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Cytotoxicity of 2′-fluoro-5-iodo-1-β-D-arabinofuranosylcytosine and its relationship to deoxycytidine deaminase

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2′-Fluoro-5-iodo-1-β-D-arabinofuranosylcytosine (FIAC) and several structural analogs were synthesized recently by Fox and coworkers [1]. Some of them were found to have good activity against herpes simplex virus (HSV) [2, 3]. The selectivity of these compounds against HSV is related to their behavior as substrates of HSV-induced thymidine kinase [4] versus cellular thymidine kinase. The mechanism of their antiviral action may be related to the inhibition of DNA polymerases by the triphosphate derivatives of those pyrimidine nucleoside analogs [2, 5]. The cytotoxicity of FIAC was found to vary with the cell lines used for examination [2, 6]. When 2′-fluoro-5-iodo-1β-arabinofuranosyluracil (FIAU), the deaminated analog of FIAC, was evaluated for its cytotoxic effects, FIAU was found to be equal to, or more toxic than, FIAC in most of the cell lines tested [2, 6]. This suggested the possibility that the FIAC cytotoxicity could, to a certain extent, be dependent on its transformation to FIAU through the action of dCyd deaminase. The deamination of FIAC to FIAU by mouse and human leukemic cell dCyd deaminase was demonstrated previously although no detailed kinetic behaviors were given [7, 8]. In view of the apparent kinetic difference between the dCyd deaminases of humans and mice [9, 10], we examined how FIAC and its analogs behaved as substrates of human dCyd deminase and whether dCyd deminase played an important role in the action of FIAC. The results are discussed in this paper.

Materials and methods

Chemicals. dCyd and most of the other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO. The [2-^{14}C]dCyd was purchased from Moravek Biochemicals, Brea, CA. The 5-ethynyl araCyd and 5-ethynyl, 2′-F-araCyd were sent by Dr. Sharma and Dr. Bobek of Roswell Park Memorial Institute, Buffalo, NY. All the other analogs were synthesized in the laboratory of Dr. J. J. Fox at Memorial Sloan Kettering Cancer Center, New York, NY.

Enzyme assay. The procedure was essentially the same as that published by Chabner *et al.* [9] for routine dCyd deaminase assay except that the components of the assay were modified. The reaction mixture contained 0.6 mM [2-^{14}C]dCyd, 45 μCi/mmole, 0.6 mg/ml bovine serum albumin, 5 mM dithiothreitol (DTT), and 2.5 mM Tris–HCl, pH 8.0, and enzyme in a total volume of 100 μl. After incubation at 37° for 30 min, 50 μl of 1.2 N trichloroacetic acid (TCA) was added. After centrifugation, 100 μl of supernatant fluid was loaded on a 0.5 × 2.5 cm Dowex 50 H^+ column. Then 1.5 ml of H_2O was used to elute the [2-^{14}C]dUrd which was mixed with aqueous counting fluid and counted in a Beckman LC 100 liquid scintillation counter. One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 nmole dUrd/min at 37°.

For those analogs which were not available in a radiolabeled form, the reaction conditions were essentially the same as above except that 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), pH 8.0 (0.01 M), was used instead of Tris–HCl buffer. These assays were terminated by addition of 4 vol. of cold methanol. The supernatant fraction was removed and mixed with equal volumes of solution containing internal standard (usually

50 μM dThd) for high performance liquid chromatographic (HPLC) analysis. Subtrate and product were determined using analytical reverse phase high pressure liquid chromatography with conditions as follows. Either a Knaver RP-8 (7 μC-8) column (Unimetrics Corp., Los Angeles, CA) or an Alltech C8 (10 μm) column was eluted with appropriate isocratic solutions containing from 1:100 to 1:9 parts acetonitrile in 0.03 N acetic acid (dependent upon analog to be analyzed). HPLC hardware consisted of a Micrometrics dual pump solvent delivery system and variable wavelength u.v. detector interfaced with an autoinjector (100 μl sample loop) and a Shimadzu Chromatopac CR-1A data processor. Both relative and absolute concentrations of substrate and product were determined by two methods: (1) comparison with internal standard after correlation with known samples, and (2) external comparison with authentic samples of known concentrations; results by these methods agreed within 5% when assaying controls. Extent of deamination at each concentration of analog was determined in duplicate, and the K_m and relative V_{max} values were derived by linear regression from double-reciprocal plots. The procedures for dCyd kinase assays were the same as described previously [11].

