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FAILURE OF 6-THIOGMP TO INHIBIT GUANYLATE KINASE IN INTACT CELLS*

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Abstract—Experiments have been performed that make necessary a modification of the hypothesis that 6-thioguanine produces cytotoxicity by a sequential blockade of the enzymes, ribosylamine-5-phosphate: pyrophosphate phosphoribosyltransferase (PRPP-amidotransferase), inosinate dehydrogenase and guanylate kinase. L5178Y murine leukemia cells (*in vivo* and *in vitro*) were pretreated with 6-thioguanine under conditions known to produce significant accumulations of the analog nucleoside 5'-monophosphate, 6-thioGMP, inhibition of purine *de novo* biosynthesis, and marked cytotoxicity. When these cells were incubated with [8-^{14}C]guanine, no inhibition in the formation of guanine nucleotides was observed in comparison with control cells not treated with 6-thioguanine. Furthermore, measurement of changes in the intracellular concentrations of GMP, GDP and GTP did not provide evidence for the occurrence of a 'cross-over' between GMP and GDP in the presence of 6-thioGMP. Thus, the predicted accumulation of GMP resulting from the postulated blockade of guanylate kinase by 6-thioGMP did not occur. L5178Y cells incubated with guanine for periods of 1 hr or less accumulated concentrations of GDP and GTP that approximated the intracellular levels of ADP and ATP. Time studies were performed with human erythrocytes in which the rate of formation of nucleotides was compared in cells incubated with guanosine or 6-thioguanosine. With guanosine, the rapid formation of guanine nucleotides was observed with the ratio of GTP:GDP:GMP approximating the ratios of the adenine nucleotides of the erythrocytes, i.e. no accumulation of GMP was observed at any time period up to 6 hr. In contrast, with 6-thioguanosine, a rapid initial formation of 6-thioGMP was observed with a gradually accelerating formation of 6-thioGTP. After 2 hr, the concentration of 6-thioGMP decreased whereas the formation of 6-thioGTP achieved a velocity of about 0.007 μmole/min/ml of erythrocytes. This velocity is about 2 per cent of that expected with saturating levels of GMP, if one assumes the intraerythrocytic activity of guanylate kinase to be 0.3 enzyme unit/ml of cells (enzyme unit: 1 unit catalyzes the conversion of 1 μmole substrate into product/min). These findings are in general agreement with the results of studies with purified guanylate kinase preparations described in the accompanying publication [R. L. Miller, D. L. Adamczyk, T. Spector, K. C. Agarwal, R. P. Miech and R. E. Parks, Jr., *Biochem. Pharmac.* **26**, 1573 (1977)], which indicate that the K_m value and V_{max} value of 6-thioGMP are approximately 2.0 mM and about 3 per cent of the V_{max} with GMP respectively. Therefore, it may be concluded that administration of 6-thioguanine does not cause significant inhibition of guanylate kinase. However, the poor reactivity of 6-thioGMP with guanylate kinase probably causes the marked intracellular accumulation of this analog nucleotide after administration of 6-thioguanine or its derivatives.

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‡ Abbreviations used are: PRPP-amidotransferase, ribosylamine-5-phosphate: pyrophosphate phosphoribosyltransferase (glutamate-amidating) (EC 2.4.2.14); inosinate dehydrogenase, IMP:NAD oxidoreductase (EC 1.2.1.14); guanylate kinase, ATP:GMP phosphotransferase (EC 2.7.4.8); 6-thioGMP, 6-thioGDP and 6-thioGTP, 6-thioguanosine 5'-mono-, di- and triphosphate, respectively; Sarcoma 180/TG, Sarcoma 180 cells resistant to 6-thioguanine: PRPP, 5-phosphoribosyl-1-pyrophosphate; nucleoside diphosphokinase (NDP kinase), ATP: nucleoside diphosphate phosphotransferase (EC 2.7.4.6); purine nucleoside phosphorylase, purine-nucleoside: orthophosphate ribosyltransferase (EC 2.4.2.1); hypoxanthine-guanine phosphoribosyltransferase, IMP-GMP pyrophosphate phosphoribosyltransferase (EC 2.4.2.8); PRPP synthetase, ATP; D-ribose-5-phosphate pyrophosphotransferase (EC 2.7.6.1); and E.U., enzyme unit (1 unit catalyzes the conversion of 1 μmole substrate into product/min).

Several years ago, this laboratory, in collaboration with colleagues from Yale University, presented the hypothesis that 6-thioguanine (6-TG) exerts its cytotoxic effects as the result of a sequential blockade involving the enzymes, PRPP-amidotransferase,‡ inosinate dehydrogenase and guanylate kinase [1]. In accord with this hypothesis were the observations of potent inhibition of *de novo* purine biosynthesis by the administration of 6-thioguanine [2] and of inhibition of PRPP-amidotransferase by 6-thioGMP [3, 4]. Also, it had been shown both with bacterial and Sarcoma 180 inosinate dehydrogenases that the enzyme is progressively and irreversibly inhibited by exposure to 6-thioGMP [1, 5]. Consistent with a significant role of the enzyme guanylate kinase in the action of 6-TG is the marked intracellular accumulation of 6-thioGMP with the delayed and relatively slow formation of the polyphosphate nucleotides [6]. Studies with purified guanylate kinase preparations from a variety of tissues indicated that 6-thioGMP can act as an inhibitor/alternative substrate with K_i values similar to the K_m values for GMP, but with V_{max}

values of less than 0.1 per cent of those for GMP [7–10]. However, studies during the past year, as reported in the accompanying paper [11], indicate that the apparent inhibition of guanylate kinase resulted from a spectrophotometric artifact and require that this portion of the hypothesis be modified.

