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THE EFFECT OF THE CYTOSTATIC AGENT 9-α-D-ARABINOFURANOSYLADENINE ON THE NUCLEIC ACID METABOLISM IN L5178Y CELLS

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Abstract—The influence of 9-α-D-arabinofuranosyladenine (α-araAdo) on cell metabolism of mouse lymphoma cells (L5178y) has been studied on cellular as well as on subcellular level. α-AraAdo strongly inhibits the cell proliferation of L5178y cells. Starting with 3×10^3 cells/ml and an incubation period of 72 hr, the drug reduces the cell proliferation to 50 per cent at a concentration of 12 μM. The cells die because of "unbalanced growth". The cytostatic effect of α-araAdo can be abolished by coincubation with deoxyadenosine but not by adenosine. At cytostatic concentrations α-araAdo inhibits selectively the incorporation rate of thymidine into DNA but not the incorporation rates of precursors into RNA or protein. α-AraAdo is intracellularly phosphorylated to α-araATP. The compound has no effect on the extent of intracellular phosphorylation of exogenous administered adenosine or deoxyadenosine. α-AraAdo is incorporated into DNA but not into RNA; one molecule of α-araAdo is incorporated per 14,000 molecules of deoxyadenosine.

All pentose moieties of naturally occurring nucleosides (both the components of nucleic acids and the nucleoside antibiotics) are always glycosidically linked to nitrogen atom of a pyrimidine or purine in the β-form. Only very few reports on the biological activity of α-nucleosides are available [1]. In one study 9-α-D-arabinofuranosyladenine (α-araAdo) and 9-α-D-arabinofuranosyl-8-azaadenine were found to exert antiviral activity [1].

Stimulated by our findings showing that α-araATP is a potent inhibitor of the mammalian DNA polymerase α and has neither an effect on the activity of DNA polymerase β nor on herpes simplex virus-induced DNA polymerase (W. E. G. Müller, submitted for publication) we started to study the biochemical influence of α-araAdo on cellular as well as on subcellular metabolic events.

MATERIALS AND METHODS

Compounds. The following materials were obtained: Adenosine and deoxyadenosine from Serva, Heidelberg, Germany; methyl[^{3}H]thymidine (sp. act. 19 Ci/m-mole), [^{3}H]uridine (generally labeled; sp. act. 6.3 Ci/m-mole), L-[^{3}H]tryptophan (generally labeled; sp. act. 1 Ci/m-mole), [G-^{3}H]deoxyadenosine (sp. act. 7.5 Ci/m-mole) and [G-^{3}H]-adenosine (sp. act. 9.6 Ci/m-mole) from The Radiochemical Centre, Amersham, England; DEAE Sephadex (A-25) from Deutsche Pharmacia, Frankfurt, Germany; micrococcal nuclease (Staphylococcus aureus) (sp. act. 8000 units/mg), spleen phosphodiesterase (bovine spleen) (sp. act. 2 units/mg) and alkaline phosphatase (bovine intestine) (sp. act. 35 units/mg) from Boehringer–Mannheim, Tutzing, Germany.

We thank Dr. W. Hanske (H. Mack, Illertissen, Germany) for the sample of α-araAdo and β-araAdo. 9-α-D-araATP was kindly supplied by Dr. L. Lee Bennett, Jr. (Kettering-Meyer Lab., Southern Research Institute, Birmingham, AL, U.S.A.). The materials were chromatographically pure, as checked by t.l.c.

Cell culture. L5178y cells were grown in Fisher's medium for leukemic cells, supplemented with 10% horse serum (Grand Island Biological Co., Grand Island, NY) in suspension culture. For the dose-response experiments, the cultures (5 ml) were initiated routinely by inoculation of 5×10^3 cells/ml and incubated at 37°C in roller tubes for 72 hr; the controls reached a cell concentration of about 3×10^5/ml. The cells needed 120 hr to reach the stationary phase. α-AraAdo was dissolved in 0.9% NaCl solution and added to the assays in a volume of 0.5 ml. The ED_{50} was estimated by logit regression. In some experiments different incubation conditions were used, as described in the text. Cell concentrations and volume distributions were determined with a Model B Coulter counter with a size-distribution plotter (Coulter Electronics, Hialeah, Fla.). The number of doublings was calculated with a previously published formula [2]. The determination of cell viability was performed as described [2].

