTP
Control	12.4±2.3	14.1±2.7	6.9±1.4	15.2±2.9
1	6.7±1.1 (46), P=0.002	8.5±1.5 (40), P=0.005	4.2±0.6 (38), P=0.005	8.8±1.4 (52), P=0.005
2	7.4±1.3 (40), P=0.005	9.0±1.6 (36), P=0.01	4.0±0.6 (42), P=0.005	9.4±1.5 (38), P=0.005
3	6.4±1.1 (48), P=0.001	6.8±1.1 (52), P=0.001	6.0±1.1 (12), NS	13.0±2.2 (14), NS
4	6.7±1.2 (46), P=0.005	12.7±2.0 (10), NS	5.4±0.9 (22), NS	12.4±2.2 (18), NS
5	7.2±1.3 (42), P=0.005	7.2±1.2 (49), P=0.001	6.3±1.2 (8), NS	14.3±2.4 (6), NS
1 plus EDTA	10.6±1.9 (14), NS	11.5±1.9 (18), NS	5.5±0.9 (20), NS	12.0±2.0 (21), NS
2 plus EDTA	10.0±1.8 (20), NS	11.0±1.9 (22), NS	5.2±0.8 (24), NS	12.6.±2.2 (17), NS
3 plus EDTA	5.3±0.9 (58), P=0.001	5.3±1.0 (62), P=0.001	3.0±0.5 (56), P=0.001	6.8±1.2 (55), P=0.001
4 plus EDTA	5.6±1.0 (55), P=0.001	6.7±1.1 (52), P=0.001	3.2±0.6 (53), P=0.001	6.1±1.0 (60), P=0.001
5 plus EDTA	6.0±1.1 (52), P=0.001	5.8±1.0 (59), P=0.001	2.0±0.5 (58), P=0.001	7.0±1.2 (54), P=0.001

and dGMP synthesis, in the presence of EDTA decrease dTTP and dGTP level by, on average, 48%. This may follow from the fact that EDTA restrains the activity of the deoxynucleoside 5′-diphosphate kinase but not inhibition of phosphorylation of THI as these compounds are not phosphorylated by the enzyme present in the tumour homogenate. This finding is also confirmed by the fact that EDTA inhibits the synthesis of dTTP and dGTP to the same degree in the presence of biologically inactive THI.

In previous studies on uracil ACN derivatives it was proved that uracil alliloxymethyl derivatives, beside moderating the synthesis of DNA precursors, inhibit the *in vivo* incorporation of labeled dN into DNA obtained from experimental tumours (Greger & Draminski, 1989; Modrzejewska *et al.,* 1996; 1999). Similar studies which were carried out on ACNs and THIs demonstrated that these compounds possess similar inhibitory properties. The results of these experiments (Table 5) confirm the conclusions which follow from the data included in Tables 2–4. The incorporation of [^{14}C]dThd and [^{14}C]dGuo into DNA of both tumours is inhibited by these ACN derivatives which restrain dTMP and dGMP synthesis, diminishing, as a consequence, the synthesis of dTTP and dGTP (on average 50.5 ± 2.7% of incorporation inhibition) being substrates for DNA polymerase. For the same reasons THI derivatives which inhibit dTMP and dGMP synthesis decrease the incorporation of [^{14}C]dThd and [^{14}C]dGuo into DNA (on average 53.4 ± 4.0% of incorporation inhibition). As was explained, while commenting on results of Table 4, the decrease of [^{14}C]dN incorporation into DNA of the tumours by on average 92% of incorporation inhibition in the presence of ACN and EDTA is a consequence of inhibition of ACN triphosphate syntesis by EDTA. Although THI derivatives in the presence of EDTA decrease the incorporation of [^{14}C]dN into DNA (58 ± 5.2% of incorporation inhibition) this is a result of decreased concentration of dTTP and dGTP and not the effect of EDTA on THI.

Table 5. Incorporation of [^{14}C]dThd and [^{14}C]dGuo (0.02 MBq) into DNA obtained from 20% homogenate (v/w) in medium A of neurofibrosarcoma (A) and ovarian cancer (B).

[^{14}C]dN were incubated separately for 1 h with homogenates of tumours with active ACNs and THIs (0.2 mM, Fig. 1 comp. 1, 2, 3, 4, and 5) alone or in the presence of 10 mM EDTA. The DNA was isolated from homogenates and incorporation was expressed in nmol of [^{14}C]dN per mg of DNA (for details see Materials and Methods). Each value: the mean ± S.D. for four separate experiments. In parentheses: % of incorporation inhibition. NS, the difference non significant.

	A		B	
Compound tested	dThd	dGuo	dThd	dGuo
Control	24.2±4.1	33.6±5.8	18.4±3.0	38.3±6.5
1	11.1±1.7 (54), P=0.002	15.8±2.7 (53), P=0.002	9.9±1.8 (46), P=0.005	18.0±3.0 (53), P=0.002
2	12.1±2.0 (50), P=0.002	16.5±2.8 (51), P=0.002	9.4±1.7 (49), P=0.005	19.9±3.4 (48), P=0.005
3	11.2±1.8 (54), P=0.002	13.8±2.2 (59), P=0.001	14.0±2.3 (24), NS	30.2±4.8 (21), NS
4	12.5±2.1 (48), P=0.005	29.5±5.0 (12), NS	14.7±2.5 (20), NS	31.8±5.2 (17), NS
5	11.7 ±1.9 (52), P=0.002	15.4±2.5 (54), P=0.002	15.1±2.7 (18), NS	34.5±6.1 (10), NS
1 plus EDTA	1.84±0.33 (92), P=0.001	2.62±0.44 (92), P=0.001	1.51±0.3 (92), P=0.001	3.1±0.6 (92), P=0.001
2 plus EDTA	1.88±0.35 (92), P=0.001	2.82±0.49 (92), P=0.001	1.4±0.25 (92), P=0.001	3.0±0.6 (92), P=0.001
3 plus EDTA	9.2±1.5 (62), P=0.001	13.4±2.2 (60), P=0.001	8.5±1.4 (54), P=0.001	20.0±3.4 (58), P=0.005
4 plus EDTA	8.7±1.4 (64), P=0.001	14.1±2.5 (58), P=0.001	9.4±1.6 (49), P=0.002	19.1±3.2 (50), P=0.002
5 plus EDTA	8.2±1.3 (66), P=0.001	12.4±1.9 (63), P=0.001	7.5±1.2 (60), P=0.001	15.7±2.8 (59), P=0.001

The inhibition of the activity of dThd and dCyd kinases by uracil ACN derivatives, i.e. inhibition of pyrimidine nucleoside kinases by pyrimidine acyclonucleosides might have been expected. It is not surprising that THI derivatives reveal similar properties as being benzene rings condensed with tertahydropyridine they are formally similar to the ACN

derivatives of tetramethylene uracil 2, 6 and 7 (Fig. 1). Recently several papers have been published which present an extension of THI biological activity spectrum. And so THI derivatives may produce parkinsonism in experimental animals (Abe *et al.,* 2001) damage DNA (Jung & Surh, 2001) participate in the regulation of methyltransferase activity in metabolism of catecholamines (Kawai *et al.*, 2000) or inhibit the binding of cGMP by respective phosphodiesterase (Kotera *et al.,* 2000).

The results of the present paper revealing novel inhibitory properties of THIs towards dN kinases will contribute to a search for further THI derivatives which will be better inhibitors of DNA component synthesis than those already described.

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