Various techniques have been developed recently that have made it possible to examine the question of guanylate kinase inhibition by 6-thioGMP in intact viable cells. Most important has been the method of high pressure liquid chromatography with monitoring at two wavelengths, which has facilitated studies of the intracellular formation and interconversion of nucleotides of guanine and 6-thioguanine. When the relative rates of synthesis of 6-thioGMP, 6-thioGDP and 6-thioGTP in intact erythrocytes were examined, a much more rapid formation of the polyphosphate nucleotides was found than was predicted by the earlier studies with isolated guanylate kinase. Also, experiments have been performed to test for the postulated biochemical 'cross-over' [1] during the synthesis of guanine nucleotides from guanine in intact tumor cells inhibited by 6-thioGMP. In this case, the postulated blockade of the guanylate kinase reaction by 6-thioGMP should have caused a temporary accumulation of GMP and a fall in the GDP and GTP concentrations. As described below, neither type of experiment gave results consistent with predictions derived from the earlier kinetic studies with purified guanylate kinases [7–10]. The findings reported below, however, appear to be in reasonable agreement with the newer results described in the accompanying manuscript, which indicate that 6-thioGMP is a substrate with a relatively high K_m value (2–5 mM) and a V_{max} of about 3 per cent of that for GMP. The latter value is at least 20-fold greater than those reported in earlier studies [7–10]. Portions of this work have been reported previously [6, 12].

MATERIALS AND METHODS

Materials

Guanosine, 6-thioguanosine, 6-thioguanine and 6-methylmercaptopurine ribonucleoside were purchased from the Sigma Chemical Co., St. Louis, MO. and guanine and glutamine were from P-L Biochemicals, Milwaukee, WI. CalBiochem Corp., La Jolla, CA, supplied dithiothreitol. Dr. S. H. Chu of this Division prepared the [^{35}S]6-thioguanine (100 counts/min per nmole) used in this study. [8-^{14}C]guanine (35 μCi/μmole) and [2-^{14}C]glycine (39 μCi/μmole) were obtained from Schwarz/Mann, Orangeburg, NY. Fresh human blood collected in acid-citrate-dextrose medium, from which the platelet-rich plasma had been removed, was obtained from the Division of Hematological Research, Pawtucket Memorial Hospital, Pawtucket, R.I. Mouse tumor cells, originally obtained from Dr. A. C. Sartorelli, Yale University School of Medicine, New Haven, CT, were maintained by weekly i.p. transplantation into mice purchased from Charles River Labs (North Wilmington, MA). In the case of Sarcoma 180 and Sarcoma 180/TG, about 2×10^6 cells were transplanted into female CD^1 mice whereas for L5178Y cells, female BDF$_1$ mice were used.

Preparation of cells

Human erythrocytes were centrifuged at 1350 *g* in a clinical centrifuge for 5 min. Buffy coat and acid-citrate-dextrose medium were discarded. Erythrocytes were washed twice with 5 vol. of 0.9% NaCl and once with incubation medium (see below) by gentle resuspension and centrifugation (as above) and then suspended to about 50% (v/v) in incubation medium.

For drug treatment *in vivo*—incubation studies *in vitro*, 6-thioguanine was administered to mice in 0.9% NaCl solution by i.p. injection 6 or 7 days after tumor implantation. Control mice received identical volumes of 0.9% NaCl solution. One hr after administration of 6-thioguanine, tumor cells were removed from two mice of each group, pooled, and washed twice with 5 vol. of 0.9% NaCl. Contaminating erythrocytes were removed by differential centrifugation. Cells were then washed once with tumor cell incubation medium (see below) and resuspended to about 20% (v/v) in the same medium. For other studies performed entirely *in vitro*, cells were removed from mice 6 or 7 days after implantation and washed as above.

Incubation and extraction procedures

Washed erythrocytes (15% suspension) were incubated with guanosine (0.5 mM) or 6-thioguanosine (1 mM) in a medium consisting of NaCl (75 mM), $MgSO_4$ (2 mM), glucose (10 mM), potassium phosphate buffer (50 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml) at a pH of 7.4. Dithiothreitol (2 mM) was also included in incubations with 6-thioguanosine. Total volumes were 10 ml and incubations were carried out in a shaking water bath (80 oscillations/min) at 37° with air as the gas phase. At appropriate times after the addition of substrate, 0.5-ml aliquots of incubation mixture were removed and placed into chilled (4°) 15-ml centrifuge tubes containing 0.25 ml of 12% perchloric acid, mixed well and allowed to stand for 15 min. After centrifugation to remove denatured protein (1350 *g* for 5 min), 0.4-ml aliquots of the supernatant fluids were neutralized with cold KOH to pH 6.5–7.5 using phenol red as the indicator. After standing at 4° for 15 min, insoluble $KClO_4$ was removed by centrifugation. The clear supernatant solutions were transferred to 10 × 75 mm tubes, frozen and kept at −20° until analyzed by high pressure liquid chromatography (see below).