Incorporation of nucleic acid and protein precursors. For the determination of DNA, RNA and protein synthesis, suspensions (volume 50 ml) of exponentially growing cells at 2×10^5 cells/ml were supplemented with α-araAdo 20 min prior to the addition of the labeled precursors 25 μCi [^{3}H]dThd, 25 μCi [^{3}H]Urd, and 25 μCi [L-^{3}H]Trp. The incubations with the precursors were continued up to 60

Abbreviations—α-araAdo = 9-α-D-arabinofuranosyladenine. α-araAMP, α-araADP and α-araATP = 9-α-D-arabinofuranosyladenosine-5′-mono, di, triphosphate.

min. Samples of 5 ml were analyzed for acid-insoluble radioactivity [3].

Preparation of [^{3}H]α-araAdo. α-AraAdo was labeled by catalytic exchange in solution with tritium gas [4]. The tritiated α-araAdo was isolated according to Tsuboi *et al.* [5]. Under the preparation conditions used the compound was labeled in 2- and 8-position [6]. The sp. act. of [^{3}H]α-araAdo was found to be 2 Ci/m-mole (7.8 mM/l.). The purity of the material was greater than 98 per cent, as checked by t.l.c. (see below).

Intracellular [^{3}H]nucleoside phosphates. Fifty-ml cultures containing 3×10^6 L5178y cells/ml were incubated for 30 min at 37° with unlabeled and radiolabeled nucleosides. After incubation, the cells were harvested and subsequently washed three times with saline by centrifugation (3000 *g* for 3 min at 0°). The cellular pellet was suspended in 5 ml of 60% aqueous methanol and homogenized by twenty strokes in a Dounce homogenizer with the tight-fitting pestle. The methanol insoluble material was removed by centrifugation (10,000 *g* for 10 min at 0°) and the resulting extract was evaporated to dryness. The residue was dissolved in 0.5 ml of 0.05 M NH_4HCO_3 and applied to a column of DEAE-Sephadex A-25 (0.5 × 5 cm) previously equilibrated with the same buffer. Subsequently the column was washed with the equilibration buffer and then eluted with a linear 0.05 M (pH 8.0) to 0.4 M (pH 9.0) bicarbonate gradient (200 ml) as described [7]. The total fractions were counted in 10 ml aquasol. The nucleosides appeared during the wash with 0.05 M NH_4HCO_3, the nucleotides at the following molarity of bicarbonate: α-araAMP, 0.30 M; α-araADP, 0.32 M; α-araATP, 0.36 M; AMP, 0.28 M; ADP, 0.34 M; ATP, 0.38 M; dAMP, 0.22 M; dADP, 0.29 M and dATP, 0.36 M.

Incorporation of araAdo into DNA. Exponentially growing cells, 2.25×10^8, were incubated in 500 ml of culture medium in the presence of 21 μg [^{3}H]α-araAdo (sp. act., 2.0 Ci/m-mole) for 24 hr. Subsequently nucleic acids from the cells were extracted by the method of Kirby and Cook [8]. The ratios of the absorbences of the following wavelengths (nm) were 260:280 = 2.0 and 260:240 = 1.6. From 2.25×10^8 cells 1.07 mg DNA were isolated. This corresponds to a yield of about 70 per cent (DNA content per lymphocyte, 6.8×10^{-12} g [9]). The nucleic acid preparation contained 58 per cent DNA, 36 per cent RNA and 2 per cent protein. This preparation was purified by CsCl density centrifugation as described [10]. Two distinct DNA bands, a small satellite band (buoyant density of 1.693 g/ml) and a main band (1.704 g/ml), and one RNA band (greater 1.8 g/ml) were obtained.

Digestion of DNA. The purified DNA from cells incubated with [^{3}H]α-araAdo was digested with micrococcal nuclease, spleen phosphodiesterase and alkaline phosphatase. The detailed experimental procedures are given earlier [10].

Analytical methods. Thin layer chromatography was performed in an ascending system on cellulose plates with the following system: *n*-butyl alcohol, saturated with distilled water; the different R_f values are: adenosine 0.29; deoxyadenosine 0.27; β-araAdo; 0.28 and α-araAdo 0.34. DNA was determined by the method of Kissane *et al.* [11], RNA by the orcinol reaction [12], and protein according to the method of Lowry *et al.* [13].

RESULTS

The studies on the biochemical basis for the cytostatic activity of α-araAdo has been studied on the cellular (cell growth) as well as subcellular level (incorporation studies into nucleic acids, phosphorylation of nucleosides).

