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STUDIES ON THE BIOCHEMICAL ACTIONS OF 6-SELENOGUANINE AND 6-SELENOGUANOSINE*

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Abstract—Survival studies were performed in mice bearing Sarcoma 180 ascites tumor treated with 6-thio and 6-seleno analogs of guanine and guanosine. The selenium-containing analogs were somewhat superior to the sulfur-containing compounds in antitumor activity and therapeutic index. The formation of 6-SeGMP from 6-seleno-guanine (6-SeG) was demonstrated in Sarcoma 180 ascites cells. Guanine, 6-thioguanine (6-TG) and 6-SeG show comparable substrate activity whereas 8-azaguanine is a much poorer substrate for hypoxanthine-guanine phosphoribosyl transferase from Sarcoma 180 cells. Both 6-TG and 6-SeG are good substrates for purine nucleoside phosphorylase from Sarcoma 180 cells. Chemically and enzymatically synthesized 6-SeGMP behaved as a competitive inhibitor (K_i 1×10^{-4} M) of erythrocytic and Sarcoma 180 guanylate kinases. Weak substrate activity was demonstrated in the presence of large amounts of erythrocytic guanylate kinase.

Mautner *et al.*[1] have shown that 6-SeG,† a close analog of 6-TG, is as effective an inhibitor as 6-TG to the growth of several experimental tumors and also is less toxic to the host and therefore has a somewhat superior therapeutic index. These guanine analogs differ chemically in that the Se atom has a significantly larger covalent radius (1·07 Å) and van der Waal's radius (2·00 Å),[2] than the S atom (0·94 and 1·85 Å, respectively); and the p*K*a values are different (8·3 and 7·6 for 6-TG and 6-SeG, respectively).[3,4] These chemical differences suggest that 6-SeG or its anabolites may react differently from 6-TG or its anabolites with specific enzymes within the cell. Therefore, the following studies were undertaken to gain further insight into the mechanism of action of 6-SeG: (1) the response of Sarcoma 180 ascites tumor to 6-SeG and its ribonucleoside; (2) the metabolism *in vivo* of 6-SeG by this tumor line; and (3) the kinetic analysis of 6-SeG and SeGMP with specific mammalian enzymes involved in purine metabolism. Preliminary reports of this work have been presented.[5]

MATERIALS AND METHODS

6-TG, 6-TGR, Clelands Reagent (DTT) and DEAE cellulose (0·85 mEq/g) were purchased from CalBiochem Corp., Los Angeles, Calif. The sources of various nucleotides, cofactors, other enzyme substrates, pyruvate kinase and lactate dehydrogenase were described earlier.[4] Erythrocytic nucleoside diphosphokinase and crystalline

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† Abbreviations used: 6-TG, 6-thioguanine; 6-TGR, 6-thioguanosine; TGMP, 6-thioguanosine-5′-phosphate; 6-SeG, 6-selenoguanine; 6-SeGR, 6-selenoguanosine; SeGMP, 6-selenoguanosine-5′-phosphate; 8-AzaG, 8-azaguanine; 8-AzaGMP, 8-azaguanosine-5′-phosphate; TGTP, 6-thioguanosine-5′-triphosphate; SeGTP, 6-selenoguanosine-5′-triphosphate; DTT, dithiothreitol; GMP kinase, guanylate kinase (EC 2.7.4.8); HGPRT, hypoxanthine-guanine phosphoribosyl transferase (EC 2.4.2.8); NDP kinase, nucleoside diphosphokinase (EC 2.7.4.6); TEAB, triethyl ammonium bicarbonate; PRPP, 5-phosphoribosyl-1-pyrophosphate; S-180, Sarcoma 180.

PNPase were supplied by Dr. R. P. Agarwal, Brown University, Providence, R.I. Dowex-1-formate, Ag-1-×8, 200–400 mesh, was purchased from Bio-Rad, Richmond, Calif. The magnesium salt of 5-phosphoribosyl-1-pyrophosphate (PRPP) was purchased from P & L Biochemical Inc., Milwaukee, Wis. All other chemicals were of the highest purity available.

Preparation of 6-selenoguanine and its ribonucleoside. 6-SeG and 6-SeGR were synthesized according to the procedures of Mautner *et al.*[1] and Chu,[6] respectively.

