INHIBITION OF DNA-DEPENDENT DNA POLYMERASE α BY ARABINOSYL-5-AZACYTOSINE AND ITS METABOLIC TRANSFORMATION IN L1210 MOUSE LEUKEMIC CELLS

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Arabinosyl-5-azacytosine is phosphorylated by the L1210 mouse leukemic cells *in vivo* as well as by the cell-free extract in the presence of ATP. The drug inhibits *in vitro* the activity of DNA-dependent DNA polymerase α from L1210 cells in a dose-dependent manner but to a lesser degree than arabinosylcytosine. When administered *in vivo*, it depresses the activity of DNA polymerase to about the same extent as arabinosylcytosine. The K_m constant for the phosphorylation of arabinosyl-5-azacytosine is 46% higher than the corresponding value for arabinosylcytosine.

Previously we have reported that 1-β-D-arabinosyl-5-azacytosine (ara-5AC) depresses the growth of L1210 mouse leukemic cells *in vivo* at the dose levels which are similar to those of arabinosylcytosine (araC). The *in vitro* 50% inhibitory concentration of ara-5AC in L1210 system is about $7{\cdot}5 \cdot 10^{-7}$ mol l^{-1}. The drug is phosphorylated by 2'-deoxycytidine kinase (NTP: deoxycytidine 5'-phosphotransferase, EC 2.7.1.74) and is incorporated into DNA. At a concentration of 10^{-5} mol l^{-1} it almost completely blocks the incorporation of thymidine into DNA of L1210 cells *in vitro*[1]. Whereas 5-azacytidine and its 2'-deoxy derivative produced a potent inhibition of DNA methylation, ara-5AC and araC primarily affected DNA synthesis[2].

It was therefore of interest to investigate the effects of ara-5AC on DNA synthesis more in detail and to gain some insight into its action on DNA-dependent DNA polymerase α (Deoxynucleosidetriphosphate: DNA deoxynucleotidyl-transferase, EC 2.7.7.7) which is generally regarded as a target enzyme for araC.

EXPERIMENTAL

Chemicals: Ara-5AC was synthesized in this Institute by Dr J. Piskala. AraC, 2'-deoxycytidine, and deoxyribonucleoside 5'-triphosphates were delivered by Calbiochem, Luzern. [6-^{3}H]Arabinosyl-5-azacytosine and [5,6-^{3}H]arabinosylcytosine were prepared as described previously[1]. Their respective specific radioactivities were 25 and 26 GBq/mmol. [2-^{14}C]2'-Deoxycytidine (1·8 GBq/mmol) and 2'-deoxy[U-^{14}C]cytidine 5'-triphosphate (13·3 GBq/mmol) were delivered by the Institute for Research, Production and Uses of Radioisotopes, Prague. Alkaline phosphatase (Type III-S, *E. coli*) was obtained from Sigma, St. Louis.

Animals and cell line: L1210 leukemic cells were implanted to inbred DBA/2 male mice (25 g) ip and 6 days later harvested by aspiration of ascitic fluid. For DNA polymerase assay *in vivo* araC or ara-5AC (40 mg/kg) were injected ip to groups of leukemic animals on day 6 following leukemic cell inoculation. Each group included 8-10 animals which were killed by cervical dislocation at different time intervals following the administration of inhibitors and the leukemic cells from each group were pooled.

Phosphorylation of ara-5AC and araC: L1210 cells were washed twice in physiological saline and suspended in 10^{-2} mol l^{-1} Tris-HCl buffer (pH 7·4) with $1{\cdot}5 \cdot 10^{-1}$ mol l^{-1} KCl and $5 \cdot 10^{-3}$ mol l^{-1} mercaptoethanol, sonicated (1 min), and spun down at 105 000 *g* in a Beckman L8-55 centrifuge for 1 h. The supernatant fraction contained 10—15 mg of protein/ml (Coomassie Brilliant Blue 250G). The reaction mixture (0·3 ml) was incubated at 37°C for 30 min in a Dubnoff shaking bath and contained: Tris-HCl buffer (pH 7·4), $5 \cdot 10^{-2}$ mol l^{-1}; ATP and $MgCl_2$, $5 \cdot 10^{-3}$ mol l^{-1}; ara-5AC, araC or dCyd, $2{\cdot}5 \cdot 10^{-5}$ — $2 \cdot 10^{-4}$ mol l^{-1}; and supernatant fraction (1 mg of enzyme protein). The reaction was terminated by immersing in the ice-cold water and aliquots were analyzed by descending chromatography on a Whatman No 1 paper in a solvent system composed of tert-butylalcohol–ethanol–formic acid–water (20 : 60 : 5 : 15, vol.%). The radioactive spots were cut out using standards and their radioactivity was measured. In some instances the chromatograms were scanned with a 2π scanner. The rate of phosphorylation was linear over the period studied and was expressed as nmol of total phosphates formed per 1 mg of protein in a 60-min incubation period.

