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TRANSFORMATION AND METABOLIC EFFECTS OF 5-AZA-2′-DEOXYCYTIDINE IN MICE

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Abstract—In an attempt to understand the preferential action of 5-aza-2′-deoxycytidine against the lymphatic system, metabolic conversions of the tritiated drug in mouse liver and spleen *in vivo* and *in vitro* were followed. Simultaneously, the degradation of the drug resulting in the formation of *N*-amidino-*N*′-deoxy-β-D-ribofuranosylurea was investigated. In the spleen the drug is extensively phosphorylated and incorporated into DNA; however, in the liver and other non-lymphatic tissues its utilization is limited. The analogue affects the formation of DNA in the spleen without concomitantly interfering with the level of cyclic AMP.

5-Azapyrimidines represent a group of highly active cytostatics [1, 2]. Clinical studies have shown that one of them, 5-azacytidine, is especially active in human acute myelogenous leukemia [3, 4]. 5-Aza-2′-deoxycytidine, preferentially affecting the lymphatic system [5, 6], suppresses the cell growth in AKR mouse with lymphatic leukemia, in P388 leukemia bearing mice and in L1210 leukemia [7].

The inhibitory action of 5-aza-2′-deoxycytidine depends on its metabolic conversion [8]. In AKR mouse leukemic cells the analogue inhibits phosphorylation of deoxycytidine and is incorporated into DNA [7]. The action of the drug against the lymphatic system is paralleled by a pronounced inhibition of antibody formation [9] and a block in thymidine incorporation into spleen and thymus DNA [10]. Simultaneously the activity of thymidine and thymidylate kinases measured in tissue extracts is markedly depressed.

In the present study changes in the metabolic conversion and incorporation of 5-aza-2′-deoxycytidine in the liver and spleen were investigated in an attempt to clarify the mechanism responsible for the preferential action of the drug against the lymphatic system. Simultaneously the changes in the synthesis of DNA and the level of cyclic AMP in the spleens of 5-aza-2′-deoxycytidine-treated mice were followed.

MATERIALS AND METHODS

Chemicals. 5-Aza-2′-deoxycytidine and *N*-amidino-*N*′-deoxy-β-D-ribofuranosylurea were prepared [11] by Dr. A. Píškala from this Institute. 5-Aza-2′-deoxycytidine-^{3}H (19.5 Ci/mmole) was prepared by Dr. B. Černý from the Isotope Laboratory (Prague-Krč, Czechoslovakia). Thymidine-2-^{14}C (48 mCi/mmole), deoxycytidine-2-^{14}C (44 mCi/mmole), cytidine-U-^{14}C (200 mCi/mole), and cytidine-2-^{14}C (48 mCi/mmole) were provided by the Institute for Research, Production and Uses of Radioisotopes (Prague, Czechoslovakia). Deoxycytidine and ATP were delivered by Calbiochem-Behring (Luzern, Switzerland).

Animals and cell-free tissue extracts. Three strains of mice (inbred AKR and DBA/2, and random-bred H) kept under standard conditions were used. The experiments were started between 8.00 and 9.00 a.m. with groups of 4–6 female mice (24–26 g body wt). 5-Aza-2′-deoxycytidine and/or radioactive compounds were injected i.p. in a maximal volume of 0.2 ml. The animals were killed by cervical dislocation, bled and the tissues under investigation were removed and homogenized in a cooled glass homogenizer with a tight-fitting Teflon pestle in 3 vol. of cold 25 mM Tris–HCl buffer (pH 7.5) containing 25 mM KCl and 5 mM $MgCl_2$. The homogenates were centrifuged (10,000 *g*, 20 min, 2°) and the supernatant fractions were used as a source of enzyme activities.

Level of cyclic AMP. The level of cyclic AMP was estimated in the homogenates of mouse spleens in 7.5% trichloroacetic acid by a modified cyclic AMP binding assay [11]. Binding protein was prepared from bovine adrenals [12] and the separation of the free and bound cyclic AMP was carried out on albumin-coated charcoal [13]. The samples were measured in triplicates with standards ranging from 0.25 to 16 pmoles and after correction for recovery the results were expressed as pmoles of cyclic AMP per mg protein.