Antitumor testing. Sarcoma 180 (ascites) strain used in this study was generously supplied by Dr. A. C. Sartorelli, Yale University, and has been carried in this laboratory for several years. Survival time studies (Table 1) were carried out with CF-1

Table 1. Effect of 6-seleno and 6-thio analogs of guanine and guanosine on the survival time of mice bearing S-180 cells*

Drug	Daily dosage (mg/kg)	Average survival (days ±S. E.)	Average change in wt (%)	50 Day survivors/No. of animals
Control		13·5 ± 0·3	+17·9	0/60
6-SeG	1·6	17·6 ± 5·5	+6·9	0/5
	3·3	16·0 ± 1·2	+15·5	0/10
	6·5	21·5 ± 1·1	+1·7	0/25
	13·0	23·8 ± 0·7	−3·2	0/39
	26·0	7·4 ± 1·4	−17·7	0/5
6-SeGR	1·0	21·2 ± 1·9	+4·7	0/5
	2·1	21·6 ± 0·4	+2·2	0/15
	4·1	21·7 ± 0·6	+4·2	0/20
	6·2	23·7 ± 0·8	+4·0	4/75
	16·6	25·9 ± 2·4	−1·0	0/10
6-TG	1·0	18·4 ± 1·1	+4·7	0/25
	2·0	21·4 ± 1·3	+0·5	0/10
	5·0	12·7 ± 1·7	−0·9	0/10
	10·0	9·7 ± 0·5	−3·3	0/10
6-TGR	2·6	20·4 ± 1·2	+4·9	0/10
	5·2	15·7 ± 1·4	+1·4	0/10
	10·5	11·8 ± 1·5	−2·2	0/15

* Treatment: two times daily for 4 days.

mice purchased from the Charles River Breeding Company, N. Wilmington, Mass. Mice were inoculated with approximately five million Sarcoma 180 cells and drug injections were begun on the second day.

6-SeG was homogenized by adding to it two drops of 20% Tween 80 and a volume of 95% ethanol equivalent to 5% (v/v) of the final volume, and then brought to the desired final volume with isotonic saline solution. 6-TG was solubilized in isotonic saline with dilute sodium hydroxide and adjusted to pH 8 with dilute HCl. 6-SeGR and 6-TGR solutions were made in isotonic saline. Fresh solutions of these drugs were prepared daily just prior to use.

The animals were divided into groups of comparable weight (22–25 g/mouse). In these experiments the drugs in volumes of either 0·25 or 0·50 ml were injected intraperitoneally twice a day at a 5-hr interval for 4 consecutive days. Tumor-bearing animals receiving isotonic saline served as controls. The per cent change in body weight from the onset to the termination of therapy was used as an indication of drug toxicity.

Metabolism of 6-selenoguanine in vivo. CF-1 female mice (Carworth, Inc., New City, N.Y.) bearing 5-day growths of S-180 cells, were injected intraperitoneally with a freshly prepared solution of 6-SeG in isotonic saline. One hr after injection of 6-SeG, the ascites cells from each mouse were removed after injecting 1–2 ml of isotonic saline into the peritoneal cavity.[7] The cells were collected in an ice-cooled cylinder, and

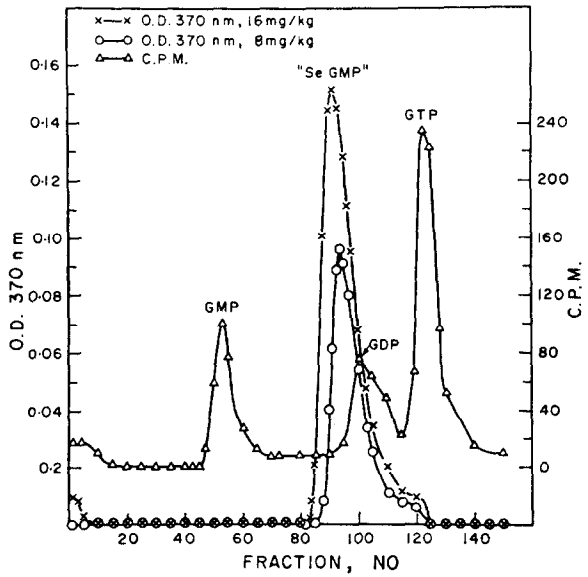

FIG. 1. Metabolism of 6-selenoguanine *in vivo*. Neutralized perchloric acid extracts of 6-SeG-treated S-180 cells from mice given dosages of 8 and 16 mg/kg were chromatographed on two separate Dowex-1-formate columns (1 × 10 cm). 8-^{14}C-GMP, GDP and GTP (80,000, 100,000 and 125,000 counts/min respectively) were added to the extract obtained from 16 mg/kg dosage before applying to the column. Other details are given under Materials and Methods.

cells that contained visible amounts of blood were discarded. The cells from each dosage level were pooled, and 0·5-ml aliquots were removed for hematocrit determination. The remaining cells were centrifuged for 10 min at 4° at 12,000 *g*. Six vol. of 4% perchloric acid were added to the pellet with rapid stirring with a Vortex mixer. This extract was centrifuged at 12,000 *g* for 20 min, and the pellet was re-extracted twice with one-half the original volume of 4% perchloric acid.