Tumor cells (Sarcoma 180 or Sarcoma 180/TG, control or 6-TG-treated), prepared as above, were suspended to a final concentration of 10% (v/v) in incubation medium consisting of Tris–HCl (40 mM), KCl (20 mM), NaCl (88 mM), $MgCl_2$ (2 mM), glucose (5.5 mM) and potassium phosphate buffer (22 mM) at a final pH of 7.4. [8-^{14}C]guanine (5.83 μCi/μmole) was added to a concentration of 100 μM. For L5178Y cell studies, cells were removed from four mice, pooled and washed as above. Cells were suspended to a final concentration of 5% (v/v) in tumor cell incubation medium and 6-thioguanine (180 μM) was added. The mixtures were incubated for 30 min, washed to remove residual 6-thioguanine, and resuspended (to a 5% concentration) in incubation medium containing [8-^{14}C]guanine (5.83 μCi/μmole; 100 μM). Other incubation conditions were as for human erythrocytes. Samples (1.0 ml) of the incubation mixtures were removed (at appropriate times after the addition

of labeled precursor) into chilled 15-ml centrifuge tubes containing 0.2 ml of 26% perchloric acid, mixed well, and allowed to stand for 15 min. Neutralization and storage conditions were as for erythrocytes. In experiments with L5178Y cells in which 6-thioguanine nucleotide synthesis was monitored, [^{35}S]6-thioguanine (500 cpm/nmole; 180 μM) was substituted for unlabeled 6-thioguanine and unlabeled guanine (100 μM) was used in place of [8-^{14}C]guanine. All other details were as above.

For studies of purine biosynthesis *de novo*, Sarcoma 180 cells were removed from two mice, pooled, washed twice with 0.9% NaCl and once with tumor cell incubation medium (see above). After incubation of cells (10% suspension) for 15 min, 6-thioguanine was added to appropriate flasks to a concentration of 150 μM and incubation was continued for 60 min. Cells were then washed twice with 0.9% NaCl, once with incubation medium, and finally resuspended to a concentration of 10% in incubation medium containing glutamine (2 mM). After 15 min of incubation, [2-^{14}C]glycine (39 μCi/μmole; 2.8 μCi/ml of incubation mixture) was added and incubations were continued. Total volume was 3.6 ml. At appropriate times after addition of radioactive precursor, 0.5-ml aliquots of reaction mixture were removed and placed into chilled (4°) 15-ml centrifuge tubes containing 0.1 ml of 26% perchloric acid. Neutralization and storage of extracts are described above.

Analysis of cell extracts

Nucleotide levels were determined in neutralized acid-soluble extracts by high pressure liquid chromatography using a Varian LCS-1000 liquid chromatograph. Usually 20 μl of the cell extract was used for chromatography. For studies with human erythrocytes, the column employed was a Reeve-Angel AS-Pellionex-SAX column (3 m × 1 mm). The low concentrate eluant was 0.002 M KH_2PO_4 (pH 4.5) and the high concentrate eluant was 0.5 M KH_2PO_4 (pH 4.5) in 1.0 M KCl. The starting volume was 40 ml and the flow rates were column, 14 ml/hr and gradient, 7 ml/hr. Eluants were monitored at two wavelengths, 254 nm to detect normal nucleotides and 350 nm to facilitate detection of 6-thioguanine nucleotides. For the tumor cell studies, high pressure liquid chromatography was carried out exactly as described by Parks and Brown [13]. In all cases, areas of peaks on the chromatographic profiles were determined by planimetry and the concentrations of nucleotides were determined by comparison with areas of standard nucleotide solutions of known concentration chromatographed under identical conditions.

To determine the incorporation of radioactivity from [8-^{14}C]guanine, [^{35}S]6-thioguanine or [2-^{14}C]glycine into nucleotides, fractions of the column eluants were collected at 2-min intervals (0.4 ml/fraction). The contents of the fraction collector tubes were rinsed with two 4.5-ml aliquots of a dioxane-based scintillation fluid prepared by dissolving two 4-g packets of Omnifluor [New England Nuclear, Boston, Mass.; contains 98% PPO (2,5-diphenyloxazole) and 2% bis-MSB(*p*-bis-[*O*-methylstyryl]-benzene)] plus 100 g napthalene in 1 liter of scintillation grade *p*-dioxane. All radioactivity measurements were made with a Packard Tri-Carb model 2000 scintillation counter at an efficiency of about 70 per cent.

RESULTS AND DISCUSSION

Rates of incorporation of guanosine and 6-thioguanosine into the nucleotide pools of human erythrocytes

It has been established in earlier studies that fresh normal human erythrocytes, in comparison with most other cell types, have very low levels of guanine nucleotides [14–17]. However, the human erythrocyte contains adequate activity levels of all of the enzymes required for the conversion of guanine or guanosine to GTP [10]; furthermore, when washed human erythrocytes are incubated with guanosine (1 mM) in the presence of high orthophosphate concentrations (50 mM, pH 7.4), and 10 mM glucose, they rapidly accumulate GTP in concentrations that far exceed the normal ATP concentrations of the cell (about 1.2 to 1.6 μmoles/ml of cells [14, 15]). In fact, in recent experiments on the formation of GTP by human erythrocytes, when reaction mixtures were supplemented with additional guanosine after several hours of incubation, GTP concentrations in the order of 7.0 μmoles/ml of cells were achieved (G. W. Crabtree, C. A. Bell and R. E. Parks, Jr., unpublished observations). Also, the initial rate of synthesis of GTP from guanosine is about 5-fold more rapid than the rate of synthesis of GTP from guanine [6]. It has been proposed that the rate-limiting step in guanine nucleotide synthesis in human erythrocytes is the formation of PRPP and that PRPP is synthesized more readily from ribose-1-phosphate (formed by the purine nucleoside phosphorylase reaction) than from glucose [18].

