Biochemical Pharmacology, Vol. 20, pp. 847–852. Pergamon Press, 1971. Printed in Great Britain

6-METHYLTHIOGUANYLIC ACID, A METABOLITE OF 6-THIOGUANINE

PAULA W. ALLAN and L. LEE BENNETT, JR.

Kettering–Meyer Laboratory,* Southern Research Institute, Birmingham, Ala. 35205, U.S.A.

(*Received* 9 *July* 1970; *accepted* 31 *July* 1970)

Abstract—Human epidermoid carcinoma (H. Ep. No. 2) cells, grown in culture in the presence of 6-thioguanine-^{35}S or methionine[methyl-^{14}C] plus unlabeled 6-thioguanine, contained a previously undescribed metabolite that was characterized by chromatographic and electrophoretic migration and by chemical and enzymatic hydrolysis as 6-methylthioguanylic acid. Two other nucleotides present in these cells had the properties expected of the di- and triphosphates of 6-methylthioguanosine. In contrast to previously reported results with 6-mercaptopurine ribonucleotide and its S-methyl derivative, 6-methylthioguanylic acid was inferior to 6-thioguanylic acid as an inhibitor of PP-ribose-P amidotransferase. Thus, the methylated derivatives of 6-thioguanylic acid probably do not contribute significantly to the marked inhibition of purine synthesis *de novo* produced in mammalian cells by 6-thioguanine.

6-THIOGUANINE (6-TG),† an analog of guanine with strong growth-inhibitory effects against both microbial and mammalian cells, is anabolized to nucleotides and incorporated into DNA and RNA.[1,2] Nucleotides derived from 6-TG produce a number of metabolic effects either by direct action on certain enzymes or as a consequence of their incorporation into polynucleotides.[1–3] We reported earlier that 6-MP, another 6-thiopurine with potent biological activity, was converted to 6-MeMP-ribonucleotide via 6-MP-ribonucleotide;[4] this methyl derivative was much more potent than 6-MP-ribonucleotide in inhibiting PP-ribose-P amidotransferase (EC 2.4.2.14), an important site of action of 6-MP.[5,6] 6-TG and other 6-thiopurines are substrates for a nonspecific transmethylase, accepting methyl groups from S-adenosylmethionine in a cell-free system.[7] Further, 6-MeTG has been isolated as a urinary metabolite of 6-TG in man.[2,8] These results suggest that 6-MeTG-ribonucleotide (6-methylthioguanylic acid) may be a metabolite of 6-TG. Such a metabolite might be responsible for some of the metabolic effects of the analog. We report here the formation from 6-TG of ribonucleotides of 6-MeTG in a cell culture line of a human epidermoid carcinoma (H. Ep. No. 2), selected because of our earlier observation that it, of the several cell types examined, formed the largest amounts of 6-MeMP-ribonucleotide from 6-MP.[9]

MATERIALS AND METHODS

Compounds. 6-Thioguanine-^{35}S was prepared by exchange between elemental ^{35}S and 6-thioguanine during refluxing in dimethylacetamide for 5 hr; this method is a

* Affiliated with the Sloan–Kettering Institute for Cancer Research, New York, N.Y.

† Abbreviations used: 6-TG, 6-thioguanine; 6-MeTG, 6-methylthioguanine; 6-MP, 6-mercaptopurine; 6-MeMP, 6-methylthiopurine.

modification of that used by Moravek and Nejedly[10] for labeling of thiopurines. The crude product was purified by recrystallization from water and by preparative thin-layer chromatography on Brinkmann silica gel F-254 plates. The final product was free of detectable amounts of radioactive impurities. 2′-Deoxythioguanosine was obtained from Dr. Harry B. Wood, Chemotherapy, NCI, NIH. 6-Methylthioguanylic acid and 6-methylthio-2′-deoxyguanosine were synthesized in our laboratories by Dr.

