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DEACETYLATION OF 2′,3′,5′-TRI-*O*-ACETYL-6-AZAURIDINE IN VARIOUS ANIMAL SPECIES AND MAN*

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Abstract—The deacetylation activity detected in blood plasma of rodents under study (rat, mouse, rabbit and guinea-pig) was high. In the investigated non-rodent species (dog, pig and man) this activity was much lower. While there were great differences between the species relatively small difference was observed between individuals within each species, with the exception of the rabbit where genetic differences in the hydrolytic activity of non-specific esterases can be expected. In the liver postmicrosomal fraction higher deacetylation activity was found in the rat than in the dog. The relation of the different rate of deacetylation activity to general toxicity is discussed.

6-AZAURIDINE,† produced by fermentation from 6-azauridine,[1] is a cytostatic agent that belongs to the group of pyrimidine antimetabolites. After its conversion in the body to 6-azauridine 5′-phosphate, it interferes with the biosynthesis *de novo*, via orotidylic acid of pyrimidine precursors of nucleic acids.[2] The rapid urinary elimination of the drug[3] and its unsuitability for oral administration—as it is split in the intestine to the more toxic and much less effective compound, 6-azauracil[4]—led to the synthesis of the tri-acetylated derivative, 2′,3′,5′-tri-*O*-acetyl-6-azauridine (TA-6-azauridine),‡ which found wider therapeutic applications in the treatment of psoriasis[5] and herpes viral infections.[6] Promising results with TA-6-azauridine also were obtained in smallpox.[7,16]

Previous studies from this laboratory, however, showed that this sort of drug "latentiation"[8] markedly influenced the chronic toxicity of the compound as compared to the tolerance of the parent molecule in some species. The rat was much more tolerant than man to free 6-azauridine,[9] but less tolerant to TA-6-azauridine.[10] On the other hand, the excellent tolerance to treatment by TA-6-azauridine which was given orally in an equimolar ratio to the intravenous therapeutic doses of free 6-azauridine indicated that the acetylation of 6-azauridine did not change its tolerance in man.[11]

It is known that after its introduction into the body TA-6-azauridine is gradually

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† Proprietary name: Riboazauracil®.

‡ Proprietary name: Azaribine®.

deacetylated by the action of non-specific esterases, which form two intermediate products, i.e. di-*O*-acetyl-6-azauridine (DA-6-azauridine), mono-*O*-acetyl-6-azauridine (MA-6-azauridine),* and finally free 6-azauridine.[3] Supposing that the differences in the toxicity might be due to the deacetylation activity resulting in the slower or faster liberation of the active product, 6-azauridine, we decided to study species differences in the deacetylation activity.

EXPERIMENTAL

In view of the fact that TA-6-azauridine is deacetylated by human blood serum,[1,2] we started our studies with the incubation of the compound with samples of plasma obtained from a wide range of species: rat (Wistar), mouse (Konárovice strain), guinea-pig (mixed breed), rabbit (Chinchilla), dog (mongrel) and man. To correlate the enzymatic deacetylation in plasma and in the tissues we also investigated the rate of the reaction in the liver postmicrosomal fraction of rats and dogs. TA-6-Azauridine and 6-azauridine were obtained from Messrs Spofa, Prague. 2′,3′-Di-*O*-acetyl-6-azauridine and 5′-*O*-acetyl-6-azauridine used as standard samples of intermediate products, were synthesized in the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague.

The incubation of TA-6-azauridine with plasma

Incubation was carried out at 37° in a medium containing 1 part of water solution of 40 mM TA-6-azauridine and 9 parts of citrate plasma of above mentioned species. The final volume of the incubation medium was 5 ml. The pH value was 7·4 and did not change during the incubation. At different time intervals the aliquots of the incubation media were removed and the enzymatic reaction was stopped by the addition of trichloroacetic acid to give a final concentration of 5%. The protein precipitate was removed by centrifugation and aliquots (0·1 ml) of the supernatant fractions were applied to chromatographic paper (Whatman No. 3). TA-6-Azauridine and its deacetylated products were separated using the solvent system butanol–acetic acid–water (10:2:5). The compounds were visualized on the chromatograms in ultraviolet light utilizing standard samples of the compounds for the identification. The absorbing zones were cut out, eluted with water and the amount of the derivatives was measured at 262 mμ by means of a spectrophotometer. No distinction was made between the possible isomers of diacetylated and monoacetylated derivatives. The stability of TA-6-azauridine during the incubation at 37° was checked by replacing the plasma in the medium by saline.