Figure 1 presents the results of a time study in which the relative rates of synthesis of GMP, GDP and GTP from guanosine (0.5 mM) were compared in the human erythrocyte. Throughout the experiment, GTP accumulated much more rapidly than

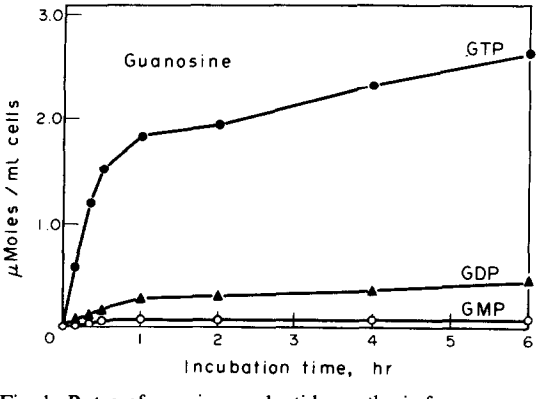

Fig. 1. Rates of guanine nucleotide synthesis from guanosine in human erythrocytes. Fresh, washed human erythrocytes (15% suspension) were incubated with guanosine (0.5 mM) at 37° in a shaking water bath with air as the gas phase. At the times indicated, samples of the reaction mixture were removed, extracted with perchloric acid, neutralized and aliquots of the neutralized solutions were analyzed for nucleotide content by high pressure liquid chromatography. For details of procedures, see Methods. Data are reproduced with the permission of the New York Academy of Sciences.

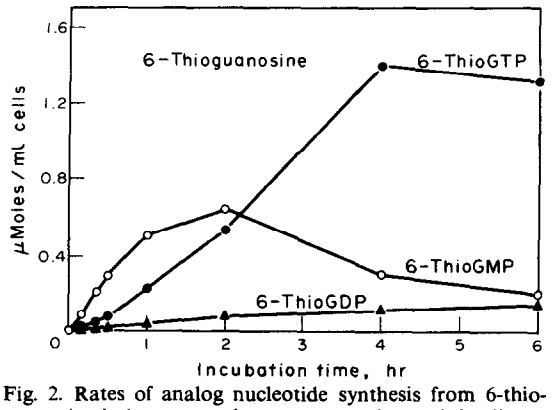

Fig. 2. Rates of analog nucleotide synthesis from 6-thioguanosine in human erythrocytes. Experimental details are as for Fig. 1, except that 6-thioguanosine was present at 1 mM and both incubation medium and perchloric acid contained dithiothreitol (2 mM). Data are reproduced with the permission of the New York Academy of Sciences.

either GDP or GMP, and at all times the GMP levels were low. In fact, through examination of high pressure liquid chromatograms in this experiment (data not shown), the ratio of GTP:GDP:GMP resembled closely the ratio of ATP:ADP:AMP. This experiment indicates that, in the erythrocyte, the rate-limiting step involves the synthesis of GMP, which is then rapidly converted into polyphosphate nucleotides by guanylate kinase and nucleoside diphosphokinase, both of which occur in the erythrocyte at reasonably high activities (guanylate kinase, about 0.3 E.U./ml of cells; NDP kinase, about 70 E.U./ml of cells [10]).

The rate of formation of guanine nucleotides during the first 20 min of incubation with guanosine was approximately 0.05 μmole/ml of cells/min. It is significant that this rate is much lower than the activities of purine nucleoside phosphorylase (13.0 E.U./ml of cells [10]), hypoxanthine-guanine phosphoribosyltransferase (0.3 E.U./ml of cells [16]), guanylate kinase (0.3 E.U./ml of cells [10]) and PRPP synthetase (0.35 E.U./ml of cells [19]).

All of the above enzymic activity measurements were performed with hemolysates by use of assays that measure maximal velocities, and it is probable that under intracellular conditions one or more of these enzymes may operate at less than maximal velocity. Other important aspects to be considered are: (1) a good estimate of the activity of phosphoribomutase in the human erythrocyte is not available, and (2) as established largely through the studies of Paterson *et al.* [20–22], there is a facilitated diffusion mechanism that efficiently delivers nucleosides such as guanosine into the erythrocyte. Although it seems unlikely that this transport mechanism could be rate limiting, it will be of interest to repeat an experiment similar to that of Fig. 1 in the presence and absence of a specific nucleoside transport inhibitor such as *p*-nitrobenzylthioguanosine [23].

As seen in Fig. 2, very different results were obtained when human erythrocytes were incubated with 1.0 mM 6-thioguanosine under conditions essentially identical to those of Fig. 1. During the first hour of incubation, much more rapid synthesis of 6-thioGMP than of 6-thioGDP or 6-thioGTP was observed. Also, the initial rate of 6-thioguanine nucleotide synthesis from 6-thioguanosine was much slower than the rate observed for the synthesis of guanine nucleotides in Fig. 1 (about 0.01 μmole/min/ml of cells from 6-thioguanosine vs about 0.05 μmole/min/ml of cells from guanosine). Especially significant is that after a lag period of about 30 min the formation of 6-thioGDP and 6-thioGTP began to accelerate, and at 2 hr, when the 6-thioGMP level has reached about 0.6 μmole/ml of cells, the rate of formation of 6-thioGTP reached a velocity of about 0.007 μmole/min/ml of cells. This latter value compares favorably with a value which can be estimated from other published works using Sarcoma 180 cells (see Table 6, Ref. 24).