TABLE 1. CHROMATOGRAPHIC AND ELECTROPHORETIC MIGRATIONS OF 6-THIOGUANINE AND RELATED COMPOUNDS

Known compounds*	R_f values in solvent†					Electrophoretic migrations‡	
	A	B	C	D	E	F (cm)	G (cm)
6-TG	0·48	0·57	0·47	0·58	0·09		
6-TGR	0·36	0·48	0·41	0·52	0·18	+ 1·9	+19·3
6-TGRP	0·14	0·13	0·21	0·32	0·29	+15·4	+31·4
6-MeTG	0·77	0·83	0·87	0·92	0·03		
6-MeTGR	0·69	0·75	0·80	0·85	0·05	+ 2·2	+ 8·6
6-MeTGdR	0·78	0·85	0·88	0·90	0·03	+ 1·7	+ 1·0
6-MeTGRP	0·26	0·38	0·58	0·62	0·10	+14·5	+26·2
Metabolites							
6-MeTGRP derived from [^{35}S]-6-TG	0·29		0·56	0·61	0·11	+14·7	+27·9
6-MeTGRP derived from [^{14}C-methyl]methionine	0·25		0·60	0·61	0·10	+15·7	+23·7
Snake venom-treated 6-MeTGRP derived from [^{35}S]-6-TG	0·65						
Snake venom-treated 6-MeTGRP derived from [^{14}C-methyl] methionine	0·66						+ 8·4
Snake venom-treated polyphosphates of 6-MeTGR derived from [^{35}S]-6-TG or [^{14}C-methyl]-methionine	0·68	0·72					
Acid-hydrolyzed 6-MeTGRP derived from [^{35}S]-6-TG	0·72						
Acid-hydrolyzed 6-MeTGRP derived from [^{14}C-methyl]-methionine	0·76						
Acid-hydrolyzed polyphosphates of 6-MeTGR derived from [^{35}S]-6-TG or [^{14}C-methyl]-methionine	0·78	0·81					

* The known compounds were detected by illumination of the chromatogram with ultraviolet light; the unknowns were detected by radioautography. Abbreviations: R, ribonucleoside; RP, ribonucleotide; 6-MeTGdR, 6-methylthio-2′-deoxyguanosine.

† Chromatographic solvents: Solvent A. Equal volumes of 93·8% aqueous *n*-butanol and 44% aqueous propionic acid. Solvent B. Isobutyric acid–glacial acetic acid–H_2O (70:0·7:35). Solvent C. 2,2,3, 3-Tetrafluoro-1-propanol–H_2O–90% formic acid (25:15:0·5).[14] Solvent D. Isobutyric acid–concentrated NH_4OH–H_2O (57:4:39). Solvent E. 0·1 M sodium phosphate, pH 6·8–solid $(NH_4)_2SO_4$–*n*-propanol (100:60 g:2).[15]

‡ Buffers and conditions for electrophoresis: Buffer F. 0·05 M formate, pH 3·5, 2000 V for 1 hr. Buffer G. 0·05 M borate, pH 9·2, 1500 V for 90 min.

J. A. Montgomery and Miss H. J. Thomas by the methylation of 6-thioguanylic acid and 2′-deoxythioguanosine. Crude snake venom (*Crotalus atrox*) was obtained from Ross Allen's Reptile Institute, Silver Springs. Fla. Methionine[methyl-^{14}C] was purchased from New England Nuclear Corp.

Cell cultures. The cell culture line used was a human epidermoid carcinoma (H. Ep. No. 2) established in culture by Moore *et al.*[11] and maintained in our laboratories.[12]

Metabolism of 6-TG. H. Ep. No. 2 cells were grown in suspension cultures (3×10^5 cells/ml) on a rotary shaker in medium that contained either 6-TG-^{35}S (83 nc, 32 nmoles/ml); or methionine[methyl-^{14}C] (1·34 nmoles, 20 nc/ml); or methionine-[methyl-^{14}C] plus unlabeled 6-TG (6 nmoles/ml). After 4 or 24 hr the cells were harvested by centrifugation and extracted with boiling 80% aqueous ethanol. The water-soluble portion of this extract was subjected to two-dimensional paper chromatography, first in 72% aqueous phenol and then in butanol-propionic acid (equal volumes of 93·8% aqueous 1-butanol and 44% aqueous propionic acid). Radioactive compounds were located by exposure of no-screen X-ray film to the chromatogram for periods of 2–4 weeks. These methods have been described in detail elsewhere.[13]

For characterization of metabolites, radioactive compounds falling in the nucleotide area of the two-dimensional chromatograms were eluted and were either rechromatographed in other solvents or subjected to enzymatic or chemical degradation. Enzymatic hydrolysis to the nucleosides was accomplished by incubation with snake venom for 4 hr at 37°, and hydrolysis to the purine base was accomplished by incubation for 1 hr at 100° with 1 N HCl. The hydrolyzed samples were then subjected to chromatography on paper in parallel with known compounds. Nucleotides were also characterized by parallel chromatography, co-chromatography and electrophoretic migration on paper. The compositions of solvents and buffers are given in Table 1.

