SENSITIVITY OF SOME ARABINOSYLCYTOSINE DERIVATIVES TO ENZYMATIC DEAMINATION BY CYTIDINE DEAMINASE FROM MOUSE KIDNEY*

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Enzymatic deamination of derivatives of arabinosylcytosine by partially purified cytidine deaminase isolated from mouse kidney is studied. The rate of deamination of 5′-chloroarabinosylcytosine (*Ia*), 5′-bromoarabinosylcytosine (*Ib*), 5′-chlorocyclocytidine (*II*), and 2′,5′-anhydroarabinosylcytosine (*III*) is compared with that of arabinosylcytosine, of its N^4-acetyl-, tri-O-acetyl-, tetraacetyl derivatives and of cyclocytidine. The deamination of the anhydro derivative *III* to the corresponding uracil derivative *IV* proceeds significantly slower than that of arabinosylcytosine, and comparably to that of cyclocytidine. The deamination of the 5′-halogeno derivatives *Ia*, *Ib*, and *II* proceeds *via* the anhydro derivative *III* to *IV*. Some of the known arabinosylcytosine derivatives, cyclocytidine, N^4-acetyl-, tetraacetyl-, and tri-O-acetyl derivatives, show a resistance to enzymatic deamination. Different nucleoside analogs are tested as inhibitors of deaminase; only 2-β-D-ribofuranosyl-1,2,4-triazol-3-one possesses some activity.

1-β-D-Arabinofuranosylcytosine (araC), the most effective agent in the therapy of acute myeloblastic leukemia, can be metabolised along two enzymatic pathways: by phosphorylation to araCTP, a potent inhibitor of DNA synthesis[1,2], or through deamination to the inert metabolite arabinosyluracil[3–5] (*cf.* ref.[6,7]). Resistance to araC was at first attributed to a decrease in the ability to convert this antimetabolite to araCTP, although it was apparent that an increased deamination of araC offered an alternative mechanism to render the drug ineffective[8].

Evidence for a role of deamination in the development of resistance to araC was provided by Steuart and Burke[9] who found higher levels of cytidine deaminase in leukocytes from leukemic patients whose disease did not respond to araC than in the cells from patients sensitive to this agent. In addition, prolonged therapy with araC resulted in an increase in nucleoside deaminase levels in bone marrow cells; it was suggested that enzyme induction could account for the alteration in nucleoside

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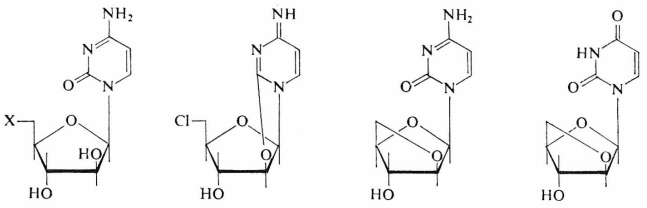
deaminase activity[9]. Indeed, it was shown[10] that HeLa cells grown in medium with added arabinosylcytosine exhibited a fourfold increase in cytidine deaminase activity. Tetrahydrouridine (THU) has been found as a potent inhibitor of cytidine deaminase activity[11], inhibiting the deamination of araC *in vitro* as well as *in vivo*[12,13]. Simultaneous application of THU to patients treated with araC potentiated significantly the antitumor activity of araC[14,15].

Recently, a specifically selected series of analogs of arabinosylcytosine was prepared and studied[16–18]. The attention was focused on the 5′-deoxy derivatives of arabinosylcytosine which were not able to form the 5′-triphosphate ester, the proposed proper active form of arabinosylcytosine[1,2]. A new group of biologically active derivatives was discovered[16,19,20]. A considerable activity was observed in particular with the 5′-chloro derivative *Ia* which was shown one of the most potent inhibitors of DNA synthesis, among all the compounds tested in our experiments[19,20]. A considerable biological activity was observed[16] also at the other 5′-halogeno derivatives *Ib*, *II*, and the anhydro derivative *III*. On the basis of the easy chemical transformation[18] of *Ia*,*b* and *II* to the anhydro derivative *III* it was suggested that their mechanism of action should differ from that of arabinosylcytosine. The suggestion was also supported by difference between both groups of compounds at their preliminary test results[16,17,20].



Ia, X = Cl
Ib, X = Br

II *III* *IV*

It was therefore interesting to know whether the compounds would differ from arabinosylcytosine also in the resistance against cytidine deaminase.

The study was completed by comparative experiments with arabinosylcytosine and its derivatives. Substances capable to inhibit cytidine deaminase activity and to prevent the deamination of arabinosylcytosine were also searched for.

EXPERIMENTAL

Cytidine deaminase was isolated from BALB/c mouse kidney tissue (20 g) and partially purified by ammonium sulfate fractionation[21,22]. Enzyme preparation dialyzed against 0·01M buffer

(pH 7·0) containing 0·005 mol l^{-1} dithiothreitol was stored at −20°C and enzymatic activity was stable for many weeks under these conditions. Protein concentration was determined according to ref.[23].