Digestion with alkaline phosphatase: Following elution from chromatogram of putative 5'-di- and triphosphates with water and evaporation to dryness, the sample was dissolved in 0·2 ml of 10^{-2} mol l^{-1} Tris-HCl buffer (pH 7) and 2 μl of alkaline phosphatase was added; the mixture was incubated at 37°C for 30 min, rechromatographed and the chromatogram was scanned for the radioactivity distribution. Upon rechromatography the undigested control did not reveal changes in the mobility of radioactive components.

Assay of DNA-*dependent* DNA *polymerase* α: As a source of the DNA polymerase activity a high-speed supernatant fraction from L1210 cell was used. The cells were washed with saline and suspended in 1 vol. of $2 \cdot 10^{-2}$ mol l^{-1} phosphate buffer (pH 7·8) with $5 \cdot 10^{-3}$ mol l^{-1} $MgCl_2$ and dithiothreitol. The cells were counted and gently homogenized by hand-operated teflon pestle in a glass homogenizer; the homogenate was centrifuged (Spinco, Beckman) at 105 000 *g* for 2 h. The resulting supernatant fraction was dialysed against the same buffer and stored at —70°C. A 0·1-ml portion corresponded to $2 \cdot 10^7$ cell equivalents. The reaction mixtures (0·3 ml) in triplicate were incubated at 37°C in a Dubnoff shaking bath and contained: Tris-HCl buffer (pH 7·8), $5 \cdot 10^{-2}$ mol l^{-1}; $MgCl_2$, $7{\cdot}5 \cdot 10^{-3}$ mol l^{-1}; dithiothreitol, $5 \cdot 10^{-3}$ mol l^{-1}; dATP, dGTP, dTTP, $1{\cdot}5 \cdot 10^{-4}$ mol l^{-1}; [^{14}C] dCTP ($2 \cdot 10^5$ cpm), 10^{-6} mol l^{-1}; supernatant fraction (0·1 ml), and 100 μg of calf thymus DNA in standard sodium citrate solution (SSC) dialyzed prior to denaturation (15 min, 100°C) in 50 vol. of 10^{-2} mol l^{-1} Tris-HCl (pH 8) with 10^{-3} mol l^{-1} EDTA and $2{\cdot}5 \cdot 10^{-2}$ mol l^{-1} NaCl. The reaction was terminated by diluting with 2 ml of ice-cold 5% TCA containing 1% pyrophosphate; the mixture was placed on the glass fibre disc filters (GF/C) and washed with cold 5% TCA with 1% pyrophosphate, twice with cold 5% TCA and once with cold ethanol. The filters were dried and assayed for the radioactivity in Omnifluor scintillant. The enzyme activity is expressed as cpm retained by the filter per $1—2 \cdot 10^7$ cell equivalents in a 30-min incubation period. Standard error was within limits of ±8%. For the assays *in vitro* with ara-5AC and araC included in the reaction mixture directly (Fig. 1) ATP ($5 \cdot 10^{-3}$ mol l^{-1}) was added simultaneously with the inhibitor, and after a 20-min preincubation period denaturated DNA primer and [^{14}C]dCTP were added for further 30 min. The reaction was linear over the range of 10^7—$4 \cdot 10^7$ cell equivalents as well as over the period studied and

was inhibited by $5 . 10^{-3}$ mol l^{-1} N-ethylmaleimide indicating only insignificant contamination with DNA polymerases γ and β.

*Phosphorylation of ara-*5AC *by intact* L1210 *cells in vivo:* L1210 leukemia bearing mice were injected ip with 1·8 MBq of tritiated ara-5AC. The animals were sacrificed after 2 and 4 h and leukemic cells were collected, washed and suspended in $2 . 10^{-2}$ mol l^{-1} phosphate buffer (pH 7·8) containing $5 . 10^{-3}$ mol l^{-1} $MgCl_2$ and dithiothreitol. The suspension was homogenized and then centrifuged as described above for the phosphorylation reactions. Aliquots of the supernatant fraction were chromatographed and analyzed by a 2π scanner.