Preparation of cellular extract. Deoxycytidine deaminase (dCyd deaminase) was extracted from human acute myeloblastic leukemia (AML) cells which were procured by leukophoresis. Packed AML cells (30 ml) were suspended in 150 ml of buffer A (0.01 M Tris, pH 7.5, containing 6 mM DTT). Cells were disrupted by three cycles of freezing and thawing. KCl solution (3 M) was added to make a final concentration of salt of 0.6 M, followed by sonication with four 15-sec bursts using a micro probe on a Branson Sonifier model 200. The suspension was centrifuged at 1500 rpm for 20 min, and deoxycytidine deaminase was purified from the supernatant fraction.

The extraction procedure for solid tumor samples was essentially the same as described above except that the tumor tissue was homogenized first by a tissue homogenizer.

Enzyme purification. The procedure was, essentially, a combined version of the procedures of Chabner *et al.* [9] and Rothman *et al.* [12] with some additional steps, all of which were performed at 4°. These included streptomycin sulfate precipitation, ammonium sulfate fractionation, and chromatography on phenyl Sepharose, DEAE-cellulose, Sephadex G-150 and hydroxylapatite columns.

The activity of highly purified human dCyd deaminase is reported to be unstable [9]. The method used in our laboratory for the storage of the purified enzyme was to keep it in 0.02 M Tris–HCl buffer, pH 8.0, containing 4 mM DTT, 0.6 mg/ml bovine serum albumin and 40% (w/v) glycerol at −70°. Under these conditions, the purified dCyd deaminase was very stable, and more than 80% of the activity was present after 1 year. The specific activity of the final preparation was between 26,000 and 32,000 units/mg for the preparations derived from several individual blast cells. Although the purity was higher than published values [9], the preparation still was not homogeneous, since multiple protein bands appeared after the sodium dodecylsulfate (SDS) gel electrophoresis. No indication of an isozyme was observed throughout the purification, and the general properties of enzyme from one individual to another were similar. Molecular weight was estimated to be 49,000 by the G 150 gel filtration technique, and the isoelectric point was found to be 4.9, using isoelectrofocusing. These values are similar to those published by Chabner *et al.* [9].

Results and discussion

Kinetic behaviors of dCyd analogs. dCyd analogs, with substitution at the 5 and 2′-up position were examined as substrates of human dCyd deaminase; the results are presented in Table 1. The nucleoside graduately lost its binding affinity with substitution of the 2′-up —H of 5-CH_3-dCyd by —F and —OH, whereas the V_{max} ratio study suggested that the 2′-F analog could be deaminated faster than 2′-H analogs if a saturating amount of the substrate was used. The substitution of —H at the 5 position by —CH_3 or —iodo decreased both the affinity and V_{max} to a similar extent, whereas substitution by —C≡C— decreased affinity and V_{max} drastically. FIAC was deaminated by human dCyd deaminase at the same rate as dCyd under optimal conditions although the K_m was about eight times higher than for dCyd.

Cytotoxicity. The growth inhibition of FIAC of human KB cells was examined; the results are summarized in Table 2. AraCyd was included as the control to demonstrate the action of tetrahydrouridine, an inhibitor of dCyd deaminase [14]. Tetrahydrouridine, which by itself had no toxicity at 5 μM, partially reversed the cytotoxicity of FIAC and potentiated the cytotoxicity of araCyd. These observations suggested that the cytotoxic effect of FIAC on this human cell line which has dCyd deaminase activity is partially dependent on the activity of the enzyme. Since the cytotoxicity of FIAU was more potent than FIAC (Table 2), the cytotoxicity of FIAC observed in the absence of deaminase inhibitor was, most likely, due to the combined action of FIAC and FIAU.