The incubation of TA-6-azauridine with the postmicrosomal fraction of the liver

The livers of three male mongrel dogs and five Wistar rats (three males and two females) were removed quickly after exsanguination of the animals. Aliquot portions of each liver were homogenized in cooled buffered sucrose (250 mM, buffered by 20 mM tris buffer to pH 7·4). The homogenate was centrifuged for 1 hr at 105,000 *g* at 0°. Six ml of the incubation medium contained 1·8 ml of the postmicrosomal fraction (35–45 mg of protein per ml), 24 μmoles of TA-6-azauridine and 300 μmoles of tris–HCl buffer pH 7·4. Further steps were analogous to the above-mentioned experiments with plasma.

* The positions of the acetyl groups were not estimated.

RESULTS

Stability of TA-6-azauridine in aqueous medium at 37°

In the absence of plasma or postmicrosomal fraction 2·5% of TA-6-azauridine added to the incubation medium was hydrolyzed during 1 hr, with the formation of DA-6-azauridine; further products of hydrolysis were not detected. This finding was taken into account in the evaluation of deacetylation capacity of plasma or liver postmicrosomal fraction.

The incubation of TA-6-azauridine with plasma

The results are summarized in Tables 1 and 2. When comparing the deacetylation capacity of the plasma in the species under study we can see that the hydrolytic cleavage of the first acetyl group occurs very rapidly in all rodents; thus with mouse plasma TA-6-azauridine was not found after the first hour of incubation, while with the plasmas of the rat, guinea-pig and rabbit, only small amounts (2·5–5·8 per cent) remained. On the other hand, in other animal species studied the conversion of TA-6-azauridine to DA-6-azauridine was not complete within 1 hr of incubation (about 55 per cent remained unchanged). The process was even slower with pig plasma, in which at the same time interval, 80 per cent of the TA-6-azauridine was found unhydrolyzed.

The second step of the deacetylation process, i.e. the conversion of DA-6-azauridine to MA-6-azauridine also is relatively rapid in rodents. After 1 hr of incubation with mouse plasma the amount of TA-6-azauridine was greater than the amount of DA-6-azauridine in the mouse; we found 59·2 per cent of MA-6-azauridine, and only 29·8 per cent of DA-6-azauridine. In the plasmas of the rat, rabbit and guinea-pig, however, within the same time period, the amount of MA-6-azauridine did not attain the level of the DA-6-azauridine. In the group of non-rodents, e.g. in the plasma of dogs and man, the conversion of DA-6-azauridine to MA-6-azauridine is slow thus, only about 6 per cent of MA-6-azauridine was found after 1 hr of incubation in the plasma of dog and man and no MA-6-azauridine in the plasma of the pig.

The deacetylation of MA-6-azauridine to free 6-azauridine is relatively more rapid in rodents. After 1 hr of incubation we observed in the plasmas of the mouse, rabbit and guinea-pig, roughly 9 per cent of free 6-azauridine and approximatively half of that amount in the plasma of the rat. In non-rodents the amount of 6-azauridine formed was still much lower; thus in the plasma of the dog 0·6 per cent was found whereas in those of the pig and man, free 6-azauridine was not detected at all after 1 hr of incubation.

As the limits of confidence indicate (Tables 1 and 2) the results are satisfactorily homogeneous in the plasmas of all species, with the exception of the rabbit, in which a greater variance was observed.

The incubation of TA-6-azauridine with postmicrosomal fraction of the liver. The results are summarized in Table 3. The hydrolysis of the first acetyl group by the postmicrosomal fraction seems to occur more rapidly in the dog, as compared to the rat, whereas the reverse is true for the splitting of the last acetyl group, resulting in free 6-azauridine formation (6-azauridine was not found with dog plasma even after incubation for 15 min whereas in that of the rat 12·4 per cent of this compound was formed during the first 5 min).