Assay of deoxycytidine kinase and cytidine deaminase. The activity of enzymes was measured in 66 mM Tris–HCl buffer (pH 7.5) at 37° in a total volume of 0.3 ml in the presence of post-mitochondrial supernatant fractions corresponding to 25 mg wet weight of individual tissues. The incubation mixture for the deaminase assay contained 0.1 mM cytidine-2-^{14}C, deoxycytidine-2-^{14}C or 5-aza-2′-deoxycytidine-^{3}H. The assay mixture for deoxycytidine kinase contained 0.1 mM substrate, 6 mM ATP with 6 mM Mg^{2+} ions and 2 mM dithiothreitol. Aliquots of the incubation mixture withdrawn during the linear course of the respective enzyme reaction (usually 10 min) were separated on Whatman No. 1 paper in the solvent system composed of isobutyric acid–ammonium hydroxide–water (44 : 1 : 22) (deam-

ination), and of ethanol–tertiary butanol–formic acid–water (60:20:5:15) (kinase reaction). The radioactivity of individual compounds, localized on chromatograms with a Frieseke–Hoepfner scanner, and identified according to the position of standards, was measured using liquid scintillation system Isocap/300 (Nuclear Chicago Division).

Incorporation of 5-aza-2′-deoxycytidine-^{3}H and of pyrimidine precursors. The tissues under investigation were homogenized in 10 vol. of cold 0.25 mM KCl, and 1-ml portions of the homogenates were mixed with 1 ml of 0.4 M $HClO_4$. The suspensions were centrifuged (5000 *g*, 10 min, 2°) and sediments extracted three times under cooling with 4 ml of 0.2 M $HClO_4$ to remove the acid-soluble low molecular weight components. Resulting sediments were extracted with 5 ml of alcohol–ether (3:1), dried and before radioactivity measurement, dissolved at 80° in 1 ml of formic acid. The incorporation of ^{14}C-labelled pyrimidine precursors into RNA was measured as described earlier [6, 10]. The rate of RNA synthesis following 5-aza-2′-deoxycytidine treatment is expressed as dpm per μmole of spectroscopically pure uridine and cytidine 2′(3′)-phosphates isolated from the total RNA. The rate of DNA synthesis [9] is expressed as the specific radioactivity of thymine (in dpm/μmole) isolated from the total DNA.

RESULTS AND DISCUSSION

5-Aza-2′-deoxycytidine, like other 5-azapyrimidine analogues, undergoes spontaneous degradation [2] resulting in the formation of relatively stable *N*-amidino-*N*′-deoxy-β-D-ribofuranosylurea [14] (Fig. 1). The time course of 5-aza-2′-deoxycytidine degradation in aqueous solutions of different pH shows that at pH 7 the drug is more stable than at pH 9 but is less stable than at pH 6. At pH 7 and 37°, approximately 7 per cent conversion occurs in 1 hr.

We focused on the question of the preferential action of 5-aza-2′-deoxycytidine against the lymphatic system [5–7]. The analogue administered at a dose of 5 mg per kg body wt does not affect the deamination of cytidine or deoxycytidine in the liver and spleen of mice. Similarly, *in vitro* the compound does not interfere with the deamination of cytidine in cell-free extracts prepared from these tissues (not published). Furthermore, a marked depression of cytidine and deoxycytidine incorporation into DNA isolated from the spleen of mice pretreated with low doses of 5-aza-2′-deoxycytidine has recently been observed [10].

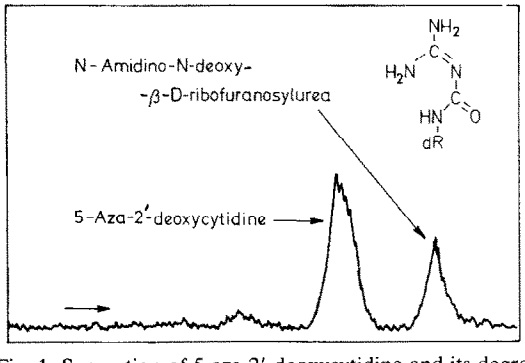

Fig. 1. Separation of 5-aza-2′-deoxycytidine and its degradation product, *N*-amidino-*N*′-deoxy-β-D-ribofuranosylurea. Separation was carried out on Whatman No. 1 paper using a solvent system composed of ethanol–tertiary butanol–formic acid–water (60:20:5:15).