The partial dependence of the cytotoxicity of FIAC on dCyd deaminase raised the possibility of the preferential killing of cells, which have become resistant to araCyd [15, 16], because they contain a high level of dCyd deaminase activity or are in a physical environment that has a high level of dCyd deaminase. Cells that have a low level of, or no, dCyd deaminase are sensitive to araCyd, provided that all other biochemical parameters are the same. Thus, the combined use of FIAC and araCyd in the clinic should be worth further investigation.

The use of araCyd in the treatment of solid tumors has not yielded good results. Among the many possible reasons

Table 1. Kinetic behaviors of dCyd analogs toward dCyd deaminase

C-5 substituent	K_m^* (relative velocity), μM		
	Parent nucleoside		
	dCyd	2′-F-araCyd	araCyd
—H	38 (1.0)	†	160 (0.85)
—CH_3	87 (0.47)	310 (1.1)	800 (0.1)
—I	60 (0.48)	310 (1.0)	500 (0.05)
—C≡CH	†	800 (0.1)	800 (0.02)‡

* K_m values are means of 1 to 3 duplicate trials; each value of relative velocity in parentheses is the V_{max} for the analog relative to the V_{max} for dCyd (V_{max} dCyd = 1.0).

† Compound not available.

‡ Mixture (1:1) of α and β anomers.

Table 2. Cytotoxicity of nucleoside analogs on human KB cell growth*

Compound	Additive	Concentration required for 50% inhibition of cell growth (μM)
FIAC		5.0
FIAC	Tetrahydrouridine (5 μM)	26.0
FIAU		1.0
AraC		1.0
AraC	Tetrahydrouridine (5 μM)	0.2
AraU		>5.0

* The growth inhibition was estimated 48 hr (two generations) after addition of compound. The culture conditions were the same as described previously [13].

Table 3. Activities of dCyd kinase and dCyd deaminase in tumor cells taken from cancer patients*

Type of tumor	Patient no.	dThd kinase	dCyd kinase	dCyd deaminase
			(nmoles·min^{-1}·mg^{-1})	
Melanoma	1	0.97	0.61	2.42
	2	0.07	0.05	0.56
	3	0.20	0.04	0.19
	4	0.17	0.05	0.91
	5	0.09	0.04	1.05
	6	0.48	0.10	0.80
Lung oat cell carcinoma	1	0.04	0.22	14.40
	2	0.11	0.06	1.25
	3	0.47	0.18	1.11
Soft tissue sarcoma	1	1.64	0.08	0.13
	2	0.32	0.24	1.69
	3	0.38	0.15	1.81
	4	0.03	0.08	0.20
	5	0.21	0.21	0.29
	6	0.30	0.26	1.60
	7	0.44	0.19	0.56

* The methodology for the preparation of cellular extract and the enzyme assays was the same as described in Materials and methods. Each cellular extract was dialyzed before being used for assays.

for the failure of araCyd was the fact that these tumors as a whole may have had high dCyd deaminase levels. Therefore, we examined the dCyd deaminase activities in surgical samples from several patients with solid tumors.

Activities of dCyd deaminase, dCyd kinase and thymidine kinase in clinic tumor samples. The enzyme activities of homogenates from tumor samples taken surgically from patients with melanoma, oat cell carcinoma, and soft tissue sarcoma were examined. The results are shown in Table 3. The activity of dCyd deaminase was high in most of these samples in comparison with dCyd kinase, the araCyd-activating enzyme. The resistance of these solid tumors to araCyd could be partly explained by high dCyd deaminase levels which break down the araCyd to araUrd. FIAC becomes more active once it is deaminated to FIAU, provided that it can be phosphorylated by thymidine kinase present in the tumor. This raises the possibility of utilizing FIAC in combination with other agents for the treatment of patients with some types of solid tumors.

It should be stated that the enzyme activities presented here only represent the activities in the solid tumor mass. These data should not be interpreted as the enzyme level of each solid tumor cell, since the tissue is heterogeneous and possibility of normal cell contamination exists even after pathologists have determined that a majority of cells are cancerous. The important point here is not whether deamination occurs in the cancerous cell or outside it, but simply that FIAC is more cytotoxic when it is deaminated to FIAU so that tissues with high levels of deaminase can be preferentially toxified.