This accumulation of 6-thioGMP with a delayed formation of 6-thioGDP and 6-thioGTP, we suggest, has special significance. In partial confirmation of earlier studies, these observations demonstrate, in intact cells, that 6-thioGMP is a poor substrate for the enzyme guanylate kinase. However, when one considers the rate of synthesis of 6-thioGTP during the period of 2–4 hr (0.007 μmole/min/ml of cells), this velocity is about 2 per cent of the maximal velocity that could have been achieved with GMP as the substrate with the amount of guanylate kinase normally found in human erythrocytes (about 0.3 E.U./ml of cells). However, this velocity is far greater than the V_{max} values for 6-thioGMP with various partially purified guanylate kinase preparations reported in earlier studies from this laboratory [7–10]. In more recent studies (documented in the accompanying manuscript [11]) the V_{max} of 6-thioGMP with partially purified guanylate kinase from human erythrocytes was about 3 per cent of the V_{max} observed with the natural substrate, GMP. Therefore, the above investigations with intact human erythrocytes are further evidence that the earlier reports of the very low reactivity of 6-thioGMP with the enzyme guanylate kinase were the result of an unfortunate artifact in the assay procedure and indicate that the conversion of 6-thioGMP to 6-thioGDP in intact cells is at least 20 times faster than predicted by the earlier erroneous kinetic data. However, the relatively slow reactivity of 6-thioGMP with guanylate kinase can result in the accumulation of this fraudulent nucleotide to remarkably high levels, e.g. about 0.6 μmole/ml of cells in Fig. 2, a concentration that far exceeds the concentrations of GMP that normally occur in intact cells.

Failure to demonstrate a biochemical cross-over in the synthesis of GTP from guanine in murine tumors inhibited by 6-thioguanine

The hypothesis has been presented [1] that potent inhibition of guanylate kinase by 6-thioGMP should cause a biochemical cross-over during the conversion of guanine or other guanine nucleotide precursors to GTP, i.e. inhibition of guanylate kinase by 6-thioGMP should produce an accumulation of GMP with a concurrent decrease in the concentrations of GDP and GTP. It was postulated that such a cross-over might be of short duration due to blockade of purine *de novo* biosynthesis and of inosinate dehydrogenase by 6-thioGMP, which would cause a lowering of the GMP pools. In order to test this hypothesis, experiments were performed in which

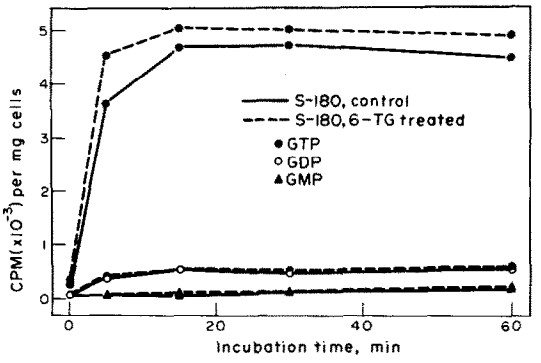

Fig. 3. Effect of 6-TG treatment on the incorporation of [8-^{14}C]-guanine into guanine nucleotides in Sarcoma 180 ascites tumor cells. Cells were removed from mice 1 hr after the i.p. administration (4 mg/kg; 6-TG treated) or 0.9% NaCl (control), washed and incubated (final cell concentration = 10%) with [8-^{14}C]guanine (5.83 μCi/μmole; 100 μM) in a final volume of 10 ml. For details of incubation, extraction, etc, see Methods.

Sarcoma 180 ascites cells, Sarcoma 180/TG cells (resistant to 6-thioguanine due to an increase in an alkaline phosphohydrolase [25]) and L5178Y cells were preincubated with 6-TG under conditions known to cause intracellular accumulations of 6-thioGMP in concentrations of about 0.1 μmole/g of cells or greater [26].

In Fig. 3 are presented the results of a time-course experiment on the incorporation *in vitro* of [8-^{14}C]guanine (100 μM) into guanine nucleotides in 6-day post-inoculation Sarcoma 180 ascites cells removed from mice 1 hr after an i.p. injection with 6-TG (4.0 mg/kg). The control consisted of cells removed from similar animals injected i.p. with an equal volume of 0.9% NaCl 1 hr prior to sacrifice. Aliquots of cells were removed at the indicated time periods during 1 hr of incubation. Perchloric acid extracts were prepared and subjected to high pressure liquid chromatography and the amounts of radioactivity in the peaks corresponding to GMP, GDP and GTP were determined. As seen in Fig. 3, no significant difference was observed in the rate or amount of radioactivity incorporated into the nucleotide pools of 6-TG-pretreated or control Sarcoma 180 cells. No evidence was observed to indicate the occurrence of cross-over, i.e. no accumulation of GMP was detected throughout the experiment that could be attributed to an intracellular inhibition of the enzyme, guanylate kinase. Essentially identical results were obtained in similar experiments with Sarcoma 180/TG ascites cells. Also, an experiment was performed with L5178Y cells removed from mice, 6 days post-implantation, and incubated *in vitro* in a medium containing 6-TG (180 μM). After 0.5 hr, the cells were washed three times in drug-free incubation medium and were resuspended in incubation medium containing [8-^{14}C]guanine (100 μM). The results of this experiment were qualitatively similar to those of Fig. 3, i.e. there was no evidence of accumulation of GMP consistent with an inhibition of guanylate kinase by 6-thioGMP. Of interest is that during the first 15 min of incubation with [8-^{14}C]guanine, the GTP levels of the L5178Y cells increased about 1.5- to 2-fold above the concentrations at zero time, as measured both by high pressure liquid chromatography and by the amount of radioactivity incorporated (data not shown). In similar experiments where L5178Y leukemia cells were pretreated for 30 min with [^{35}S]6-thioguanine (180 μM; 500 cpm/nmole) prior to treatment with non-labeled guanine (100 μM), the total acid-soluble 6-thioGMP incorporated intracellularly approximated 0.084 μmole/ml of cells. This concentration of intracellular 6-thioGMP is consistent with the amounts found in other experiments where significant tumor inhibition occurred.

