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DEMETHYLATION OF PURINE ANALOGS BY MICROSOMAL ENZYMES FROM MOUSE LIVER*

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Abstract—Purines and purine analogs with methylthiol, methylamino, methoxy, ring-carbon-methyl, and ring-nitrogen-methyl substituents have been tested for their ability to serve as substrates for the microsomal demethylases of mouse liver. Ring-methylated 6-thiopurines appeared to be stable, whereas other ring-methylated purine derivatives and most of the methylthiol and methylamino derivatives were demethylated. A microsomal plus soluble fraction of Ehrlich ascites tumor cells was unable to demethylate any of the compounds tested.

IN THE search for effective anticancer agents, many methylated purines and methylated purine analogs have been synthesized and tested for their biological activity.[1–4] Several compounds methylated on ring nitrogens at position 1 or 9 have inhibited tumor or bacterial growth, whereas those methylated at positions 3 or 7 have been without this activity. Compounds with methylated amino or thiol groups at position 6 have also inhibited growth, as have compounds with methylated amino groups at position 2. Likewise, the compound with a methyl group alone at position 6 has antitumor activity.

The identity of the active forms of these compounds has been a problem of continuing interest. If such compounds are demethylated, then their active forms and basic mechanisms of carcinostasis would be expected to be the same as those of the parent unmethylated compounds, although their absorption, distribution, and excretion patterns might be different. In contrast, if the methyl groups should prove to be stable, then these compounds might constitute one or more new classes of carcinostatic drugs with new mechanisms of action.

The study of the metabolism of methylated purines and purine analogs has therefore been pursued by several workers. Because this subject has not previously been considered as a whole, the pertinant literature is here reviewed in some detail. Hansen *et al.*[5] found no demethylation products in the urine of rats receiving 9-methyl-6-thiopurine. LePage and Jones[6] showed that ascites tumors did not demethylate 9-methyl-6-thioguanine to allow nucleotide formation. Similarly, demethylation of 1-methyl-6-thioguanine could not be shown to occur.[6] The frequent observations of altered biological and biochemical effects produced by methylation also suggest that little if any demethylation occurs.[1–4, 7] In contrast, methyl groups are readily removed

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from the 1, 3, 7, and 9 nitrogens of caffeine, theobromine, and theophylline, and large numbers of demethylated metabolites of these compounds are excreted.[8-14] In a similar fashion, various methylated uric acids are also demethylated *in vivo*.[9]

Demethylation of methylated amino or thiol groups at the 6 position of purine analogs has been demonstrated. Jamison *et al.*[15] and Duggan and Titus[16] showed that 6-methylaminopurine was demethylated and converted to adenine and guanine to equal extents in normal and neoplastic mammalian tissues *in vivo* and by bacteria. Likewise, the demethylamination of 6-methylaminopurine was catalyzed in *Escherichia coli* by adenosine deaminase.[17] The demethylation of 6-dimethylamino-9-(3'-amino-3'-deoxy-3-D-ribofuranosyl) purine (aminonucleoside) by rats may occur by dedimethylamination.[18] The demethylation of 6-methylthiopurine in man was demonstrated by Elion *et al.*[19, 20] by the detection of urinary 6-mercaptopurine and 6-thiouric acid. Sarcione and Stutzman[21] have detected the demethylation of 6-methylmercaptopurine in rats and postulated that undetectable amounts of methylmercaptan may be an intermediate in the formation of sulfate from 6-methylmercaptopurine. The demethylation of 6-methylthiopurine by rat liver microsomal enzymes in the presence of $NADPH_2$ and oxygen has recently been demonstrated.[22, 23] This observation raised the possibility that other methylated purines and purine analogs might also be demethylated by the S-, N-, and O-demethylases which are found among the microsomal drug-metabolizing enzymes of liver. Purines and purine analogs with methylthiol, methylamino, methoxy, ring-carbon-methyl, and ring-nitrogen-methyl substituents have therefore been tested as substrates for these enzymes. Caffeine, theobromine, and theophylline have also been included in this study, since the demethylation of caffeine by rat liver microsomes has previously been demonstrated.[24]

MATERIALS* AND METHODS

Female ICR Swiss mice, 20 to 25 g, were decapitated and the livers immediately removed and homogenized with 2 volumes of ice-cold isotonic KCl. Ehrlich ascites carcinoma was maintained and grown in female ICR Swiss mice by the weekly intraperitoneal inoculation of approximately 10^6 cells. Ascites tumor cells were removed from the peritoneal cavity by capillary pipet after laparotomy and the ascitic fluid removed after centrifugation. Tumor cells were suspended and homogenized in ice-cold distilled water, after which the homogenate was made isotonic in KCl of the same concentration as the liver homogenate. The homogenates were centrifuged at $9{,}000 \times g$ for 10 min to remove unbroken cells, nuclei, and mitochondria. The supernatant fraction from this preparation was used in these experiments. All procedures were carried out at 4° or below.