The combined extracts were adjusted to pH 5–6 at 4° by the addition of potassium hydroxide. After 20 min in an ice bath the mixture was centrifuged to remove the insoluble potassium perchlorate. Chromatography of the neutralized extracts was carried out on a Dowex-1-formate column by a modification of the procedure of Moore and LePage.[8] All eluting solutions contained 5 mM DTT. The flow rate was 1 ml/min, and the effluent was monitored at 370 nm. Radioactive ^{14}C-guanine nucleotides were added as markers to the extracts prior to chromatography (Fig. 1).

Enzymatic synthesis of 6-selenoguanosine-5'-monophosphate (SeGMP). The procedure for the enzymatic synthesis of SeGMP was a modification of that of Miech *et al.*[9] Added to 15 ml of distilled water was 6·5 mg of 6-SeG (30 μmoles). The suspension was boiled gently to dissolve most of the solid, quickly cooled to room temperature, and 0·4 ml of 0·1 M DTT was added. To this 6-SeG solution was added 1 μmolar unit of HGPRT (see below) from the dialysed S-180 ascites cell extract, and this solution was diluted to 20 ml with water. This mixture was then added to 20 ml of a solution containing 100 mM, Tris–HCl (pH 7·5); 10 mM, M_gSO_4; and 1 mM, PRPP in a 50-ml Erlenmeyer flask. The flask was tightly stoppered and incubated overnight at 37°. On the following day, the reaction mixture was centrifuged at 12,000 *g* for 20 min at 4°, diluted 1:4 with 1 mM DTT, and immediately placed on a DEAE–cellulose–bicarbonate column.

The eluted fractions that contained SeGMP were concentrated in a flash evaporator which removed the excess TEAB and left the triethyl-ammonium salt of the analog nucleotide as described by Miech *et al.*[9] This salt was dissolved in a small volume of water to yield a concentration of nucleotide of approximately 1–2 mM. This solution was partitioned into aliquots of 1 μmole and lyophilized to a viscous orange residue which was stored under nitrogen at −20°. Such storage is recommended since the selenohydryl group (–SeH) can be oxidized in the presence of air to the corresponding diselenide. The yield of the nucleotide from 6-SeG was about 60 per cent. Most of the DTT was removed from the nucleotide upon evaporation and lyophilization.

Chemical synthesis of 6-selenoguanosine-5-phosphate. A suspension of 2-amino-6-seleno-9-β-D-ribofuranosyl purine (6-selenoguanosine), 1·4 g in 15 ml of dry trimethyl phosphate, was stirred in an ice water bath, and to this was added dropwise 2·5 ml of freshly distilled phosphorus oxychloride, followed by 55 μl of water. The resultant yellow solution was stirred at 0° for 6 hr. At the end of this period, 800 ml of ether was added to the reaction mixture, the resultant yellow precipitate was filtered off, and the filtrate saved. Barium acetate (2·5 g) was added to the filtrate, and the mixture (pH 8·5) was allowed to stand overnight at room temperature. The precipitated barium phosphate was removed by centrifugation, washed with 10 ml water, and the combined supernatant fluids were evaporated to 40 ml in vacuum. The barium salt of the seleno nucleotide was precipitated by addition of 80 ml of ethanol. The light yellow precipitate was collected by centrifugation, washed with 10-ml portions of ethanol–water (2:1), then with 20 ml of ethanol followed by 20 ml of acetone. The crude nucleotide was dried over phosphorous pentoxide at room temperature under vacuum, and yielded 1·1 g of yellow solid. This barium salt of the crude nucleotide preparation was converted to the sodium salt by passage of a water solution of it through the sodium form of Dowex 50 (100–200 mesh). The sodium salt of SeGMP solution was subjected to flash evaporation, and the residue was dried as described for the barium salt.