In summary, the deamination of FIAC and its structural analogs by highly purified human deoxycytidine deaminase was examined. FIAC was found to be as good a substrate as dCyd under optimal conditions, wtih a K_m eight times higher than dCyd. The change of the substrate behaviors of dCyd with modification of —H at the 5 and/or 2′ (up) position by other substituents was also evaluated.

The potency of FIAC against human KB cells was found to be partly dependent on the activity of deoxycytidine deaminase, which is high in solid tumor samples taken from patients. The potential use of agents such as FIAC that are activated by deoxycytidine deaminase in the treatment of patients with tumors that have high thymidine kinase and deoxycytidine deaminase activity was discussed.

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Norepinephrine and prostaglandin biosynthesis by iris smooth muscle and iris microsomes

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Catecholamines have been reported to stimulate prostaglandin (PG) biosynthesis in a variety of tissues including spleen [1, 2], heart [3], phrenic diaphragm [4], kidney [5], brain [6], rabbit mesenteric blood vessels [7], rabbit iris smooth muscle [8–10], and cultured cells [11, 12]. The mechanism underlying the stimulatory effect of these amines on PG synthesis is poorly understood. It has been suggested that: (a) they act as cofactors for the cyclooxygenation of arachidonic acid (AA), as shown by their stimulatory effects on PG synthetase activity [13]; and (b) they stimulate PG synthesis through receptor-mediated mechanisms; thus, treatment with the α-adrenergic receptor blocking agent phenoxybenzamine inhibited the appearance of PGs from dog spleen [1] and rabbit kidney [5]. Furthermore, PG synthesis in cell cultures from dog kidney cells [11] and from rabbit splenic pulpa [12] is mediated through α-adrenoreceptors.

In a previous communication from this laboratory [9], we reported that, in rabbit iris smooth muscle, norepinephrine (NE) stimulated PG synthesis in a dose-dependent manner. The NE stimulation of PG synthesis was blocked by indomethacin [10]. To throw more light on the mechanism of NE stimulation of PG release in this tissue, we have compared the effects of NE and other catecholamines on conversion of [1-^{14}C]AA into PGs by iris muscle and iris microsomes.

Materials and methods

[1-^{14}C]AA (sp. act. 56.5 mCi/mmole) was purchased from the Amersham Corp., Arlington Heights, IL; NE and other catecholamines were purchased from the Sigma Chemical Co., St. Louis, MO.

In general, two rabbit irides from pairs were incubated (of the pair, one was used as control) in 1 ml of isoosmotic medium that contained 0.25 μCi of [1-^{14}C]AA (sp. act. 56.5 mCi/mmole) bound to 0.1 mg albumin at 37° for 1 hr. Catecholamines and other agents were added as indicated. At the end of incubation the medium was analyzed for PGs.

Microsomes were prepared from iris muscle as previously described [14]. Microsomes, equivalent to 0.6 mg protein, were incubated in 1 ml of 0.1 M phosphate buffer, pH 7.8, containing 0.25 μCi AA, in the presence and absence of the drug as indicated, at 37° for 1 hr. At the end of incubation the PGs were extracted and analyzed.

The medium was analyzed for prostaglandins. It was acidified with 10% formic acid to pH 3.5 and extracted three times with 3 ml of ethylacetate. The solvent was evaporated under nitrogen. The residue was dissolved in chloroform–methanol (2:1), spotted on Whatman precoated silica gel LK6DF plates, and developed in a solvent system [15] of ethylether–methanol–acetic acid (90:1:2, by vol.). After visualization of the PG standards by exposure to iodine vapor, the radioactive PG spots were located with autoradiography, and their radioactive contents were measured by counting in a Beckman liquid scintillation counter. In the present study, we have analyzed for $PGF_{2\alpha}$ and PGE_2. Data are reported as ^{14}C-radioactivity (cpm) of AA converted into PGs/two rabbit irides, or as ^{14}C-radioactivity converted into PGs/mg of iris microsomal proteins.