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INHIBITION OF THYMIDINE PHOSPHORYLASE BY 6-AMINOTHYMINE AND DERIVATIVES OF 6-AMINOURACIL*

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Abstract—6-Aminosubstituted derivatives of thymine and of 5-bromo-, 5-iodo- and 5-fluorouracil are powerful inhibitors *in vitro* of thymidine phosphorylase; 6-hydrazinothymine and, particularly, 6-azidothymine are significantly less active. The inhibitory action of the 6-aminosubstituted derivatives seems to be based on a fortification of the product inhibition of this enzyme brought about by thymine and the 5-halogenated uracils. 6-Aminosubstitution enhances 2- to 9-fold the inhibitory properties of these compounds. In view of the fact that in man 5-fluorodeoxyuridine and 5-iododeoxyuridine are rapidly converted by thymidine phosphorylase into an inactive product (5-iodouracil) or more toxic product (5-fluorouracil), the type of inhibitors described may be expected to be of practical importance.

GREAT practical importance should be attributed to the inhibition of pyrimidine-deoxyribonucleoside phosphorylases found in human and animal tissue. These enzymes are responsible for the fact that 5-iododeoxyuridine and 5-fluorodeoxyuridine, two well-known cytostatic agents, are rapidly cleaved in animals and man into an inactive compound (iodouracil) or a more toxic compound (fluorouracil) (Prusoff[1,2]; Heidelberger[3,4]). Hence, the use of compounds preventing this cleavage could be expected to result in a considerable increase in the therapeutic effectiveness of the before-mentioned nucleosides. Previous experiments on this problem carried out in our laboratory[5] had shown that two types of pyrimidine-nucleoside phosphorylases exist, which differ in their sensitivity to different inhibitors: (1) uridine-deoxyuridine phosphorylase (E.C.2.4.2.3)[6] and (2) thymidine phosphorylase (E.C.2.4.2.4).[7] Uridine-deoxyuridine phosphorylase is inhibited by various unnatural nucleosides, viz. deoxyglucopyranosylthymine[5,8], deoxyxylopyranosylthymine,[9] 5′-deoxy-5′-mercapto-thymidine[10] and 5′-deoxy-5′-iodothymidine.[10] One of the most effective compounds is deoxyglucopyranosylthymine. When used in cats, the tissues of which contain only uridine-deoxyuridine phosphorylase, the compound produced a 2 to 14-fold increase in the extent of the incorporation of 5-iododeoxyuridine into the DNA of different tissues, depending upon the conditions used.[11,12] Thus, evidence was provided that inhibitors of nucleoside phosphorolysis can be used effectively to enhance the biochemical processes underlying the therapeutic action of these agents.

Contrary to uridine-deoxyuridine phosphorylase, thymidine phosphorylase is not inhibited by any of the above-mentioned unnatural nucleosides.[5,9,10] Considering,

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however, that it is just this enzyme which occurs in man,[8, 13] its inhibition would be of particular importance. Our studies of the inhibition of this enzyme began with the observation that free pyrimidine bases, liberated during the phosphorylase reaction, bring about a relatively weak inhibition of the enzyme.[7] By introducing an amino group into the uracil ring in position 6, we have been able to increase this effect as much as 9-fold. This is to say, inhibitors of thymidine phosphorylase *in vitro* are now available with an effectiveness comparable to that of deoxygluco- and deoxyxylopyranosylthymine on the uridine-deoxyuridine phosphorylase. A report of these investigations is given in this paper.

METHODS

Thymidine phosphorylase was prepared from horse liver by the method of Friedkin and Roberts.[7] Purity grade V was used. The influence of the different compounds on enzymatic arsenolysis of 1 mM thymidine was measured by a previously described technique.[5] The extent of thymidine cleavage was followed by the diphenylamine reaction:[14] 1 μmole of deoxyribose liberated corresponds in our assay system to an extinction of 0·835 at 601 mμ.