Enzyme assays. Assays for inhibition of P-ribose-PP amidotransferase were carried out as described in Table 2.

TABLE 2. INHIBITION OF 5-PHOSPHORIBOSYLPYROPHOSPHATE AMIDOTRANSFERASE BY 6-THIOPURINE NUCLEOTIDES*

Nucleotide	Concn (mM) required for 50 per cent inhibition of PP-ribose-P amidotransferase
6-MP-ribonucleotide	1·11†
6-MeMP-ribonucleotide	0·090†
6-TG-ribonucleotide	0·27
6-MeTG-ribonucleotide	1·15

* The enzyme preparation used was 100-fold purified from cultured mouse adenocarcinoma 755 cells as described elsewhere.[5] The standard assay mixture consisted of 3·33 mM L-glutamine-^{14}C, 42 mM tris–maleate (pH 7·5), 1 mM PRPP, 2 mM magnesium ion, and enzyme in a final volume of 150 μl. After 20 min the reaction was stopped by boiling for 1 min. Portions of the reaction mixture were subjected to paper chromatography in a solvent consisting of ethanol–*t*-butylalcohol–formic acid–water (12:4:1:3, by vol.), and the extent of the reaction was determined by measurement of the amount of glutamate-^{14}C formed.[5]

† These values are from earlier experiments.[5] However, 6-MeMP-ribonucleotide was also included in the experiments with the nucleotides of 6-TG and 6-MeTG and has the same degree of inhibition as in the earlier experiments; hence the values given are a valid index of the relative inhibitory capacity of the four nucleotides.

RESULTS AND DISCUSSION

As expected, the autoradiograms from cells exposed to 6-TG-^{35}S showed heavy labeling in the areas to which nucleotides migrate. Since 6-TG is incorporated into nucleic acids, mono-, di-, and triphosphates of both the ribonucleoside and deoxyribonucleoside of 6-TG must be formed. The autoradiograms from cells exposed only to methionine[methyl-^{14}C] showed no radioactivity in the nucleotide area, but three spots in this area appeared when unlabeled 6-TG was also present (Fig. 1). When 6-TG-^{35}S was the precursor, among the many radioactive spots on the chromatogram were three with the same R_f values as those appearing with labeled methionine plus unlabeled 6-TG as the precursors. The radioactivity in these spots was about 4-fold greater at 24 hr than at 4 hr. Autoradiograms for experiments with 6-TG-^{35}S are not shown because they show the same new nucleotide spots as do the experiments with labeled methionine plus unlabeled 6-TG and because the new methylated nucleotides can be seen better on the autoradiograms when no labeled nucleotides of 6-TG itself are present.