About 40 mg of the sodium salt of the SeGMP was dissolved in 20 ml of 0·01 M triethylammonium bicarbonate (TEAB) solution and added to a DEAE-cellulose-bicarbonate column (2·5 × 14 cm, 0·88 mEq/g capacity). The column was washed with about 200 ml of 0·01 M TEAB solution. A linear gradient of TEAB (0·05–0·25 M over a volume of 1 l) was applied to the column, and SeGMP emerged from the column between 0·14 and 0·17 M TEAB. Fractions containing SeGMP as detected by absorbance at 360 nm, were pooled, taken to dryness by flash evaporation and re-

dissolved in 10–15 ml of water, and flash evaporation was repeated three times to remove most of the TEAB. Finally the residue was dissolved in 10 ml of water and divided into appropriate volumes, lyophilized and stored under nitrogen at −20° until required for study. Immediately prior to the studies, a solution of SeGMP was prepared and quantitated by assuming its molar absorbance equal to selenoguanosine ($\epsilon = 22{\cdot}3 \times 10^3$ at 357 nm in water).[6]

Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and purine nucleoside phosphorylase (PNPase) from sarcoma 180 *cells.* CF-1 mice bearing 6–8 days growths of S-180 cells were killed by cervical dislocation. The ascitic cells were isolated and

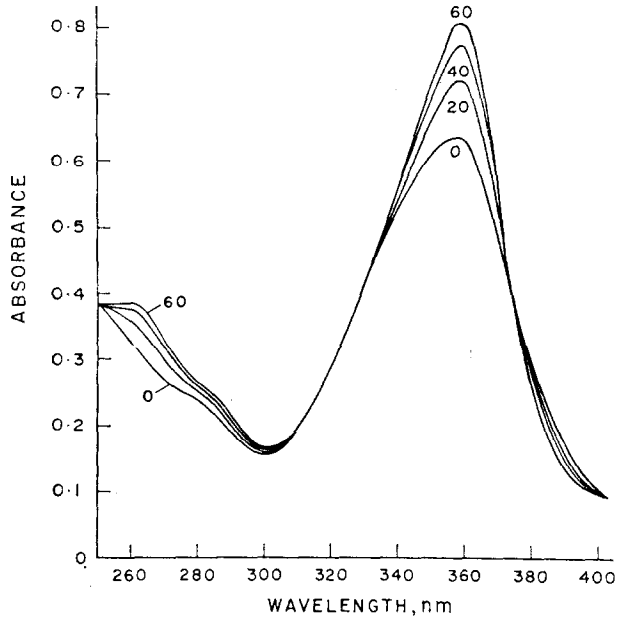

FIG. 2. Conversion of 6-selenoguanine to 6-selenoguanosine-5′-phosphate by HGPRT from S-180 Cells. The reaction mixture contained in 1 ml: 10 μl of the S-180 cell extract (see Materials and Methods); Tris–HCl (pH 7·5), 40 mM; PRPP, 0·6 mM; $MgCl_2$, 1 mM and DTT, 1 mM. Spectra were recorded against appropriate blank at room temperature by a Bausch & Lomb spectronic "505". The time sequence is from 0 to 60 min. No further change in the spectrum occurred after 60 min.

freed of traces of blood by isotonic saline washes and low speed centrifugations (ca. 100 rev/min, 6–8 min). The washed cells were centrifuged at 12,000 *g* for 5 min at 4° and the packed cells were stored at −80° until further use.

The cell pellets were thawed, homogenized and extracted with 1·5 vol. of 0·15 M KCl and 1 mM EDTA. This extract contained both PNPase and HGPRT activities and was employed in kinetic studies after exhaustive dialysis against solutions containing penicillin and streptomycin, 8 mM $MgCl_2$ and 50 mM Tris (HCl) pH 7·5.

Determination of the kinetic parameters of HGPRT with guanine and its analogs. The assay procedure was a modification of that described by Atkinson and Murray[10] using guanine as the substrate, and was essentially similar to a procedure described earlier by Way and Parks[11] in which 8-AzaG was the substrate.

Initial velocities were determined by measuring the increase in absorbance which occurs when the purine base or its analog is converted to its respective nucleotide. Figure 2 shows the increase in absorbance that occurs when 6-SeG is converted to its nucleotide. The change in molar absorbancy values at pH 7·5 (Δ O.D.) was as follows: guanine to GMP at 260 nm, 4900; 6-TG to TGMP at 344 nm, 3600; 6-SeG to SeGMP at 360 nm, 3700;* 8-AzaG to AzaGMP at 260 nm, 6100.[12] All reactions were carried out at 37°. One μmolar unit of enzyme is the amount that converts 1 μmole of guanine to GMP/min at 37° under the conditions of assay (Table 2).