Figure 4 illustrates the nucleotide profiles of L5178Y cells from this experiment. The bottom profile of this figure was obtained by removing samples of the incubation mixture at 0, 5, 15, 30 and 60 min after addition of unlabeled guanine, extracting the samples with perchloric acid, combining the neutralized extracts and subjecting an aliquot of the mixture to high pressure liquid chromatography. This procedure was followed both to obtain adequate sample volume for chromatography and to obtain a profile which represents an 'average' for the incubation period. These profiles emphasize that not only do 6-TG-containing nucleotides accumulate in these cells (see radioactivity profile), but also that the GTP levels are markedly increased over normal levels after incubation with guanine. Therefore, in the presence of substantial levels of 6-thioguanine-containing nucleotides, these cells were apparently capable of unim-

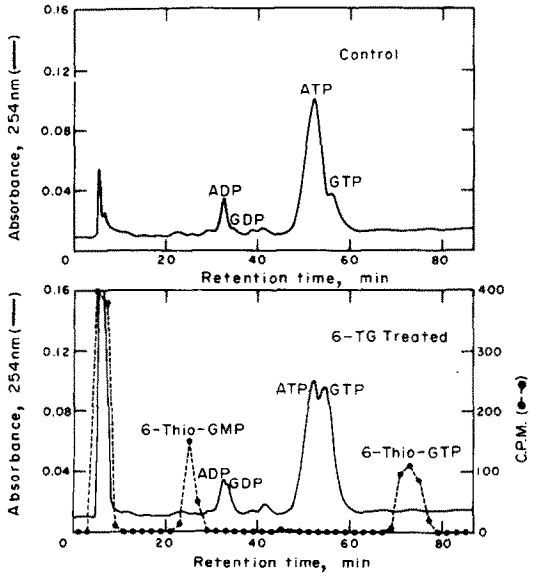

Fig. 4. High pressure liquid chromatographic profiles of L5178Y cells incubated with [^{35}S]6-thioguanine and guanine. Washed L5178Y cells were incubated for 30 min with [^{35}S]6-thioguanine (180 μM; 500 cpm/nmole), washed to remove residual labeled 6-thioguanine, and then incubated with unlabeled guanine (100 μM). Details of incubation, extraction, and chromatography are described under Methods. The upper profile represents a normal profile of L5178Y cells, i.e. cells which had not been incubated with either 6-thioguanine or guanine. For the lower profile, samples of the incubation mixture were removed at 0, 5, 15, 30 and 60 min after addition of unlabeled guanine, extracted, neutralized and the extracts were pooled. For high pressure liquid chromatography (HPLC), 20 μl of this "pooled" material was utilized. Eluates were monitored for radioactivity by collecting 2-min samples after HPLC.

peded and rapid synthesis of large quantities of guanine nucleotides from guanine.

In other experiments reported elsewhere, substantial antineoplastic synergism was observed between 6-thioguanine and 6-methylmercaptopurine ribonucleoside (MMPR) [26]. It was demonstrated that when animals were pretreated with MMPR, the analog nucleotide, 6-thioGMP, was synthesized more rapidly and to a significantly higher concentration than was observed with animals not pretreated with MMPR. In addition, the subsequent rate of decline in the 6-thioGMP levels (presumably due to the action of an alkaline phosphohydrolase) increased from a $T_{1/2}$ of about 7 hr to about 10–11 hr [26]. Therefore, an experiment similar to that of Fig. 3 was performed on Sarcoma 180 cells removed from mice that had been pretreated with MMPR (4 mg/kg) and 6 hr later, 6-TG (10 mg/kg). In this experiment also, no evidence of a GMP:GDP cross-over consistent with an inhibition of guanylate kinase was detected.

In order to demonstrate that under the conditions employed in these experiments the levels of 6-thioGMP which accumulated were capable of reproducing previously established [3] metabolic inhibitions in tumor cells, Sarcoma 180 ascites cells removed from mice 6 days after inoculation were thoroughly washed and incubated with 6-TG (150 μM). After 1 hr, the cells were washed free of extracellular 6-TG and were suspended in incubation medium containing glutamine (2 mM) and [2-^{14}C]-glycine (39 μCi/μmole, 2.8 μCi/ml of incubation mixture). Control cells were treated similarly, but were not preincubated with 6-thioguanine. After 1 hr of incubation with labeled glycine, the cells were washed and extracted as above. Aliquots of the neutralized extracts were subjected to high pressure liquid chromatography with collection of individual fractions of column eluate for counting of radioactivity. The greatest amount of radioactivity was present as ATP. As seen in Fig. 5, after incubation with 6-thioguanine, the incorporation of radioactivity from [2-^{14}C]glycine into ATP was significantly reduced.