Effect on cell proliferation. α-AraAdo is an effective inhibitor of cell proliferation. In dose-response experiments with L5178y cells, starting with 5000 cells/ml and an incubation period of 72 hr, the cell growth was reduced to 50 per cent (= ED_{50}) by 3.2 ± 0.4 μg/ml (= 11.9 μM). To check the influence of the compound on cell proliferation of exponentially growing cells, the drug was added for 24 hr to cultures grown to a density of 100,000 cells/ml. Under these conditions the ED_{50} has been determined to be 6.7 ± 0.6 μg α-araAdo per ml.

The concentration range in which the compound acts cytostatically is limited; complete cytostasis is observed up to 2.3 times the ED_{50} (for an incubation period of 24 hr with cell cultures of a density of 100,000 cells/ml). After exposure of cells to four times the ED_{50} of α-araAdo, followed by the procedures of washing the cell suspension once to remove the drug, of diluting and plating, viability (expressed by the ratio of the plating efficiency of the treated cells to the plating efficiency immediately before addition of α-araAdo) drops to 85 per cent. At six times the ED_{50} the survival rate decreases to 47 per cent.

After incubation of L5178y cells with α-araAdo the cell volume alters significantly. In the presence of the ED_{50} concentration during a 24 hr period, the cell volume increases from 1270 ± 150 μm^3 to 1670 ± 170 μm^3.

The α-araAdo-caused inhibition of cell proliferation could be negated by coincubation with the natural nucleoside dAdo in the roller tube assay (Table 1); the cell doublings increase from 0.76 in the absence of dAdo to 1.63 in the presence of the natural nucleoside. Hence by coincubation of the cells with dAdo almost the control value for the cell doublings is reached (1.79). The inhibitory potency of the compound can not be abolished by coincubation with Ado (Table 1). The natural nucleosides were added at concentrations that had no influence on cell proliferation.

Influence on synthesis of macromolecules in vivo. α-AraAdo exerts a strong inhibitory influence on the incorporation rate of [^{3}H]dThd into DNA (Fig. 1); the incorporation rate is reduced by 50 per cent at a concentration of about 45 μM. α-AraAdo has no effect on the incorporation rate of Urd or Trp into RNA or protein (Fig. 1). In control experiments (without α-araAdo) the incorporation rates into acid-insoluble material were found to be as follows: 24,000 cpm [^{3}H]dThd, 6400 cpm [^{3}H]Urd or 7100 cpm [^{3}H]Trp per 100,000 cells.

Intracellular phosphorylation. α-AraAdo is rapidly phosphorylated in L5178y cells (Table 2). In exponentially growing cells 63 per cent of the α-

Table 1

α-AraAdo (μM)	Additional compound (μM)	Cell concentration after incubation (cells × 10^3/ml)	Cell doublings	Alteration of α-araAdo effect by additional compound (in doubling steps) (increase [+]; decrease [−])
—	—	345	1.79	—
20	—	170	0.76	—
—	dAdo: 30	340	1.76	—
—	Ado: 10	305	1.59	—
20	dAdo: 30	310	1.63	+ 0.87
20	Ado: 10	165	0.73	− 0.03

Negation of the α-araAdo-caused inhibition of cell growth by coincubation with dAdo. The experiments were performed for 24 hr with logarithmically growing cultures at a cell concentration of 1 × 10^5/ml. The degree of cell proliferation is expressed in doubling steps.

araAdo uptaken is present as α-araATP after an incubation time of 30 min. Only 7 per cent was found to be α-araAdo; 14 per cent araAMP and 9 per cent araADP. Seven per cent of the radioactive material derived from α-araAdo did not elute from the DEAE column with the authentic compound; this means that at the most 7 per cent of the α-araAdo taken up by the cells has been degraded metabolically. It was not attempted to identify these products.

Effect on phosphorylation of dATP and ATP. In a series of experiments it was checked whether α-araAdo affects the phosphorylation of the naturally occurring nucleosides Ado and dAdo (Table 2). In the presence of a 10-fold excess of exogenous α-araAdo, Ado as well as dAdo are phosphorylated in the cells to the same amount as in the control cultures (in the absence of α-araAdo). On the other hand exogenously administered Ado and dAdo have no influence on intracellular phosphorylation processes of α-araAdo as well.