Cytidine Deaminase Assay

$4 . 10^{-3}$ mol l^{-1} Solutions of nucleosides in water were incubated each in three separate tubes. The incubation mixture contained 0·1 ml (0·4 μmol) of arabinosylcytosine (or derivative of arabinosylcytosine), 50 μl of 0·05M sodium–potassium phosphate buffer (pH 7·4), and 50 μl of cytidine deaminase preparation (8 mg protein per assay) in a final volume of 0·2 ml. The enzyme was added to the ice-cooled samples and the mixtures were then incubated at 37°C in Hoeppler thermostate for 30 min, 60 min, and 5 h. The reaction was stopped by an equal volume of 1·0M perchloric acid and the sample was cooled in ice-bath for 10 min. The precipitated protein was removed by centrigufation (2 000 r.p.m., 10 min), the supernatant was neutralized with 1·0M-KOH to pH 7·0 and cooled in ice-bath for 5 min. The insoluble $KClO_4$ was pelleted by centrifugation (4 000 r.p.m., for 15 min). The supernatant containing the nucleoside substrates and the products of enzymatic deamination was analysed by liquid chromatography on silica gel columns[24].

Chromatographic Analysis

The high performance liquid chromatography (HPLC) equipment has been described recently[24]. For normal-phase chromatography (A) the silica packing was LiChrosorb SI 100, 5 μm particle size (E. Merck, Darmstadt, G.F.R.), and the mobile phase was prepared from 74 volume parts of dichloromethane, 22 parts of methanol and 3 parts of an aqueous solution containing 2·65 mol formic acid and 0·5 mol ammonium formate in 1 liter (pH 2·5), *cf.*[24]. Reversed-phase liquid chromatography (B) was performed on Separon SI C 18 octadecyl-silica, 10 μm particle size (Laboratorní přístroje, Prague, Czechoslovakia), with a mobile phase consisting of 0·1M acetic acid (98%) and dimethylformamide (2%). Stainless steel columns, 25 cm long, 4·2 mm inner diameter, were used in both cases. Inlet pressure, 7·6 MPa (A) and 7·0 MPa (B); flow rate, 1·0 ml/min (A) and 0·85 ml/min (B). The detection was performed spectrophotometrically at 254 nm. The retention (capacity) factors k (*cf.*[24]) for the compounds of interest are given in Table II.

RESULTS AND DISCUSSION

The rates of enzymatic deamination of arabinosylcytosine and of its derivatives by mouse kidney deaminase *in vitro* are given in the Table I. The natural nucleoside cytidine and arabinosylcytosine are substrates for cytidine deaminase[22] and are deaminated very rapidly, with a rate preference for cytidine; cytidine is completely deaminated within 30 min, while 22% of arabinosylcytosine remain unchanged under the same conditions. In the case of cytidine, the deaminated product was measured in the form of uridine (86%) in admixture with uracil (14%). The percentage of uracil increases in time. Arabinosyluracil (araU) was the only deaminated product in the case of arabinosylcytosine.

In contrary to that, the N^4-acetyl derivatives of arabinosylcytosine (N-acetyl-, tetraacetyl-), possessing an amidic nitrogen instead of an amino group in position 4,

cannot serve as substrates for cytidine deaminase before a prior cleavage to the unsubstituted arabinosylcytosine what limits their rate of deamination. Arabinosyluracil was the only deaminated product observed. At N^4-monoacetylarabinosylcytosine, 4% of arabinosyluracil were found after 5 h of incubation. With tetraacetylarabinosylcytosine, no arabinosyluracil was observed after 5 h. Similarly, the lipophilic character of the triacetyl derivative restricts the rate of its deamination. 16% of arabinosyluracil were found after 5 h of incubation. Our results correspond to the observation[25,26] *in vitro* with the N^4-acyl derivatives of arabinosylcytosine which were found to be resistant against cytidine deaminase from mouse kidney. One of them N^4-behenoylarabinosylcytosine, seems to become a potent antitumor agent[26], which should be administered in oil solution, due to its lipophilic character. The cyclocytidine also remains resistant against cytidine deaminase unless it is cleaved to arabinosylcytosine what is in agreement with the result reported.[12] Arabinosyluracil was the only deaminated product detected in the experiment. The amounts of 4%, 7% and 22% of arabinosyluracil were found in the measured interval. Cyclocytidine has been found relatively non-toxic in mice with L1210 leukemia[27], however it did not find any utilization in human chemotherapy for serious side-effects (see[7]). In both types of compounds, the N-acetyl derivative and cyclocytidine, the rate

TABLE I

Rates of enzymatic deamination of arabinosylcytosine derivatives after incubation at 37°C

Compound	Per cent of deaminated nucleoside		
	30 min	60 min	5 h
Cytidine	100	100	100[a]
AraC (ref.[28,35–37])	78	95	100[b]
Cyclocytidine (ref.[35])	4	7	22[b]
N^4-Acetyl-araC (ref.[38])	4	4	4[b]
Tetraacetyl-araC (ref.[41])	0	—	0
Tri-O-acetyl-araC (ref.[40])	0	—	16[b]
Ia (ref.[17])	40	73	100[c]
Ib (ref.[16,18])	22	39	99[c]
II (ref.[17])	0	11	53[c]
III (ref.[16,18])	2	4	28[c]
AraC + 2-β-D-ribosyl-1,2,3-triazol-3-one	2	20	58[b]
AraC + thioguanine	64	—	87[b]

[a] Measured as uridine + uracil; [b] measured as arabinosyluracil; [c] measured as *IV*.

of deamination is diminished when compared with that of arabinosylcytosine. The experiments indicated a slight inhibitory effect of both compounds against cytidine deaminase. A more detailed study, both *in vitro* and *in vivo*, of their inhibitory activity is under investigation.