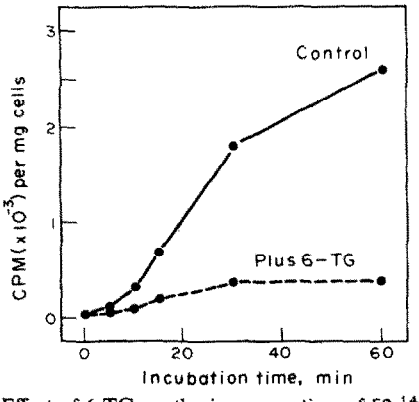

Fig. 5. Effect of 6-TG on the incorporation of [2-^{14}C]glycine into ATP in Sarcoma 180 ascites tumor cells. Cells were removed from mice, washed and incubated (10% final concentration) with 6-TG (150 μM) for 1 hr before addition of glutamine (2 mM) and [2-^{14}C]glycine (39 μCi/μmole; 2.8 μCi/ml) to give a total volume of 3.6 ml. Incubation medium was as for Fig. 3. Samples were removed at the indicated times and radioactivity present in ATP was determined as in Methods.

Therefore, it may be concluded that the quantities of 6-thioguanine nucleotides formed in the above experiment were sufficient to produce the expected block of purine *de novo* biosynthesis, presumably at the PRPP-amidotransferase step [3].

COMMENTS

The data presented above conclusively demonstrate that the intracellular accumulation of significant levels of the analog nucleotide, 6-thioGMP, does not produce a detectable interference with the interconversion of GMP and GDP, mediated by the enzyme, guanylate kinase. Since the appearance of our preliminary report [12], various groups of workers have documented similar findings with other types of cells [24, 27, 28]. At the time of our preliminary report, a satisfactory explanation was not at hand for the failure to demonstrate the postulated biochemical cross-over between GMP and GDP due to inhibition of guanylate kinase. Previously, there was no reason to question the earlier kinetic studies performed on numerous occasions with guanylate kinase preparations from various tissues and different species [7–10]. Now, however, as documented in the accompanying manuscript [11], it is apparent that the prior kinetic studies yielded erroneous data due to a spectrophotometric artifact [29]. The kinetic parameters (K_m, K_i and V_{max} values) measured by the use of several assay procedures are reasonably consistent with the intracellular findings presented above. As noted in the accompanying paper [11], when the corrected K_i of 6-thioGMP (2.3 mM) is used to calculate the degree of intracellular inhibition of guanylate kinase, a value of about 2 per cent is obtained rather than 46 per cent inhibition as estimated previously [24].

The marked accumulation of GTP and GDP in L5178Y cells (see Fig. 4) and erythrocytes after incubation with guanosine or guanine deserves further examination. In studies to be documented elsewhere, this laboratory has shown that, if the GTP level of erythrocytes is markedly elevated by preincubation with guanosine, subsequent incubation of these cells with 6-thioguanosine leads to a marked accumulation of 6-thioGMP with a striking inhibition in the rate of formation of 6-thioGDP and 6-thioGTP, in contrast to the results presented in Fig. 2 above. These observations suggest that accumulation of intracellular guanine nucleotides can result in inhibition of guanylate kinase. If similar results are found with tumor cells, i.e. L5178Y murine leukemia, one might expect that preincubation of the cells with guanosine or guanine under conditions that result in a large accumulation of GDP and GTP might markedly inhibit the incorporation of 6-thioguanine into cellular nucleic acids. Therefore, future studies are planned to examine the influence that modulations in the guanine nucleotide levels of cells may have on the cytotoxicity produced by 6-thioguanine or its derivatives.

The results presented above are in general agreement with the kinetic data in the accompanying manuscript [11], which demonstrate that K_m and V_{max} values of GMP and 6-thioGMP with guanylate kinases from various sources differ markedly, i.e.

K_m of GMP, 0.01 to 0.02 mM; K_m of 6-thioGMP $\simeq$ 2.0 mM; and V_{max} with 6-thioGMP about 3 per cent of that with GMP. In the experiment shown in Fig. 2, however, when the 6-thioGMP level reached about 0.5 to 0.6 μmole/ml of cells, the formation of 6-thioGTP proceeded at a rate approximately 2 per cent of that possible in the presence of saturating levels of GMP, if one assumes that the activity of guanylate kinase is 0.3 E.U./ml of cells [10]. To have achieved the observed rate of formation of 6-thioGTP with this amount of intracellular guanylate kinase, one would have expected a 6-thioGMP concentration in the order of four to five times greater than the K_m value, i.e. 8–10 mM. Possible explanations of this discrepancy include: (a) the enzymatic activity of guanylate kinase within the cell is considerably greater than the assumed value of 0.3 E.U./ml of erythrocytes; and (b) a form of compartmentation, or perhaps protein–protein aggregation, occurs that makes local concentrations of 6-thioGMP markedly greater than those measured with intact cells with the assumption that the 6-thioGMP is uniformly distributed throughout intracellular water.

Although the data above and in the accompanying manuscript [11] indicate that 6-thioGMP does not significantly impair the intracellular interconversion of guanine nucleotides, one may not conclude that guanylate kinase is not involved in the mechanism of cytotoxicity of 6-thioguanine. In fact, this enzyme may play a crucial role, since it is established that 6-thioGMP is a poor substrate for guanylate kinase, i.e. it has relatively high K_m and low V_{max} values compared to GMP. This fact can account for the marked accumulation of the analog nucleotide, 6-thioGMP, that is observed within the first hour after incubation of various cells with 6-thioguanine or 6-thioguanosine (see Fig. 2). Although intracellular GMP concentrations vary from one cell type to another and are usually too low to measure conveniently by techniques such as high pressure liquid chromatography, the intracellular concentrations of GMP are usually estimated to fall in the range of 1–10 nmoles/ml of cells. This range of GMP concentrations is only about 1–10 per cent of the 6-thioGMP levels regularly shown to accumulate in susceptible tumor cells. In view of the well-established metabolic inhibitions caused by nucleoside 5′-monophosphates [30], it remains a distinct possibility that this marked accumulation of 6-thioGMP causes metabolic effects that are responsible for certain of the cytolytic manifestations of drugs of this class. In support of this possibility, the closely related purine analog, 6-selenoguanosine, has been shown to possess antitumor and cytotoxic actions which are virtually indistinguishable from those exhibited by 6-thioguanosine [31]. When cells are treated with 6-selenoguanosine under conditions which result in cell death, this analog accumulates intracellularly at the nucleoside 5′-monophosphate (6-selenoGMP) level with no detectable formation of analog nucleoside 5′-di- or triphosphate. Accordingly, 6-selenoguanosine appears to exert its metabolic effects as 6-selenoGMP [31, 32]. However, for 6-thioguanine, incorporation into cellular DNA may also serve as a mechanism of cytotoxicity in some types of cells [24], but this mechanism may not hold for all cells [31, 32].