In the two-dimensional solvent system, the three new radioactive compounds moved farther than expected for most nucleotides; it has been observed earlier that 6-MeMP-ribonucleotide migrates in this system much faster than 6-MP-ribonucleotide.[4] For this reason, and also since these metabolities were labeled from either 6-TG-^{35}S or methionine[methyl-^{14}C], they were suspected to be the mono-, di-, and triphosphates of 6-MeTG-ribonucleoside. The quantities isolated were insufficient for determination of phosphorous:sugar:base ratios. Therefore, characterization was carried out by chromatographic and autoradiographic procedures in conjunction with chemical and enzymatic degradations and comparison with known compounds. The most extensive characterization was made of the metabolite which moved fastest in the two-dimensional solvent system and which was suspected to be 6-MeTG-ribonucleotide. The R_f values in this solvent system (0·46 in phenol–water and 0·26 in butanol–propionic acid) for the unknown were the same as those for an authentic synthetic sample of 6-MeTG-ribonucleotide. In other solvent systems, the R_f values were also similar (Table 1). In addition, close coincidence of ultraviolet absorption and radioactivity was noted when a sample of 6-MeTG-ribonucleotide was added to the cell extract prior to chromatography and its position on the chromatogram determined by scanning with an ultraviolet lamp prior to exposure of the chromatogram to X-ray film. The electrophoretic migrations of the unknown in two buffers were similar also to that of synthetic 6-MeTG-ribonucleotide. The unknown was converted by treatment with snake venom to a compound migrating like 6-MeTG-ribonucleoside and by acid hydrolysis to a compound migrating like 6-MeTG. The possibility that the unknown might be the deoxynucleotide of 6-MeTG is ruled out by the quite different migrations of 6-MeTG-ribonucleoside and 6-MeTG-deoxyribonucleoside, particularly upon electrophoresis in borate buffer. This compound thus appears to be 6-MeTG-ribonucleotide.

In the two-dimensional solvent system, known nucleoside mono-, di-, and triphosphates migrate in a characteristic fashion; the addition of each phosphate results in a slower migration in each solvent. The two compounds suspected to be the di- and triphosphates of 6-MeTG-ribonucleoside migrated to positions expected of the polyphosphates. After treatment with snake venom essentially all of the radioactivity in these spots migrated like 6-MeTG-ribonucleoside, and after acid hydrolysis essentially