RESULTS

The phosphorylation of radioactive ara-5AC in the presence of cell-free extract from L1210 cells (*a*) is shown in Fig. 1. The peaks **1** and **2** located at the origin of the chromatogram were eluted, digested with alkaline phosphatase and rechromatographed (*b*); the mobility of the radioactive input corresponded to that of ara-5AC whereas the radioactivity from undigested control remained at the origin (results not given).

The inhibition of DNA-dependent DNA polymerase α from L1210 cells by ara-5AC *in vitro* is given in Fig. 2. In parallel experiments the effect of araC on the activity of this enzyme was also assayed. Both drugs inhibited the polymerase, though to a different extent. At the concentration of $2·5 . 10^{-4}$ mol l^{-1} ara-5AC inhibited

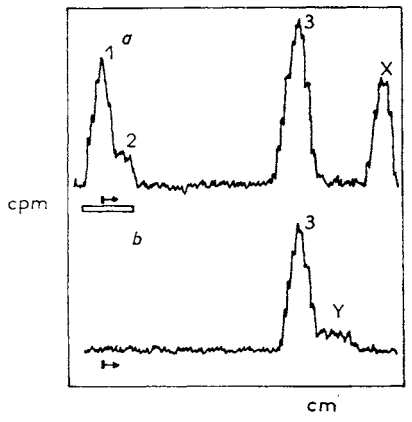

FIG. 1

Phosphorylation (*a*) of [^{3}H]ara-5AC (peak 3) *in vitro* and digestion (*b*) of newly formed 5′-di- and triphosphates (1, 2) with alkaline phosphatase followed chromatographically; X, Y unidentified peaks. cpm, amount of radioactivity; cm, relative distance from origin. For further details see Experimental

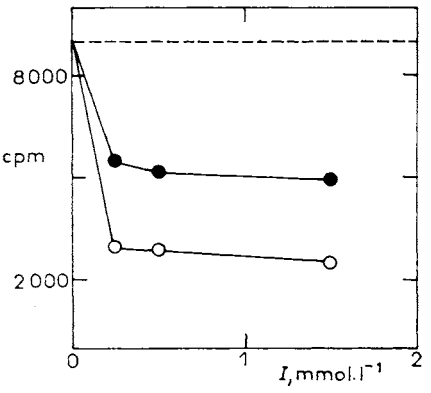

FIG. 2

Inhibition of DNA-dependent DNA polymerase α by ara-5AC (●) and araC (○) *in vitro*. cpm, enzyme activity; *I*, mmol l^{-1}, concentration of inhibitors; (— — —), control

the polymerase activity by 30%, while araC by 60%. At the concentration of $1{\cdot}25 \cdot 10^{-3}$ mol l^{-1} ara-5AC inhibited by 45% and araC by 75%. In the absence of ATP added to the reaction mixture no inhibition occurred with the implication that the phosphorylation of ara-5AC is a requisite for its inhibitory action and that the level of endogenous ATP was not sufficient for the formation of biologically active ara-5AC 5'-triphosphate.

The K_m constants for the phosphorylation of ara-5AC and araC (Table I) indicated that the K_m constant for ara-5AC is by 46% higher than that for araC suggesting lower affinity of ara-5AC for 2'-deoxycytidine kinase; this may explain the more efficient inhibition of DNA polymerase *in vitro* by araC (Fig. 2). For the sake of comparison the Michaelis–Menten constant is given also for 2'-deoxycytidine. It is by 44% less than for araC.

The time course of the phosphorylation of ara-5AC *in vivo* by intact L1210 cells at 2- and 4-h time intervals after the administration of the drug to leukemic mice (Fig. 3) shows that at 2 h prevalently 5'-monophosphate (the R_F value 0·48) is formed, and that the level of higher 5'-phosphates is low; after 4 h the amount of ara-5AC and its 5'-monophosphate diminishes considerably and the prevailing radioactivity is accounted for by 5'-triphosphate.

