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STUDIES ON 6-AZAURIDINE AND 6-AZACYTIDINE—III.
THE FATE OF 6-AZACYTIDINE IN VARIOUS ANIMAL SPECIES

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Abstract—The elimination of parenterally administered 6-azacytidine, 100 mg per kg of body wt, was studied in the urine of mice, rats, guinea pigs, rabbits, cats and dogs. The deamination of 6-azacytidine *in vivo* and *in vitro* is extremely low in rats, Within 24 hr the drug is completely eliminated in all investigated species, either in the original or the deaminated form. The deaminated product, 6-azauridine, accounts for about one-third of the total amount. In mice, the kidney is the organ with the highest enzymatic activity for the deamination of 6-azacytidine; in guinea pigs, the kidney and ileum; in rabbits, the ileum; in cats, the ileum and liver, while in dogs only the liver appears to be involved. The results are discussed from the point of view of 6-azauridine being the effective metabolite and from a phylogenetic aspect.

6-AZACYTIDINE, a member of the 6-azapyrimidine group, has been synthesized as another antimetabolic compound with cytostatic activity.[1–3] Its metabolic and biochemical effects have been compared with the effects of 6-azauridine.[4] The active metabolite, 6-azacytidine-5′-phosphate, possesses only about 10 per cent of the inhibitory effect of 6-azauridine-5′-phosphate on orotidylic acid decarboxylase.

In previous communications of our group,[5, 6] histological as well as neurotoxic side-effects in mice of both antimetabolites were found to be virtually the same, but more expressed for 6-azauridine. Because of the partial conversion of 6-azacytidine to 6-azauridine described by Handschumacher, Škoda and Šorm[4] in Ehrlich ascites tumor-bearing mice it was felt necessary to deal with the deamination of 6-azacytidine in more detail. As there are differences in the neurotoxic side-effects of 6-azacytidine between mice and rats,[7] the dynamics of deamination and elimination of 6-azacytidine in different animal species, with some correlation to man, become more important.

MATERIALS AND METHODS

Experimental animals

In these studies the following animals were used: male and female albino mice (Konárovice strain) of 18–20 g body wt; male and female rats (Wistar) of 200–250 g body wt; male rabbits (Chinchilla) 2·2–2·8 kg body wt; male and female guinea pigs (mixed breed) of 600–800 g body wt; male and female cats of 2·2–2·7 kg body wt; and male and female dogs (mongrel) of 6·8–10·0 body wt.

6-Azapyrimidines

6-Azacytidine was obtained through the courtesy of Academician F. Šorm from the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences; 6-azauridine was generously supplied by Messrs Spofa, Praha. Both preparations were checked for chromatographic purity.

Experimental procedure

The toxicity of 6-azauridine was determined in all animal species using the intraperitoneal route of administration. A shortage of 6-azacytidine limited for this compound the tests to mice and rats.

For the metabolic experiments *in vivo*, 100 mg (409·3 μmoles) of 6-azacytidine per kg of body wt were administered to all animals intraperitoneally, except dogs which were injected intravenously. The excreted urine was collected for 24 hr from all animals. For mice, a special metabolic cage was used; the urine was collected quantitatively on paper (Whatman No. 3) through a plastic mesh bottom, and was quantitatively eluted with water. The urine from rats and guinea pigs was collected in metabolic cages. The urine of rabbits was collected from the urinary bladder by means of catheters. Dogs with a urinary bladder fistula (Pavlov) were used. The urine from such dogs, trained to stay in a stand, was collected directly into containers. In cats, a polythene cannula was fixed in the bladder, the external end was screwed into a polythene bottle.[8] With this arrangement the cats could move freely in the cage during the entire experimental period. The frequency of determination of the amount of excreted compounds varied with different species.