TABLE 1. TIME COURSE OF THE DEACETYLATION OF 2′,3′,5′-TRI-*O*-ACETYL-6-AZAURIDINE BY BLOOD PLASMA OF SOME NON-RODENTS

Species*	Derivative	Minutes of incubation			
		8	16	32	64
Man (6)	2′,3′,5′-Tri-*O*-acetyl-6-azauridine	81·3 (75·0–87·6)†	74·3 (66·9–81·7)	66·3 (62·1–70·5)	56·7 (52·7–60·7)
	Di-*O*-acetyl-6-azauridine	17·2 (11·0–23·4)	25·3 (18·2–32·4)	31·3 (28·2–34·4)	37·7 (34·5–40·9)
	Mono-*O*-acetyl-6-azauridine	0	0·2 (0–0·6)	2·3 (0–5·0)	6·7 (5·0–8·4)
	6-Azauridine	0	0	0	0
Dog (5)	2′,3′,5′-Tri-*O*-acetyl-6-azauridine	85·2 (83·0–87·4)	76·6 (72·7–80·5)	66·5 (57·5–77·5)	52·4 (46·0–56·8)
	Di-*O*-acetyl-6-azauridine	14·8 (12·6–17·0)	22·0 (18·0–26·0)	30·6 (23·1–38·1)	41·8 (36·4–47·2)
	Mono-*O*-acetyl-6-azauridine	0	1·4 (0–3·0)	2·9 (0·7–5·1)	5·4 (4·0–6·8)
	6-Azauridine	0	0	0	0·6 (0–2·3)
Pig (5)	2′,3′,5′-Tri-*O*-acetyl-6-azauridine	94·4 (88·8–100·0)	91·0 (88·1–93·9)	84·4 (84·2–84·6)	79·6 (75·1–84·1)
	Di-*O*-acetyl-6-azauridine	5·6 (0–11·2)	9·0 (6·1–11·9)	15·7 (15·5–15·9)	20·4 (15·9–24·9)
	Mono-*O*-acetyl-6-azauridine	0	0	0	0
	6-Azauridine	0	0	0	0

* The numbers in parentheses represent the numbers of individuals studied.

† The values are expressed as the mean percentage of absorbance with limits of confidence for P = 0·95 (the absorbance of the amount of 2′,3′,5′-tri-*O*-acetyl-6-azauridine added to the incubation medium is taken for 100%).

TABLE 2. TIME COURSE OF THE DEACETYLATION OF 2′,3′,5′-TRI-*O*-ACETYL-6-AZAURIDINE BY BLOOD PLASMA OF RODENTS

Species*	Derivative	Minutes of incubation			
		8	16	32	64
Mouse (Three pooled samples)	2′,3′,5′-Tri-*O*-acetyl-6-azauridine	35·8 (29·0–42·6)†	15·7 (6·3–25·1)	0	0
	Di-*O*-acetyl-6-azauridine	53·0 (48·5–57·5)	62·5 (53·8–71·2)	46·7 (27·9–65·5)	29·8 (23·6–36·0)
	Mono-*O*-acetyl-6-azauridine	10·3 (5·9–14·7)	19·8 (10·3–29·3)	46·7 (30·2–63·2)	59·2 (51·2–67·2)
	6-Azauridine	0·8 (0–2·7)	2·0 (0–6·3)	6·7 (2·9–10·5)	11·0 (6·6–15·4)
Rabbit (4)	2′,3′,5′-Tri-*O*-acetyl-6-azauridine	46·8 (16·3–77·3)	28·6 (0–66·8)	14·0 (0–38·7)	2·0 (0·3–3·7)
	Di-*O*-acetyl-6-azauridine	45·0 (16·8–73·2)	54·0 (36·7–71·3)	59·6 (0–129·1)	51·8 (0–134·9)
	Mono-*O*-acetyl-6-azauridine	7·7 (0–19·5)	15·9 (0–31·5)	23·4 (0–78·6)	38·1 (0–77·9)
	6-Azauridine	0·5 (0–2·1)	1·5 (0–3·6)	3·0 (0–10·4)	8·1 (0–18·3)
Guinea-pig (3)	2′,3′,5′-Tri-*O*-acetyl-6-azauridine	58·7 (52·3–65·1)	52·2 (44·9–59·5)	26·2 (18·9–35·5)	5·8 (0–15·4)
	Di-*O*-acetyl-6-azauridine	30·5 (21·1–39·9)	38·5 (30·8–46·2)	53·8 (45·4–62·2)	62·8 (54·5–71·1)
	Mono-*O*-acetyl-6-azauridine	5·0 (0–10·7)	5·8 (0–11·9)	12·8 (4·5–21·1)	22·5 (15·6–29·4)
	6-Azauridine	5·8 (0–12·6)	3·5 (0·3–6·7)	7·2 (0–17·2)	9·0 (0–19·8)
Rat (4)	2′,3′,5′-Tri-*O*-acetyl-6-azauridine	48·5 (41·6–55·4)	34·4 (29·9–42·9)	13·0 (4·6–21·4)	2·5 (0–6·5)
	Di-*O*-acetyl-6-azauridine	46·5 (36·1–57·0)	55·9 (50·4–61·4)	69·3 (57·5–81·1)	68·3 (58·0–78·6)
	Mono-*O*-acetyl-6-azauridine	5·0 (1·1–8·9)	9·4 (8·4–10·4)	17·8 (17·5–18·1)	24·9 (16·1–33·7)
	6-Azauridine	0	0	2·0 (1·8–2·2)	4·4 (0–10·3)

* The numbers in parentheses represent the numbers of individuals studied.