Table 1. Incorporation of 5-aza-2′-deoxycytidine-^{3}H in different mouse strains*

Tissue	Incorporation†		
	H	DBA/2	AKR
Liver	1.00	1.00	1.00
Kidney	1.03	0.97	1.16
Heart	0.33	0.30	0.35
Muscle	0.37	0.42	0.35
Spleen	24.60	22.80	25.90
Thymus	17.20	16.40	14.85

* Groups of five to six female mice (22–26 g body wt) received 5-aza-2′-deoxycytidine-^{3}H (80 μCi/0.02 μmole) i.p. 2 hr before being killed.

† Incorporation of the drug (expressed as dpm/A_{260}) into the acid-insoluble fraction relative to the liver (111 for H, 96 for DBA/2, and 103 dpm/A_{260} for AKR strain, respectively).

The data presented in Table 1 indicate differences in the incorporation of 5-aza-2′-deoxycytidine-^{3}H in three mouse strains. The highest uptake of the label into the acid-insoluble fraction was observed in the spleen and the thymus. In an attempt to explain the cumulative toxicity of 5-aza-2′-deoxycytidine [7], the mice were pretreated with the cold drug prior to the administration of tritiated analogue, resulting in a significant depression of the uptake of radioactivity into spleen DNA (Table 2).

Similar depression was observed using ^{14}C-labelled thymidine to measure the synthesis of DNA in the spleen. The block in thymidine incorporation was accompanied by low thymidine and thymidylate kinase activities in cell-free extracts from the spleen of the drug-treated animals [10]. However, no apparent connection has been observed in the mouse spleens between the depression of DNA synthesis and the change in the level of cyclic AMP. Although in the

Table 2. Reduced incorporation of 5-aza-2′-deoxycytidine-^{3}H into DNA in the liver and spleen of analogue pretreated mice*

5-Aza-2′-deoxycytidine pretreatment (hr)	Incorporation, dpm/A_{260}		
	Liver	Spleen	(%)
Control	13	271	(100)
3	18	188	(69.3)
24	16	111	(40.9)
48 and 24	10	96	(35.5)

* Groups of six female mice (H strain, 24–26 g body wt) received the unlabelled drug (3.2 mg/kg) i.p. at various time intervals before 5-aza-2′-deoxycytidine-^{3}H (30 μCi/0.03 μmole). Two hours later the animals were killed and incorporation of the label into the acid-insoluble fraction of liver and spleen was measured as described in Materials and Methods.

Table 3. Synthesis of DNA and level of cyclic AMP in the spleen of mice following 5-aza-2′-deoxycytidine*

Animals	Synthesis of DNA (dpm/μmoles T ± S.E.)	Level of cyclic AMP (pmoles/mg protein ± S.E.)
Males		
Control	31,550 ± 1560	21.95 ± 1.78
5-Aza-2′-deoxycytidine	8370 ± 310	22.60 ± 2.41
Females		
Control	37,485 ± 4420	19.14 ± 2.04
5-Aza-2′-deoxycytidine	7423 ± 1048	28.90 ± 3.46

* Groups of six male and female mice (25 g body wt) received 5-aza-2′-deoxycytidine i.p. 23 hr before thymidine-2-^{14}C (2 μCi/0.1 μmole) 5-aza-2′-deoxycytidine (4 mg/kg) or 0.9% NACl. The animals were killed 90 min later, and the rate of DNA synthesis [10] and the level of cyclic AMP [11] in the spleens were measured as described in Materials and Methods.

Table 4. Higher incorporation of cytidine into RNA in the spleen of 5-aza-2′deoxycytidine-treated mice*

Inhibitor (mg/kg)	Cytidine-U-^{14}C	Incorporation, dpm/μmol ± S.E. (%) UMP	CMP
0	1 μCi/0.005 μmole	1250 ± 170 (100)	4250 ± 206 (100)
4		2020 ± 215 (162)	8600 ± 722 (203)
0	3 μCi/0.015 μmole	7800 ± 430 (100)	20,640 ± 1210 (100)
4		10,250 ± 1210 (132)	28,750 ± 2780 (139)

* Groups of four mice (H strain, 25 g body wt) received i.p., 2 hr before killing and 24 hr after 5-aza-2′-deoxycytidine (4 mg/kg), cytidine-U-^{14}C as indicated. The incorporation of the precursor into UMP and CMP of total spleen RNA was measured as described in Materials and Methods.

spleens of males treated with 5-aza-2′-deoxycytidine the level of cyclic AMP is not changed (or slightly depressed in some experiments) there is a slight and repeated increase in the level of cyclic AMP in the spleens of drug-treated females (Table 3). However, the extent of the depression of DNA synthesis was similar in both males and females.