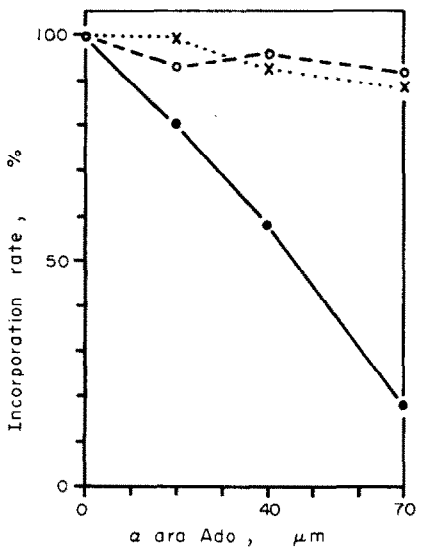

Fig. 1. Effect of α-araAdo on the synthesis of macromolecules in exponentially growing cells. The procedures were performed as described in Methods. The incorporation rate of the control experiment is set to 100 per cent. Each point represents means of counts of 6-fold sample. Standard deviation does not exceed 10 per cent. Incorporation of [^{3}H]dThd into DNA (●——●), of [^{3}H]Urd into RNA (O---O) and of [^{3}H]Trp into protein (×·····×).

Incorporation into DNA. Exponentially growing L5178y cells were incubated in the presence of [^{3}H]α-araAdo for 24 hr. Subsequently the nucleic acids were extracted from the cells and then DNA was separated from RNA by CsCl density gradient centrifugation, as described in Methods. No radioactivity was found in the RNA fraction. However the DNA fraction contained a remarkable amount of radioactivity; the sp. act. was found to be 293 × 10^3 dpm/mg DNA. This DNA was digested enzymatically with micrococcal nuclease, spleen phosphodiesterase and alkaline phosphatase and chromatographed. As shown in Fig. 2, 81 per cent of the radioactivity was detected on the α-araAdo spot. No radioactivity was found on the R_f region of β-araAdo. This finding is a strong indication that it is α-araAdo which is incorporated into DNA. Basing on the (Ade + Thy):(Gua + Cyt) ratio of 1.46, determined from Ehrlich ascites tumor cells [14], 14,000 moles of deoxyadenosine-moieties are incorporated per mole of α-araAdo.

DISCUSSION

The present study is an approach to elucidate the mode of action of a nucleoside in the unusual α-glycosidically linked form on the biochemical level. We selected D-arabinofuranosyladenine (araAdo) because of the well documented fact, that the β-form of this compound exerts both strong cytostatic and antiviral activity (surveys: 10, 15). The studies were performed with mouse lymphoma cells (L5178y), growing in suspension.

α-AraAdo is an inhibitor of the cell proliferation. The ED_{50} concentration was found to be 12 μM. Under comparable conditions the ED_{50} concentration of β-araAdo is 2.9 μM [10] and for deoxyadenosine 55.8 μM (unpublished result). The α-araAdo inhibitory effect is reversible up to 2.3 times the ED_{50}; this limited concentration range has also been observed for β-araAdo [10]. The experiments described here clearly demonstrate that α-araAdo causes cytostasis by a selective inhibition of DNA synthesis. The evidence is as follows: First, during treatment with the drug the average cell volume

Table 2

Labeled compound (μM)	Additional compound (μM)	Distribution in the methanol-soluble fraction (per 10^6 cells) Total (pmoles)	Nucleoside (pmoles)	Monophosphate (pmoles)	Diphosphate (pmoles)	Triphosphate (pmoles)
[^{3}H]α-AraAdo: 0.5	—	0.9	0.06	0.13	0.08	0.57
	Ado: 5	1.1	0.20	0.18	0.07	0.61
	dAdo: 5	0.9	0.11	0.10	0.07	0.59
[^{3}H]Ado: 0.5	—	6.9	1.7	0.1	0.3	4.7
	α-araAdo: 5	6.3	1.2	0.2	0.4	4.3
[^{3}H]dAdo: 0.5	—	1.3	0.49	0.03	0.04	0.69
	α-araAdo: 5	1.5	0.61	0.07	0.02	0.74

Phosphorylation of radiolabeled α-araAdo, Ado and dAdo in L5178y cells in the absence as well as presence of additional unlabeled nucleosides. The procedures are described in Methods. The specific radioactivity was: α-araAdo 4.4×10^3 dpm/pmole; Ado 9×10^3 dpm/pmole and dAdo 9×10^3 dpm/pmole.