The most interesting result was observed with 2',5'-anhydroarabinosylcytosine[18] (*III*). Recently it was found that the 5'-chloroarabinosylcytosine[17] (*Ia*), one of the most potent inhibitors of NA synthesis observed in our experiments,[16,19,20] was easily converted to the anhydro derivative *III* by the action of alkali[16,18]. A similar conversion[16,18] and biological activity[16,19,20] as well was observed with the other 5'-halogeno derivatives, 5'-bromo derivative[18] *Ib* and 5'-chlorocyclocytidine[17] *II*. In the present experiments, the rate of deamination of 2',5'-anhydroarabinosylcytosine *III* was found significantly lower than that of arabinosylcytosine. 2%, and 4% 28% of anhydroarabinosylcytosine *III* were deaminated when compared with 78%, 95% and 100% of arabinosylcytosine. Anhydroarabinosyluracil *IV* was the only deaminated product observed. The rate is one of the slowest within the group of arabinosylcytosine analogs tested, what considerably enhances the biological importance of *III*. The rates of deamination of the other 5'-halogeno derivatives *Ia,b* and 5'-chlorocyclocytidine (*II*) are also remarkably lower than that of arabinosylcytosine but

TABLE II
High performance liquid chromatography, k values

Compound	Normal phase	Reversed phase
Uracil	0·75	0·43
Uridine	1·66	0·74
AraU (ref.[28])	1·53	1·06
Cyclouridine (ref.[34])	3·45	0·22
Cytidine	4·57	1·51
AraC (ref.[28,35–37])	4·20	1·84
Cyclocytidine (ref.[35])	12·2	1·22
N^4-Acetyl-araC (ref.[38])	0·84	6·26
Ib (ref.[16,18])	1·35	1·97
Ia (ref.[17])	1·94	10·5
III (ref.[16,18])	2·19	2·24
II (ref.[17])	6·51	11·6
5'-Chloro-araU (ref.[39])	0·54	11·9
Tri-O-acetyl-araC (ref.[40])	0·11	13·0
Tetraacetyl-araC (ref.[41])	0·05	16·8
IV (ref.[16,18])	0·50	1·47

substantially higher than with anhydroarabinosylcytosine (*III*). The deamination of the compounds *Ia*, *Ib* and *II* proceeds mostly *via* the anhydro derivative *III*, too, as the uracil anhydroderivative *IV* was the only deaminated product observed. Chloroarabinosylcytosine (*Ia*) was deaminated in the rate 40%, 73%, 100%; bromoarabinosylcytosine (*Ib*) was deaminated in the rate 22%, 39%, 99%; chlorocyclocytidine (*II*) was deaminated in the rate 0, 11%, 53% in the measured intervals.

The results on deamination support the suggestion[16], based on chemical transformation[18] and on preliminary test results[16,19,20], that the mechanism of action of the 5′-halogeno derivatives *Ia*, *Ib*, and *II* and of anhydroarabinosylcytosine (*III*) as well should differ from that of arabinosylcytosine and that the anhydro derivative *III* might be the proper active form of the 5′-halogeno derivatives *Ia*, *Ib* and *II*.

The experiments were completed by an effort to find out a new inhibitor of cytidine deaminase. A series of different nucleoside analogs were tested, namely 6-mercaptopurine, 6-thioguanine, 4-thiouridine[28], 4-thioarabinosyluracil[28], arabinosylisocytosine[29], S-DHPA (ref.[30]), 1,2,4-triazol-3-one[31], 2-β-D-ribofuranosyl-1,2,4-triazol-3-one[31], 2,4-bis-(β-D-ribofuranosyl)-1,2,4-triazol-3-one[31], pyrimid-2-one[32]; a particularly attention was paid to the thio derivatives. In an equivalent mixture with arabinosylcytosine the rate of deamination to arabinosyluracil was measured. Of all compounds tested, only two compounds possessed some inhibitory activity. A higher and more significant activity was expressed by 2-ribofuranosyl derivative of 1,2,4-triazol-3-one, as the only one of the triazolone derivatives tested. A very low activity was observed with 6-thioguanine. According to the reported clinical experience[33], the combination of arabinosylcytosine and 6-thioguanine has the ability to extensive cell kill even in patients with late refractory acute lymphocytic leukemia, however, the combination was severely myelotoxic[33].

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