6-Aminothymine was prepared by three different procedures:
(a) by alkaline cyclization of α-methylcyanoacetyl urea;[15]
(b) by cyclization of ethyl methylcyanoacetate with urea in one step;[16] and
(c) by the reaction of 6-chlorothymine with ammonia.[16]
The preparation (a) described by Bergmann and Johnson[15] as well as the new syntheses (b) and (c) gave an identical product with a decomposition point of 312° (Lit.:[15] 355°. Anal. Calcd. for $C_5H_7N_3O_2$: C, 42·55 H, 4·99 N, 29·78. Found: C, 42·50 H, 5·01 N, 29·63). An unequivocal indication of its structure was provided by acid-hydrolysis to give the known compound 5-methyl-barbituric acid.[17]

6-Hydrazinothymine was prepared from 6-chlorothymine and hydrazine hydrate.[18] By the same method 6-azidothymine was obtained by treatment of 6-chlorothymine with sodium azide. (decomp. 268–270°, fast heated. Anal. Calcd. for $C_5H_5N_5O_2$: C, 35·93 H, 3·01 N, 41·91. Found: C, 36·32 H, 3·50 N, 41·69).[16] 5-Halogeno-6-aminouracils were synthesized either by direct halogenation of 6-aminouracil (iodo-, bromo- and chloro-derivative)[19] or by cyclization of the corresponding ethyl halogenocyanoacetate with urea. (5-fluoro-derivative: Calcd. for $C_4H_4FN_3O_2$: C, 33·10 H, 2·77 N, 28·95. Found C, 33·07 H, 2·53 N, 28·77; decomp. $> 300°$).[16] Hydrolysis of 5-fluoro-6-aminothymine with 1 N HCl gives the known 5-fluoro-barbituric acid.

In addition to the proof of purity by elementary analysis all compounds synthesized by different routes have been compared with authentic specimens by siligagel thin-layer chromatography in different solvent systems.

RESULTS AND DISCUSSION

Fig. 1 shows the inhibition of thymidine phosphorylase by thymine, 6-aminothymine, 6-hydrazinothymine and 6-azidothymine. It can be seen that the inhibitory effect of the thymine molecule is markedly increased by introducing an amino group in position 6. Judged by the concentrations required to produce a 50 per cent inhibition of the enzyme, 6-aminothymine was 9 times as active as thymine. For comparison, it should be mentioned that the inhibitory action of deoxyglucopyranosylthymine on the uridine-deoxyuridine phosphorylase is 7 times stronger than that of

thymine.[20] With an increased size of the nitrogen-containing substituent at the C-6 position, the inhibitory action of the compounds decreases, although 6-hydrazinothymine is still as effective as thymine. With 6-azidothymine, the inhibitory effect relative to thymine is considerably reduced. Methyl-barbituric acid (6-hydroxythymine) proved to be ineffective in the same concentration range. Investigations on the kinetics of the inhibition by 6-aminothymine showed a partially competitive type of inhibition, similar to that described by Friedkin and Roberts[7] for the inhibition by thymine.

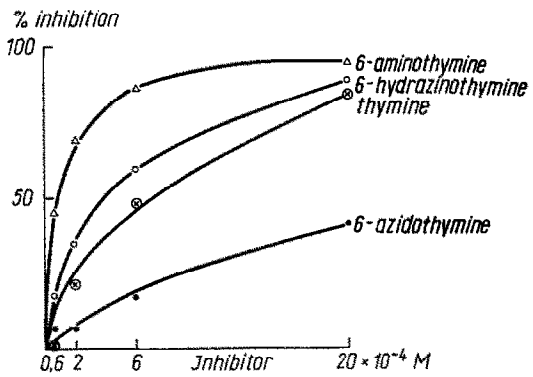

FIG. 1. Inhibition of enzymatic arsenolysis of thymidine by thymine and thymine derivatives with nitrogen-containing substituents at C-6, as dependent upon inhibitor concentrations. The reaction mixture contained: 120 mM arsenate; 200 mM Tris-HCl, pH 7·4; 1 mM thymidine; 0·1 ml of enzyme solution. Final volume 0·5 ml. The reaction was started by the addition of the enzyme. Incubation: 1 hr at 37°. Thymidine cleavage in the uninhibited sample was 0·26 μmole.