Further studies revealed that in mice pretreated for a 2-hr period with 5-aza-2′-deoxycytidine, incorporation of cytidine into UMP and CMP of RNA in the spleen was higher than in control untreated animals (Table 4). The reason for this enhancement is not clear. Using orotic acid as the label there was no increase in the labelling of RNA in the spleen (and the liver) of the analogue-treated animals. Accordingly the administration of 5-aza-2′-deoxycytidine (5 mg per kg) *in vivo* did not affect the activity of liver and spleen enzymes taking part in the conversion of orotate to UMP.

5-Aza-2′-deoxycytidine is only slightly deaminated in liver and spleen extracts *in vitro*. The data presented in Fig. 2 indicate a marked difference between the deamination of deoxycytidine and the analogue; apparently deamination in the liver is much higher than in the spleen. On the other hand, the rate of 5-aza-2′-deoxycytidine phosphorylation in the spleen is enhanced relative to that in the liver (Fig. 3).

The differences in the rate of 5-aza-2′-deoxycytidine phosphorylation and deamination seem to be responsible for the preferential action of this drug against the lymphatic system, characterized by low cytidine deaminase and high deoxycytidine kinase activity [15]. It is supposed that the biological effect of 5-aza-2′-deoxycytidine is due to its incorporation into DNA [16] which is preceded by the phosphorylation of the drug catalysed by deoxycytidine kinase. The deamination as well as phosphorolytic cleavage of the analogue resulting in the formation of 5-azauracil and its metabolites [6] cannot be excluded and might contribute to the overall inhibitory effect of the drug.

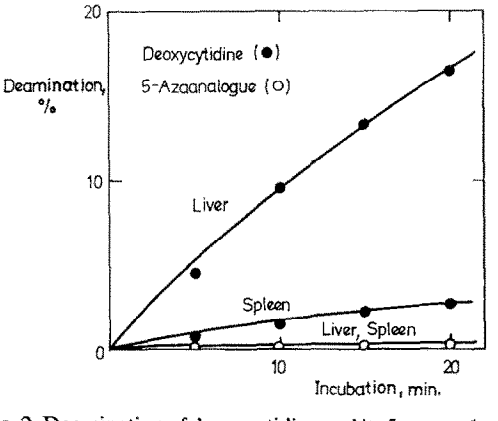

Fig. 2. Deamination of deoxycytidine and its 5-aza analogue in cell-free extracts from mouse spleen and liver. Incubation of tritium-labelled substrates (1 μmole) in 0.2 M Tris–HCl buffer (pH 7.4) was carried out at 37° with the cell-free extracts corresponding to 50 mg wet weight of the liver or spleen. Aliquots of the reaction mixtures were separated chromatographically.

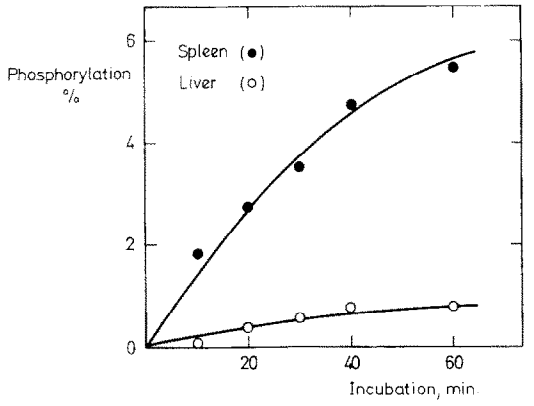

Fig. 3. Phosphorylation of 5-aza-2′deoxycytidine-^{3}H in cell-free extracts from the liver and spleen of mice. Incubation of the drug (0.1 mM) with 6 mM ATP and equimolar Mg^{2+} ions was carried out at 37° in 50 mM Tris–HCl buffer (pH 7.4) and 2 mM dithiothreitol in a total volume of 0.3 ml of the reaction mixture containing 1.2 mg protein in the added cell-free extracts prepared from the liver or spleen. Separation of the newly formed phosphates was carried out chromatographically using the solvent system as in Fig. 1.

In contrast to 5-azacytidine, the deoxy analogue does not affect enzyme induction and has no effect on gastric secretion [17] but displays a severe immunosuppressive effect [9]. The increased affinity of 5-aza-2′-deoxycytidine for the lymphatic system indicates a potential usefulness of the drug against malignancies of the lymphatic system. Clinical trials with this drug in patients with lymphatic leukemia are in progress [18].

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