† The values are expressed as the mean percentage of absorbance with limits of confidence for P = 0·95 (the absorbance of the amount of 2′,3′,5′-tri-*O*-acetyl-6-azauridine added to the incubation medium is taken for 100%).

TABLE 3. TIME COURSE OF THE DEACETYLATION OF 2′,3′,5′-TRI-*O*-ACETYL-6-AZAURIDINE BY POST-MICROSOMAL FRACTION OF THE LIVER

Species*	Derivative	Minutes of incubation 5	10	15
Dog (3)	2′,3′,5′-Tri-*O*-acetyl-6-azauridine	32·0 (17·4–46·6)†	19·3 (6·8–31·8)	9·7 (0–21·2)
	Di-*O*-acetyl-6-azauridine	46·8 (33·9–59·7)	52·7 (39·4–66·0)	56·2 (42·2–70·2)
	Mono-*O*-acetyl-6-azauridine	21·2 (19·3–23·1)	28·0 (20·5–35·5)	34·2 (24·7–43·7)
	6-Azauridine	0	0	0
Rat (5)	2′,3′,5′-Tri-*O*-acetyl-6-azauridine	34·0 (25·6–42·4)	25·7 (19·8–31·6)	17·5 (10·0–25·0)
	Di-*O*-acetyl-6-azauridine	33·6 (26·8–40·4)	39·6 (35·9–43·3)	36·4 (29·5–43·3)
	Mono-*O*-acetyl-6-azauridine	19·7 (13·6–25·8)	17·4 (10·6–24·2)	19·0 (12·7–25·3)
	6-Azauridine	12·4 (6·3–18·5)	15·0 (9·4–20·6)	26·6 (13·8–39·4)

* The numbers in parentheses represent the numbers of individual studied.

† The values are expressed as the mean percentage of absorbance with limits of confidence for P = 0·95 (the absorbance of the amount of 2′,3′,5′-tri-*O*-acetyl-6-azauridine added to the incubation medium is taken for 100%).

DISCUSSION

Our experiments have revealed that there is a considerable difference between the rates of deacetylation of TA-6-azauridine by the plasmas of different animal species; the most pronounced differences are observed between the group of rodents and some non-rodent species, e.g. the pig, dog and also man. Moreover, our observations indicate that there were no greater individual differences in the scope of each species. Only the rabbit seems to represent an exception since considerable variance in deacetylation activity of plasma was observed between individual animals. It is known that in this species certain genetic differences exist in the activity of atropinesterase.[13] It is thus probable that a relatively high variance in our observations in rabbit plasma could be attributed to differences in the activity of non-specific esterases. It is obvious that the species differences in the deacetylation activity are not restricted to the plasma because in the two species studied (dog and rat) parallel behaviour was observed in the deacetylation activity of the postmicrosomal fraction of the liver. This view is supported also by the fact that in urine obtained from rats treated with TA-6-azauridine there was more free 6-azauridine than in the urine of men.[14]

With regard to the fact that repeated oral treatment by TA-6-azauridine was better tolerated in man[11] as well as in pig,[10] than it is in the rat, it seems that the chronic toxicity of this compound is greatly dependent on the rate of deacetylation of the compound.

It is known that the cytostatic activity of 6-azauridine is dependent upon its conversion to 6-azauridine 5′-phosphate, an inhibitor of the activity of orotidylic acid decarboxylase.[2] The results of our experiments suggest that also the chronic toxicity of TA-6-azauridine is determined by the level of free 6-azauridine, which can be con-

verted to 6-azauridine 5′-phosphate. Further support for this concept is to be found in the character of the toxic effects of TA-6-azauridine,[10] which can be related apparently to the inhibition of mitotic activity, e.g. that of the intestinal epithelium. The desquamation of the epithelium, the result of such inhibition, is considered to be in a causal relation to the development of the chronic toxicity during the administration of cytostatic drugs.[15] It is thus highly probable that the toxic reactions occurring in the course of the repeated administration of TA-6-azauridine were dependent upon the rate of liberation of free 6-azauridine.

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