Assay conditions are described in Table 1. Demethylation was followed by determining the amount of formaldehyde formed in the reaction. Formaldehyde was

* Nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate were obtained from Sigma Chemical Co.; 6-methylthiopurine, from Nutritional Biochemicals Corp.; 1-methyl-2-amino-6-purinethione and 1-methyl-4-amino-pyrazolopyrimidine, from the Cancer Chemotherap-National Service Center; 2-amino-6-methylthiopurine, 1-methyl-6-purinethione, 3-methyl-6-thiopurine, 2-methylamino-6-thiopurine, and 2-amino-9-methyl-6-thiopurine, from Dr. R. K. Robins; caffeine, theophylline, theobromine, and 6-methylpurine, from California Corp. for Biochemical Research; 2-methylthio-4-hydroxytrimethylenepyrimidine and 2-amino-4-methoxytrimethylene pyrimidine were synthesized by Dr. B. R. Baker and furnished by Dr. J. Greenberg; 6-dimethy--aminopurine, from Dr. H. G. Mandel; 7-methyl-6-thiopurine and 9-methyl-6-thiopurine were synthesized in this laboratory by unpublished procedures kindly furnished by Miss G. B. Elion.

determined in an aliquot of protein-free extract from incubztion mixtures by the method of Nash[25] as modified by Cochin and Axelrod.[26] The process measured under the conditions was true demethylation, as neither methylmercaptan or methylamine substituted for formaldehyde in the assay. None of the compounds tested either reacted with the formaldehyde reagent or produced formaldehyde in the absence of enzyme. Further details will be published elsewhere.[22, 23]

TABLE 1. DEMETHYLATION OF PURINES AND PURINE ANALOGS

Substrate	Formaldehyde formed (μg)
S-Demethylation	
6-Methylthiopurine	45·8
2-Amino-6-methylthiopurine	21·4
2-Methylthio-4-hydroxytrimethylenepyrimidine	13·8
O-Demethylation	
2-Amino-4-methoxytrimethylenepyrimidine	0·8
C-Demethylation	
6-Methylpurine	0
N-Demethylation: Amino N	
2-Methylamino-6-thiopurine	0
6-Dimethylaminopurine	77·4
N-Demethylation: Ring N	
1-Methyl-6-purinethione	0
1-Methyl-2-amino-6-purinethione	0
3-Methyl-6-thiopurine	0
7-Methyl-6-thiopurine	0
9-Methyl-6-thiopurine	0
2-Amino-9-methyl-6-thiopurine	0
1-Methyl-4-aminopyrazolopyrimidine	8·6
Caffeine	2·8
Theophylline	2·7
Theobromine	0·9

Microsomes plus soluble fraction from 1 g of female ICR Swiss mouse liver were incubated for 1 hr at 37° in an air atmosphere with 2 mg substrate, 500 μg NADP, 500 μg glucose-6-phosphate, 6 mg nicotinamide, 5 mg neutralized semicarbizide hydrochloride, 25 μmoles $MgCl_2$, and 3·0 ml of 0·5 M phosphate buffer, pH 7·4, in a total volume of 6·0 ml. Each figure is the average of separate analyses of results from duplicate flasks. The average variation between duplicate formaldehyde analyses was 3·4 per cent. This experiment has been repeated with essentially the same results.

RESULTS

The ability of several methylated purines and purine analogs to serve as substrates for mouse liver microsomal demethylases is shown in Table 1. Compounds methylated on thiol or amino groups were demethylated by this system at moderate rates, with the exception of 2-methylamino-6-thiopurine. The one O-methylpurine analog tested, 2-amino-4-methoxytrimethylenepyrimidine, was demethylated to a small extent, but 6-methylpurine was not. Thiopurines methylated at the 1, 3, 7, and 9 positions were not demethylated to any degree, but a similar compound 1-methyl-4-aminopyrazolopyrimidine, was an active substrate. All three methylated xanthines were demethylated.

Because of this demonstration of the ability of certain purine analogs to serve as substrates for liver microsomal demethylases, it was of interest to determine whether tumor cells also had the ability to perform this reaction. The capacity of a microsomal plus soluble fraction preparation of Ehrlich ascites tumor cells to demethylate 6-methylthiopurine, 6-dimethylaminopurine, and 1-methyl-4-aminopyrazolopyrimidine under the conditions described above was therefore tested. No demethylation of any of these substrates was observed.

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DISCUSSION

These experiments have demonstrated that liver microsomal S-, N- and O-demethylases can metabolize some, but not all, methylated purines and purine analogs. Ring-methylated 6-thiopurines appeared to be stable, whereas other ring-methylated purine derivatives and most of the methylthio and methylamino derivatives were demethylated.

The inability of tumor cells to demethylate purine analogs confirms and extends previous findings that demethylation of this type is confined mostly to liver.[22, 23] These results suggest that methylated purine analogs may act as such in tumor cells unless previously converted to the unmethylated parent compound in the liver. This reaction must then be followed by the circulation of the parent compound from liver to tumor. The full significance of this reaction for an understanding of the mechanism of action of those compounds that are demethylated necessarily awaits detailed studies of the liver and tumor content of methylated and unmethylated forms of such a compound.

The significance of the observation that at least some antimetabolites can be metabolized by the relatively nonspecific, drug-metabolizing enzymes that are localized in liver microsomes has been discussed previously.[22, 23] That other reactions may lead to similar results is suggested by the finding of Duggan and Titus[16] that liver microsomes did not demethylate 6-methylaminopurine but that this compound was demethylaminated. The relative importance of these two types of demethylation for a wider series of compounds awaits further study.

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