In addition to thymine, 5 bromo-, 5-iodo- and 5-fluoro-uracil are known to be inhibitors of pyrimidine-nucleoside phosphorylases.[21] Their inhibitory actions decrease in the following order: 5-iodouracil = 5-bromouracil > 5-methyluracil (thymine) > 5-fluorouracil > uracil. Hence, we investigated whether the same order of effectiveness is observed with the corresponding 6-aminosubstituted derivatives. This was found to be true, as can be seen in Table 1; however, the degree of this increase in effectiveness obtained by amino-substitution at position 6 varies with the different compounds. As stated above, it is about 9-fold with 6-aminothymine, 6-fold with 6-amino-5-bromouracil and 6-amino-5-iodouracil, but only 2-fold with 6-amino-5-fluorouracil. No increase in effectiveness relative to uracil was observed with 6-aminouracil. The reason for the increases in effectiveness induced by 6-amino-substitution is still unknown. It might be supposed that the fortified activity reflects a higher acidity of 6-aminosubstituted uracil derivatives. The increase of acidity also could provide an explanation for the fact that 5-bromouracil and 5-iodouracil are more active than thymine and that the inhibitory effect of 5-fluorouracil is greater than that of uracil. The pK_a values of the different 6-aminosubstituted compounds obtained by u.v. spectroscopy are as follows:[16] 6-aminothymine = 9·0, as against 9·9 for thymine;[22] 6-amino-5-bromouracil = 7·5, as against 8·05 for 5-bromouracil;[23] 6-amino-5-iodouracil = 7·7, as against 8·25 for 5-iodouracil;[23] 6-amino-5-

fluorouracil = 7·3, as against 8·0 for 5-fluorouracil[23] and 6-aminouracil = 8·7, as against 9·5 for uracil.[22] However, since there is an uniform increase in acidity, this assumption does not provide any explanation for the varying degree of gain in activity brought about by 6-aminosubstitution, particularly in the case of uracil, with which there is no increase at all.

TABLE 1. INHIBITION OF THYMIDINE PHOSPHORYLASE BY THYMINE AND VARIOUS 5-HALOGENO-URACILS OR 6-AMINO DERIVATIVES OF THEM. SAME CONDITIONS AS GIVEN IN FIG. 1. THYMIDINE CLEAVAGE IN THE UNINHIBITED SAMPLE WAS 0·18 μMOLE

	Concentration required for 50 per cent inhibition (μmolar)		
	1 C-6 unsubstituted	2 C-6 aminosubstituted	2/1
5-Bromouracil	170	30	6
5-Iodouracil	200	40	5
5-Methyluracil(thymine)	630	70	9
5-Fluorouracil	760	370	2
Uracil	370*	530	0·7

* Concentration necessary for 25 per cent inhibition.

It is remarkable that 5-iodocytosine is completely inactive, although, due to the symmetry of the C-4 and C-6 atoms of the ring, it may be regarded as an 6-amino-5-iodouracil without an OH-group (respectively, oxo-group) at the C-4 atom.

Uridine-deoxyuridine phosphorylase also is inhibited by 6-aminothymine, although it is only 1·7 times more active than thymine.

Preliminary studies on the action of 6-aminothymine *in vivo* were carried out with mice, since in this species the liver, which is primarily responsible for the phosphorolytic cleavage of nucleosides, contains only thymidine phosphorylase. By the i.v. administration of 88 μmole of 6-aminothymine, in addition to 44 μmole of thymidine or of 5-iododeoxyuridine, the degree of ^{3}H-thymidine or ^{131}I-iododeoxyuridine incorporation into the DNA of bone marrow, spleen or intestine was found to be doubled. This is a relatively slight increase when compared with the corresponding action of deoxyglucopyranosylthymine in cats.[11, 12] The reason for this result is still unknown and subject to further investigation. Moreover, studies are under way to investigate the extent to which other biochemical reactions are affected by 6-aminothymine, 6-amino-5-halogenouracils or derivatives of this type. At present, experiments are in progress to prepare the ribonucleosides and deoxyribonucleosides of these compounds, which are of particular interest in this context.

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