Estimation of 6-azacytidine and 6-azauridine in the urine

One- and two-dimensional (descending) paper chromatography was used; the solvent systems were (a) isopropanol–ammonia–water (70 : 15 : 15); (b) butanol saturated with water; and (c) butanol–acetic acid–water (10 : 2 : 5). The azapyrimidine compounds were detected with u.v. light. The spots excised from the chromatogram were eluted quantitatively and the amounts of both nucleosides were determined spectrophotometrically.[4]

Estimation of 6-azacytidine and 6-azauridine in the blood

Three ml heparinized blood were deproteinated with 0·5 ml of 7 M perchloric acid. After centrifugation, the supernatant fluid was neutralized with 2 N KOH at 0–2°; a cooled centrifuge was used to remove the potassium perchlorate. Further manipulation was identical with the above-described method for the estimation of 6-azacytidine and 6-azauridine in the urine.

The deamination of 6-azacytidine in vitro

The activity for enzymatic deamination was tested in brain, kidney, liver, terminal ileum and spleen tissue of mice, rats, rabbits, guinea pigs, dogs and cats. The reliability of the method was checked in experiments with mouse kidney tissue. The animals were killed by decapitation, the organs immediately removed, washed in cold saline, weighed and homogenized at 0–2° in a glass-homogenizer for 3 min. Tissue homogenates, 5 and 10 per cent, were prepared in 0·2 M citrate-phosphate buffer at pH 4·8 and 7·0, respectively. To 4 ml of the homogenate warmed to 37°, 1 ml of 6-azacytidine solution (30 μmoles of 6-azacytidine) was added and incubated at 37°. At 0, 5, 10, and 20 min, samples of 1 ml were pipetted into tubes containing 0·1 ml of 7 M perchloric acid. Further manipulation was the same as with the estimation of 6-azacytidine and 6-azauridine in the blood.

The values in the linear part of the kinetic curve were used to estimate the amounts of 6-azacutidine that were deaminated, per 100 mg of tissue within 1 hr.

RESULTS

Table 1 presents the acute toxicity data. From the results it is apparent that in mice and rats the toxicity of 6-azauridine is somewhat lower than that of 6-azacytidine (25–35 per cent); the differences are at the border of significance. In agreement with Welch and co-workers,[9] the toxicity of 6-azauridine in dogs was found to be several times higher than in rodents. The experiments revealed the same for cats.

TABLE 1. ACUTE TOXICITIES OF 6-AZAURIDINE AND 6-AZACYTIDINE IN VARIOUS ANIMAL SPECIES AFTER INTRAPERITONEAL ADMINISTRATION

The mean lethal doses (LD_{50}) are expressed in g per kg of body weight, with corresponding limits o confidence (p = 0·95).

	Mouse	Rat	Guinea pig	Rabbit	Cat	Dog
6-azauridine	11·25 (9·34–13·56)	8·80 (7·21–10·74)	>8	>8	2·40 (1·47–3·90)	3·40 (3·2–3·6)
6-azacytidine	14·00 (11·1–17·64)	11·90 (10·87–13·03)	—	—	—	—

Figure 1 shows the results of the studies of the deamination and elimination of 6-azacytidine (dose: 100 mg per kg of body wt) within 24 hr in all species studied. Some data from a paper of one of us[10] are added concerning similar findings in human children with leukemia. From these data it is evident that the elimination of the amount of 6-azacytidine given is practically completed within 24 hr in all animal species studied, whereas the elimination in human subjects is less rapid; thus, in children only 80 per cent is eliminated during 24 hr. Rats and humans differ considerably from the other species investigated. In rats, the extent of deamination is very low (4 per cent) whereas in humans it reaches almost 60 per cent. The values for deamination in guinea pigs, rabbits, dogs, cats, and mice range from 25 to 38 per cent of the dose administered and excreted in the urine. Although the differences among these