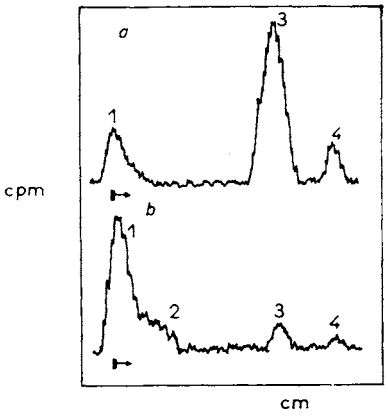

FIG. 3

Phosphorylation of [^{3}H]ara-5AC (peak **4**) by L1210 mouse leukemic cells *in vivo* 2 (*a*) and 4 h (*b*) after administration of tritiated drug followed chromatographically. **1**, **2**, **3**, 5'-tri-, di-, and monophosphates, respectively

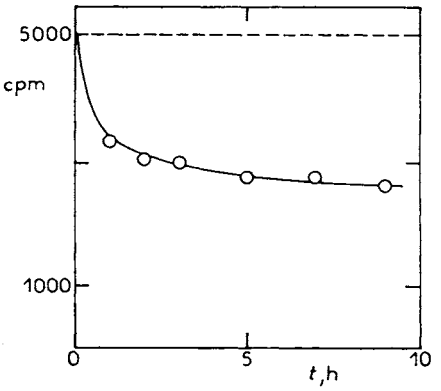

FIG. 4

Time course of the inhibition of DNA-dependent DNA polymerase α by ara-5AC *in vivo* in L1210 mouse leukemic cells. Ara-5AC (40 mg/kg) was administered to groups of leukemia L1210 bearing mice. The animals were sacrificed at different times after administration (*t*, h) and the enzyme activity (cpm) was assayed; (— — —), control

The time course of the inhibition of DNA polymerase α by ara-5AC *in vivo* is given in Fig. 4. Following the administration of the drug to leukemic mice there was a considerable inhibition of DNA polymerase activity from L1210 cells already after 1 h and a 50% inhibition was obtained approximately 4 h later; the partial block was still persisting after 9 h. In a parallel experiment araC was applied at the same dose level (40 mg/kg). In this instance the DNA polymerase activity was depressed approximately to the same extent as by ara-5AC (results not given).

DISCUSSION

The synthesis of ara-5AC was reported by Beisler and coworkers[3] and later on we have described a simplified procedure[4]. In terms of chemical structure ara-5AC possesses elements of both araC and 5-azacytidine. However, in distinction to the latter compound it is phosphorylated in mammalian tissues by deoxycytidine kinase and thus behaves as an analog of 2'-deoxycytidine. The K_m value for the phosphorylation of ara-5AC is higher than that for 2'-deoxycytidine and araC (Table I) in agreement with its more considerable structural modifications in the sugar as well as in the pyrimidine moieties to compare with araC where the cytosine ring remains unchanged.

The biological end-effect of ara-5AC resides primarily in the inhibition of the DNA synthesis[2]. The phosphorylation of the drug in the cell-free system and in intact L1210 cells (Figs 1 and 3) as well as the inhibition of DNA polymerase (Figs 2 and 4) comparable to that produced by araC provide strong circumstantial evidence that ara-5AC similarly to araC inhibits the synthesis of DNA in eukaryotic cells by interfering with the activity of DNA polymerase α. Nevertheless, in distinction to araC the incorporation of 5-azacytosine ring into DNA results in the impaired DNA methylation presumably by blocking the activity of 5-methylcytosine transferase[5] (S-Adenosyl-L-methionine: DNA (cytosine-5-)-methyltransferase, EC 2.1.1.37). Whether other mechanisms are involved, *e.g.* the formation of terminal linkages of the drug with a growing DNA strand[6], altered interactions of nuclear proteins with the substituted DNA[7] or DNA alkali-labile lesions[8] as a consequence of the incorporation of 5-aza-

TABLE I
Michaelis constants of deoxycytidine kinase for ara-5AC, araC, and dCyd

Compound	$K_m \cdot 10^{-5}$ mol l^{-1}	v_{max} . nmol
Ara-5AC	7·0	19·2
AraC	4·8	8·7
dCyd	2·7	5·8

cytosine remains to be elucidated. To obtain the direct evidence that ara-5AC is responsible for the inhibition of DNA-dependent DNA polymerase α the availability of its 5'-triphosphate is required; however, the synthesis of this compound has not yet been achieved.

Earlier we have reported that following the implantation of 10^4 L1210 leukemic cells to DBA/2 mice ara-5AC increases the life span of treated animals to the same extent as araC but in distinction to araC in a manner which is schedule-independent[1], *i.e.*, the total dose was equally effective regardless whether given once or divided into 5 equal portions. This phenomenon cannot be explained in terms of different stability of the drugs in question since ara-5AC in solution is considerably more labile than araC[3]. Further work is necessary to clarify the mechanism of action of ara-5AC and to elucidate its relation to the effects of araC.

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