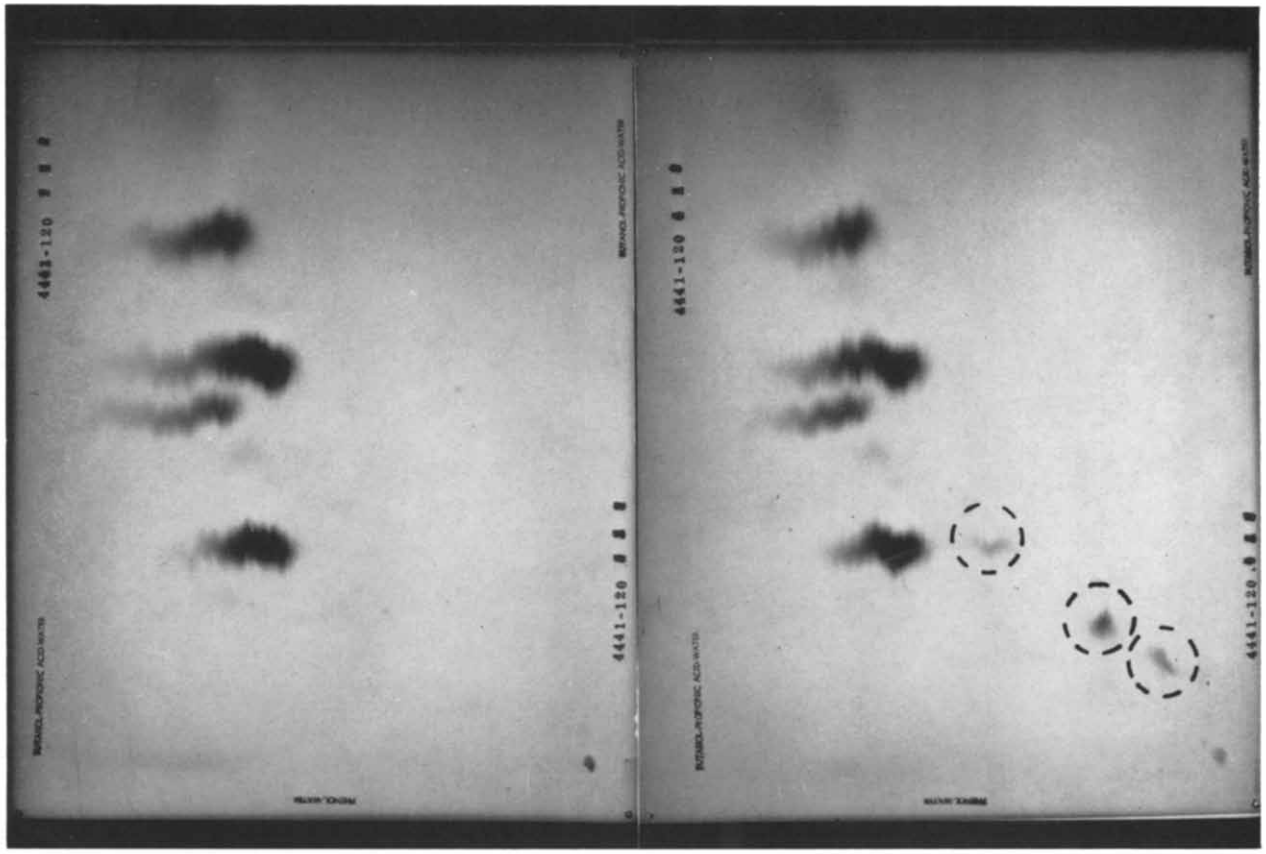

FIG. 1. Metabolism of methionine[methyl-^{14}C] and methionine[methyl-^{14}C] plus 6-thioguanine in H. Ep. No. 2 cells in culture. The figure is a reproduction of autoradiograms prepared from extracts of cells grown for 4 hr in the presence of methionine[methyl-^{14}C] (left figure) or methionine[methyl-^{14}C] plus unlabeled 6-thioguanine (right figure). The three dense spots on the left side of each figure were identified, by co-chromatography and parallel chromatography with knowns in several solvents, as methionine sulfoxide, methionine, and 5′-deoxy-5′-methylthioadenosine. The three spots appearing on the right figure are compounds identified as nucleotides of 6-methylthioguanine.

all of the radioactivity migrated like 6-MeTG. These compounds thus behave like the polyphosphates of 6-MeTG-ribonucleoside.

The conversion of 6-MP to 6-MP-ribonucleotide has been studied in H. Ep. No. 2 cells under the same conditions as those used for the present study of 6-TG;[4] therefore, a direct comparison can be made of the extents of formation of the S-methyl derivatives of the nucleotides of these two precursors. Such a comparison shows that about the same amounts of 6-MeTG-ribonucleotide and of 6-MeMP-ribonucleotide were present in cells grown for 24 hr in the presence of labeled 6-TG or 6-MP. However, it should be noted that 6-TG is converted to nucleotides (mono-, di-, and triphosphates of 6-TG-nucleosides) much more extensively than is 6-MP, which is converted chiefly to the monophosphate of 6-MP-ribonucleoside. For this reason the nucleotides of 6-MeTG are a relatively smaller proportion of the total nucleotide metabolities of 6-TG.

6-MeTG-ribonucleotide conceivably could be formed by methylation of 6-TG-ribonucleotide or via methylation of 6-TG or 6-TG-ribonucleoside and conversion of the resulting methyl derivatives to the nucleotide. Although there is no direct evidence as to the route of formation, indirect evidence against the latter route is provided by the observation that 6-MeTG and 6-MeTG-ribonucleoside were essentially nontoxic to cultures of H. Ep. No. 2 cells.[16] If one assumes that 6-MeTG-ribonucleotide is toxic [as would be expected from its capacity to inhibit PP-ribose-P-amidotransferase (see below)], then the fact that the methylated base and nucleoside are nontoxic indicates that they are not converted to the nucleotide.

The isolation of a new nucleotide metabolite is of potential importance to an explanation of the complex metabolic effects of 6-TG for which several loci of action have been defined.[1-3,17-19] One of the potent effects of 6-TG, after conversion to the nucleotide, is blockade of *de novo* purine biosynthesis by a feedback inhibition of PP-ribose-P amidotransferase.[5,17-19] This site of action is also a site for 6-MP-ribonucleotide and 6-MeMP-ribonucleotide.[5,6,18,19] Because this latter compound is much more effective than 6-MP-ribonucleotide as an inhibitor of the amidotransferase,[5,6] it was of interest to determine if methylation of 6-TG-ribonucleotide similarly increased its effectiveness. The results are shown in Table 2, which also contains, for comparison, previously published[5] data for 6-MP-ribonucleotide and 6-MeMP-ribonucleotide. 6-MeTG-ribonucleotide was an inhibitor of the amidotransferase but was inferior to 6-TG-ribonucleotide. Thus, whereas the S-methyl derivative 6-MP-ribonucleotide may account for most or all of the effects of 6-MP on purine biosynthesis *de novo*, the S-methyl derivative of 6-TG-ribonucleotide does not contribute significantly to the inhibition of purine biosynthesis produced by 6-TG. It is possible, of course, that the nucleotides of 6-MeTG may contribute to other effects exerted by 6-TG by virtue of incorporation into polynucleotides,[1] by inhibition of GMP kinase,[3] or perhaps at sites yet undefined.