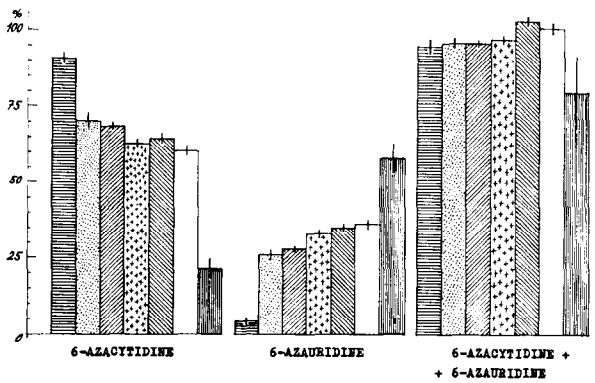

FIG. 1. The elimination and deamination of 6-azacytidine, after the administration of 100 mg of the compound per kg of body wt, within 24 hr in the rat ▤, guinea pig ⬚, rabbit ▨, dog ▦, cat ▧, mouse □ and man ▥.

species are small, they are significant. This makes the low deaminating activity in rats and the high activity in humans even more important. The kinetics of elimination and deamination of 6-azacytidine in all animal species studied are presented in Fig. 2. It is evident that both the elimination of 6-azacytidine and 6-azauridine reaches 50 per cent after 3–4 hr.

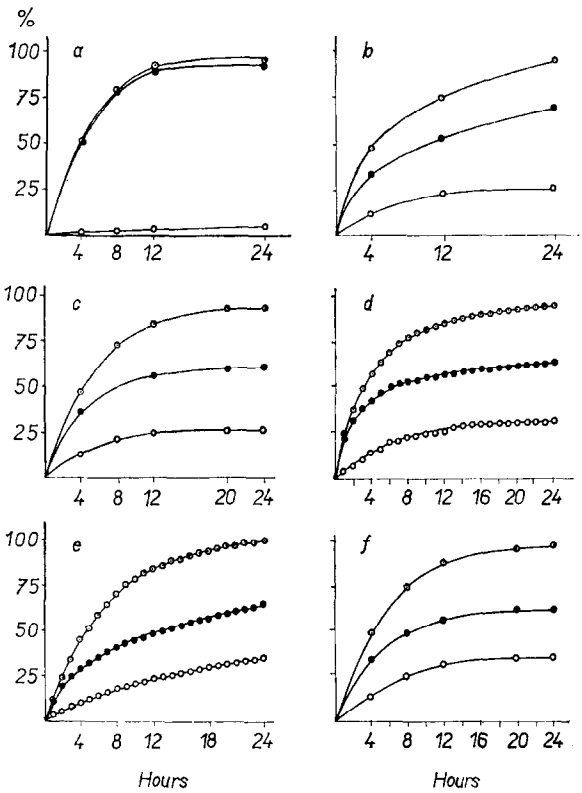

FIG. 2. The kinetics of deamination and elimination of 6-azacytidine in (a) the rat, (b) the guinea pig (c) the rabbit, (d) the dog, (e) the cat, (f) the mouse; the values are expressed as percentages of the total amount administered (100 mg of 6-azacytidine per kg of body wt).

Figure 3 represents the dynamics of the blood levels of 6-azacytidine and 6-azauridine within 6 hr after intravenous administration of 100 mg of 6-azacytidine per kg of body wt in dogs. Within the first hour there is the most rapid decline in the blood level of 6-azacytidine; thus, one hour after the injection the level of 6-azauridine reaches its maximum. In the subsequent period, up to 6 hr, the rate of decline of the blood levels of both compounds runs parallel.

The results of the experiment *in vitro* are presented in Table 2. The homogenates of brain and spleen of all species studied are without any apparent deaminating activity at pH 7·0; moreover, no activity could be found in homogenates of any of the tissues of rats that were examined. Deaminating activity was found in some tissue homogenates of mice, guinea pigs, rabbits, cats and dogs. The activity of homogenates of