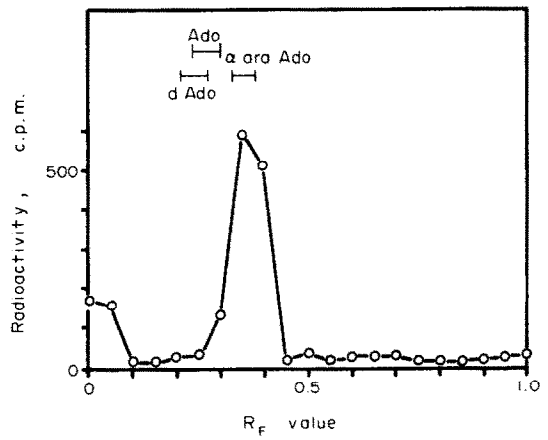

Fig. 2. Chemical nature of the incorporated radioactivity in DNA, purified from L5178y cells incubated with [^{3}H]α-araAdo. The DNA was digested to the nucleoside level and chromatographed as described in Methods. A 50 μl aliquot of the DNA digest (corresponding originally 10 μg DNA) was chromatographed. y-Axis: Radioactivity of the spots (0.5 × 1.0 cm).

increases and thus shows the phenomenon of "unbalanced growth" [16]; second, the cytostatic effect of α-araAdo can be abolished by coincubation with deoxyadenosine but not by adenosine and third, the compound inhibits only the incorporation rate of thymidine into DNA and has no effect on the incorporation rates of precursors into RNA or protein. The latter effect is also known for β-araAdo with the same cell system [10].

A further step on the way of the elucidation of the mode of action of the compound on subcellular level was the determination of the capacity of the nucleoside and nucleotide kinases to use α-araAdo and its phosphorylated derivatives as substrate. The results revealed that α-araAdo is rapidly phosphorylated to α-araATP without affecting the phosphorylating capacities of the kinases for deoxyadenosine and adenosine. From the data of Shipman *et al.* [17] it seems to be likely that β-araAdo also inhibits a step in the phosphorylation chain of Ado. This finding confirms and extends the findings published by Bennet *et al.* [18] (1, discussion remark), using the 9-α-D-arabinofuranosyl-8-azaadenine and analogue.

The intracellularly formed α-araATP could be inhibitor and/or substrate for a series of enzymes. From previous reports it is known that the arabinosylnucleotides are inhibitors for DNA polymerase(s): 1-β-D-arabinofuranosylcytosine-5'-triphosphate is an effective inhibitor of cellular as well as viral (from oncogenic RNA viruses) DNA polymerases *in vitro* [19–21] and 9-β-D-arabinofuranosyladenine-5'-triphosphate a potent inhibitor of cellular as well as viral (from oncogenic RNA viruses and herpes simplex viruses) DNA polymerases *in vitro* [19, 10, 15] as well as *in vivo* [7]. RNA polymerases are not affected by these two analogues [22]. In the light of these data it seemed conceivable to prove at first whether α-araATP exerts also an inhibitory activity on DNA polymerase(s). In fact α-araATP has been found to be a potent inhibitor of cellular DNA polymerase α (the DNA replication enzyme; cf. [23] with a K_i to K_m ratio of 0.48, while the cellular DNA polymerase β (the DNA repair enzyme; cf. [23]) as well as the herpes simplex virus-induced DNA-dependent DNA polymerase are insensitive towards α-araATP (W. E. G. Müller, submitted for publication). Therefore at the present stage of knowledge we assume that the α-araAdo-caused reduction of cell proliferation is a competitive inhibition of cellular DNA polymerase α-araATP.

α-AraATP is also used, to a small extent, as substrate for the DNA polymerase. This conclusion stems from the observation that α-araAMP moieties are incorporated into DNA during incubation of the cells with labeled α-araAdo. The data presented show that α-araAdo is not converted to the β-anomer form because after digestion of the araAdo-DNA, no radioactivity is found on the R_f range of β-araAdo; 81 per cent of the radioactivity was found on the α-araAdo spot. The clear-cut evidence that aranucleotides are incorporated into DNA is known since 1972 (e.g. [24, 25]). Future studies must stress the question about the biological consequence(s) of the incorporated α-araAMP moieties into DNA, an observation which is also known from studies with other arabinosyl nucleotides (e.g. [10, 26]). However, the fact that α-araAdo inhibits cell prolifer-

ation of L5178y cells reversibly is a strong hint that the incorporated α-araAMP moieties are incorporated in the interior of the DNA.

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