Acknowledgements—This work was supported by Grant T-13K from the American Cancer Society, by NIH, NCI, Chemotherapy Contract PH43-66-29, and by grants from the C. F. Kettering Foundation and the Alfred P. Sloan Foundation. We are indebted to Dr. D. L. Hill for assays of inhibition of PP-ribose-P amidotransferase, Miss H. J. Thomas for synthesis of 6-TG-^{35}S, Miss D. Adamson and Miss F. Chesnutt for provision of cell cultures, and Mr. T. C. Herren and Mr. H. Finch for assays of radioisotopes.

REFERENCES

1. G. A. LEPAGE, *Cancer Res.* **23,** 1202 (1963).
2. G. B. ELION and G. H. HITCHINGS, in *Advances in Chemotherapy*, (Eds. A. GOLDIN, F. HAWKING and R. J. SCHNITZER), p. 91. Academic Press, New York (1965).
3. R. P. MIECH, R. E. PARKS, JR., J. H. ANDERSON, JR. and A. C. SARTORELLI, *Biochem. Pharmac.* **16,** 2222 (1967).
4. P. W. ALLAN, H. P. SCHNEBLI and L. L. BENNETT, JR., *Biochim. biophys. Acta* **114,** 647 (1966).
5. D. L. HILL and L. L. BENNETT, JR., *Biochemistry, N.Y.* **8,** 122 (1969).
6. B. S. TAY, R. M. LILLEY, A. W. MURRAY and M. R. ATKINSON, *Biochem. Pharmac.* **18,** 936 (1969).
7. C. N. REMY, in *Transmethylation and Methionine Biosynthesis*, (Eds. S. K. SHAPIRO and F. SCHLENK), p. 107. University of Chicago Press (1965).
8. G. B. ELION, S. W. CALLAHAN, G. H. HITCHINGS, R. W. RUNDLES and J. LASZLO, *Cancer Chemother. Rep.* **16,** 197 (1962).
9. P. W. ALLAN and L. L. BENNETT, JR., *Proc. Am. Ass. Cancer Res.* **9,** 2 (1968).
10. J. MORAVEK and Z. NEJEDLY, *Chem. Ind. London* 530 (1960).
11. A. E. MOORE, L. SABACHEWSKY and H. W. TOOLAN, *Cancer Res.* **15,** 598 (1955).
12. G. G. KELLEY, M. H. VAIL, D. J. ADAMSON and E. A. PALMER, *Am. J. Hyg.* **73,** 231 (1961).
13. E. P. ANDERSON and R. W. BROCKMAN, *Biochem. Pharmac.* **12,** 1335 (1963).
14. A. J. TOMISEK, A. P. V. HOSKINS and M. R. REID, *Cancer Res.* **25,** 1925 (1965).
15. P-L Biochemicals, Inc., *Bulletin*, p. 5. Milwaukee, Wisconsin (July, 1965).
16. L. L. BENNETT, JR., R. W. BROCKMAN, H. P. SCHNEBLI, S. CHUMLEY, G. J. DIXON, F. M. SCHABEL, JR., E. A. DULMADGE, H. E. SKIPPER, J. A. MONTGOMERY and H. J. THOMAS, *Nature, Lond.* **205,** 1276 (1965).
17. R. W. BROCKMAN and E. P. ANDERSON, *A. Rev. Biochem.* **32,** 463 (1963).
18. G. A. LEPAGE and M. E. JONES, *Cancer Res.* **21,** 642 (1961).
19. R. J. MCCOLLISTER, W. R. GILBERT, JR., D. M. ASHTON and J. B. WYNGAARDEN, *J. biol. Chem.* **239,** 1560 (1964).