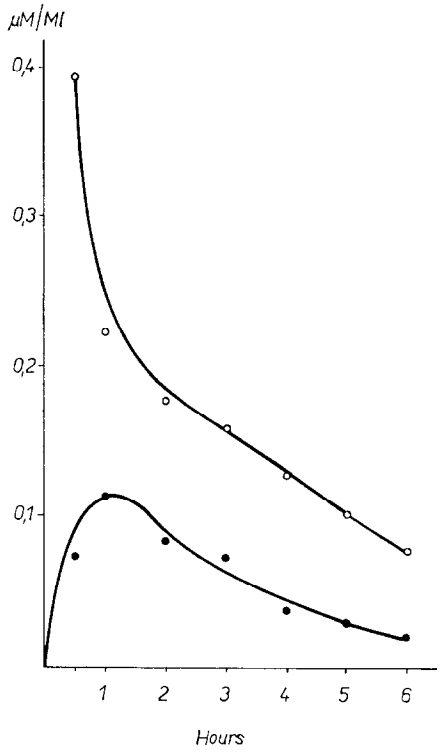

FIG. 3. Blood levels of 6-azacytidine and 6-azauridine within 6 hr after intravenous administration of 100 mg (409·3 μmoles) of 6-azacytidine per kg of body wt in dogs.

the organs differs in various species. In the mouse and the guinea pig, this enzymatic activity was found only in the kidney and ileum; in the rabbit, only in the ileum; in the cat and the dog, the kidney tissue homogenates revealed no activity, although activity was present in the liver. Whereas the cat had activity in the ileum, in the dog the only organ homogenate with 6-azacytidine-deaminating enzymatic activity was the liver.

TABLE 2. DEAMINATION OF 6-AZACYTIDINE IN VARIOUS TISSUES OF DIFFERENT SPECIES *in vitro* (pH 7·0)

The activities are expressed as μmoles of substrate deaminated per 100 mg of tissue, in 1 hr, with corresponding limits of confidence (p = 0·95).

	Mouse	Rat	Guinea pig	Rabbit	Cat	Dog
Kidney	8·96 ± 2·31	0*	4·56 ± 1·08	0	0	0
Ileum	5·18 ± 1·52	0	3·50 ± 1·08	4·10 ± 1·20	2·50 ± 0·92	0
Spleen	0	0	0	0	0	0
Liver	0	0	0	0	2·50 ± 0·80	3·10 ± 1·04
Brain	0	0	0	0	0	0

* For the sensitivity of method see text.

The maximum of the enzymatic deamination of 6-azacytidine in the mouse kidney homogenate appears at pH 4·8 to 5·0 (Fig. 4). As the sensitivity of our method is limited (about 1·5 μmoles of deaminated 6-azacytidine within 1 hr), the experiments were performed at the optimal pH for the enzymatic deamination of 6-azacytidine. The quantity of deaminated 6-azacytidine increased for all positive values found at pH 7·0 (Table 2), in good agreement with the curve presented (Fig. 4). The majority of negative results obtained at pH 7·0, also were negative at pH 4·8; this latter pH-level revealed low activity only in mouse liver, rat ileum, guinea pig spleen and rabbit spleen.