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REFERENCES

1. R. P. Miech, R. E. Parks, Jr., J. H. Anderson, Jr. and A. C. Sartorelli, *Biochem. Pharmac.* **16**, 2222 (1967).
2. A. C. Sartorelli and G. A. LePage, *Cancer Res.* **18**, 1329 (1958).
3. R. J. McCollister, W. R. Gilbert, Jr., D. M. Ashton and J. B. Wyngaarden, *J. biol. Chem.* **239**, 1560 (1964).
4. P. W. Allan and L. L. Bennett, Jr., *Biochem. Pharmac.* **20**, 847 (1971).
5. A. Hampton, *J. biol. Chem.* **238**, 3068 (1963).
6. R. E. Parks, Jr., G. W. Crabtree, C. M. Kong, R. P. Agarwal, K. C. Agarwal and E. M. Scholar, *Ann. N.Y. Acad. Sci.* **255**, 412 (1975).
7. R. P. Miech and R. E. Parks, Jr., *J. biol. Chem.* **240**, 351 (1965).
8. R. P. Miech, R. York and R. E. Parks, Jr., *Molec. Pharmac.* **5**, 30 (1969).
9. K. C. Agarwal and R. E. Parks, Jr., *Molec. Pharmac.* **8**, 128 (1972).
10. R. P. Agarwal, E. M. Scholar, K. C. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **20**, 1341 (1971).
11. R. L. Miller, D. L. Adamczyk, T. Spector, K. C. Agarwal, R. P. Miech and R. E. Parks, Jr., *Biochem. Pharmac.* **26**, 1573 (1977).
12. G. W. Crabtree, J. A. Nelson and R. E. Parks, Jr., *Proc. Am. Ass. Cancer Res.* **14**, 123 (1973).
13. R. E. Parks, Jr. and P. R. Brown, *Biochemistry* **12**, 3294 (1973).
14. E. M. Scholar, P. R. Brown, R. E. Parks, Jr. and P. Calabresi, *Blood* **41**, 927 (1973).
15. P. R. Brown, R. P. Agarwal, J. Gell and R. E. Parks, Jr., *Comp. Biochem. Physiol.* **43B**, 891 (1972).
16. R. E. Parks, Jr., P. R. Brown, Y.-C. Cheng, K. C. Agarwal, C. M. Kong, R. P. Agarwal and C. C. Parks, *Comp. Biochem. Physiol.* **45B**, 355 (1973).
17. G. R. Bartlett and G. L. Brewer, *Red Cell Metabolism and Function*, p. 245. Plenum Press, New York (1970).
18. C. M. Kong and R. E. Parks, Jr., *Biochem. Pharmac.* **24**, 807 (1975).
19. I. H. Fox and W. N. Kelley, *J. biol. Chem.* **246**, 5739 (1971).
20. J. M. Oliver and A. R. P. Paterson, *Can. J. Biochem.* **49**, 262 (1971).
21. C. E. Cass and A. R. P. Paterson, *J. biol. Chem.* **247**, 3314 (1972).
22. M. A. Pickard, R. R. Brown, B. Paul and A. R. P. Paterson, *Can. J. Biochem.* **51**, 666 (1973).
23. J. M. Oliver and A. R. P. Paterson, *Can. J. Biochem.* **49**, 271 (1971).
24. J. A. Nelson, J. W. Carpenter, L. M. Rose and D. J. Adamson, *Cancer Res.* **35**, 2872 (1975).
25. M. K. Wolpert, S. P. Damle, J. E. Brown, E. Sznycer, K. C. Agrawal and A. C. Sartorelli, *Cancer Res.* **31**, 1620 (1971).
26. J. A. Nelson and R. E. Parks, Jr., *Cancer Res.* **32**, 2034 (1972).
27. J. A. Nelson and J. Kuhns, *Pharmacologist* **16**, 209 (1974).
28. K. F. Lau and J. F. Henderson, *Cancer Chemother. Rep.* (Pt. 2) **3**, 95 (1972).
29. R. L. Cavalieri and H. Z. Sable, *Analyt. Biochem.* **59**, 122 (1974).
30. A. R. P. Paterson and D. M. Tidd, in *Handbook of Experimental Pharmacology*, XXXVIII/2—*Antineoplastic and Immunosuppressive Agents* (Eds. A. C. Sartorelli and D. G. Johns), Vol. II, p. 384. Springer, New York (1975).

31. R. E. Parks, Jr., B. Robinson, M.-Y. Chu, J. B. Melvin and E. H. Leduc, *Proc. Am. Ass. Cancer Res.* **17**, 213 (1976).

32. B. S. Robison, M-Y. Chu and R. E. Parks, Jr., *Cancer Res.* in press.