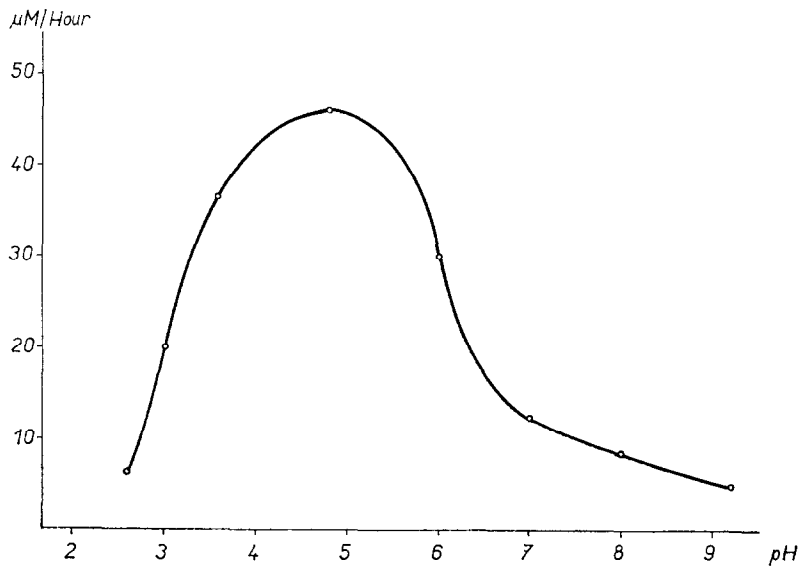

FIG. 4. The relationship between pH and enzymatic deamination activity of 6-azacytidine in homogenates of mouse kidney The values shown indicate the μmoles of 6-azacytidine that were deaminated per 100 mg of tissue within 1 hr.

DISCUSSION

Comparisons of the deamination of 6-azacytidine in various animal species *in vivo* and *in vitro* revealed several interesting points. Rats have a distinctly different metabolism of 6-azacytidine. No deamination activity could be revealed in any of the homogenates of rat tissues studied *in vitro* at pH 7·0. Only negligible amounts of 6-azauridine appeared in the urine of rats given 6-azacytidine parenterally. These findings are in good agreement with the very low levels of deoxycytidylic acid deaminase in rats.[11–13] Also, 5-iodo-2′-deoxycytidine is poorly deaminated by various rat tissue homogenates.[14] Marked deamination activity was observed in all other animal species investigated and was even more striking in humans.[10, 14]

The elimination of 6-azacytidine has parallel dynamics to that of 6-azauridine. Thus, 6-azauridine is very quickly eliminated by glomerular filtration and tubular excretion, as shown by studies in hens[15] and in man.[16] In rats, the shape of the elimination curve (Fig. 2) of 6-azacytidine is the same as that of the summation curve of

6-azauridine and 6-azacytidine in the other species. The high deamination ratio in man differs from that of all other species studied. In all animal species the drug is practically eliminated within 24 hr; within 2 to 4 hr after parenteral administration, about 50 per cent of the compound is excreted. In leukemic children, however, only 80 per cent of the drug was eliminated within 24 hr.[10]

Although the velocities of elimination of drug and of deamination appear to be very close in the various species investigated, this becomes more complicated when the relation of experiments *in vivo* and *in vitro* are more closely analyzed. The deamination *in vivo* is highest in the mouse, followed by the cat; *in vitro*, however, both species deaminated in two of the investigated organs, i.e., the mouse very strongly in the kidney, less in the ileum, the cat in the ileum and in the liver. In the dog and the rabbit, on the other hand, deamination has been found in only one organ, i.e. the liver and the ileum, respectively. An apparent exception to this correlation is found in the guinea pig, in which deaminating activity occur both in the kidney and in the ileum. These differences among species indicate further that different tissues are responsible for the final degree to which administered 6-azacytidine is converted to 6-azauridine, and that the differences found between the mouse, cat, dog, guinea pig, and rabbit, although small, are real. It is also interesting, although that multiorgan deamination occurs in some of the species studied, deamination in dogs appears to be localized in the liver. This observation needs further attention from a phylogenetic point of view.

Another point concerns the mechanism of action of 6-azacytidine. As 6-azacytidine is deaminated in various species and in man, it could be that it exerts its activity mainly as 6-azauridine; this possibility is taken into account by Handschumacher, Škoda and Šorm, who do not exclude another not yet known mechanism.[4] If our suggestion is correct, the activity of the drug in rats, in which the deamination is extremely low, also should be very low. Correlations between cytostatic and neurotoxic activities, as studied in our laboratory, show that in the rat readily reproducible neurological effects of 6-azauridine occur in the mouse but not in the rat; accordingly, the expectation concerning the lack of activity of 6-azacytidine proved to be correct.[7]

The necessity of comparative pharmacological studies of new drugs is now generally advocated. The present study certainly supports the thesis of Brodie[17, 18] that in studying new drugs intended for use in man, animal species should be chosen in which the metabolism resembles that of man clearly, for 6-azacytidine the use of the common laboratory animal, the rat, would not have any predictive value for human subjects.

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