Determination of the kinetic parameters of PNPase with guanine, hypoxanthine and guanine analogs. PNPase was assayed by measuring the increase in absorbance at a specific wavelength with time which occurs with the conversion of the purine base to its nucleoside. This spectrophotometric assay for PNPase was developed specifically for the study of the conversion of guanine analogs to their respective ribonucleosides.

Table 2. Kinetic parameters of S-180 HGPRT with guanine and its analogs*

Substrate	$K_m \pm$ S. E. (μM)	$V_{max} \pm$S. E. (nmoles/min)
Guanine	5·4 ± 0·9	3·6 ± 0·2
8-Azaguanine	300 ± 170	2·0 ± 0·9
6-Thioguanine	4·0 ± 0·6	3·7 ± 0·3
6-Selenoguanine	5·1 ± 0·6	3·6 ± 0·1

* One ml of the reaction mixture contained: HGPRT activity 3–8 mmolar units (extract containing HGPRT activity, described in Materials and Methods section); Tris–HCl buffer (pH 7·5), 45 mM; $MgSO_4$, 5 mM; PRPP, 0·5 mM and the substrate 5–110 μM. K_m and V_{max} values were calculated by using Cleland's computor program.[13] V_{max} values are expressed in terms of nanomoles per minute per 3·6 nmolar units of HGPRT.

The conversion of guanine to guanosine was used routinely to assay the activity of the enzyme, and the linearity of relative enzyme concentration (microliters of H.S.S.) extract enzyme vs. the rate of ribosylation of guanine is seen in Fig. 3. The reaction mixture is described in Table 3. One μmolar unit of enzyme is defined as the amount of enzyme that will catalyze 1 μmole of guanine to guanosine/min under the conditions specified in the legend to Table 3. The change in molar absorbancy for guanine, hypoxanthine, 8-AzaG, 6-TG and 6-SeG in conversion to their respective ribonucleosides at pH 6·5 was 5100 at 252 nm, 1400 at 248 nm, 6200 at 258 nm, 3700 at 344 nm and 3600 at 360 nm respectively.[4]

A recording Gilford spectrophotometer with a temperature regulator was used to determine all kinetic data. The velocities at various substrate concentrations were linear only over the first 1·5–2·0 min of the reaction. Enzyme concentrations and rates were adjusted so that the consumption of substrate was restricted to 20 per cent or less during the period of measurement.

* C. M. Kong and R. E. Parks, Jr., unpublished results.

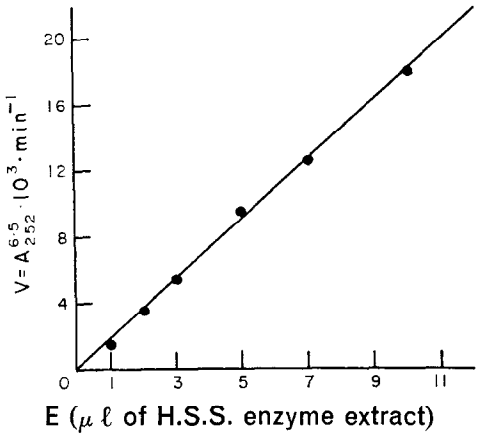

FIG. 3. Rate of conversion of guanine to guanosine by S-180 PNPase. The reaction constituents were similar to those described in Table 3, except that 0·1 mM guanine was used in each case with varying amounts of S-180 PNPase activity (see Materials and Methods). The velocity is expressed as the change in absorbance at 252 nm at pH 6·5.

Preparation of guanylate kinase (GMP kinase) from human erythrocytes. GMP kinase was isolated and purified by methods described by Agarwal and Parks.[14] The specific activity of the purified GMP kinase was 3·3 units/mg protein.

Guanylate kinase assay. GMP kinase activity was measured by recording the decrease in absorbance at 340 nm by the coupled pyruvate kinase-lactate dehydrogenase procedure described earlier.[14] One μmolar unit of the enzyme activity is defined as the

TABLE 3. KINETIC PARAMETERS OF S-180 PNPASE WITH GUANINE, HYPOXANTHINE AND GUANINE ANALOGS*

Substrate	$K_m \pm$ S. E. (μM)	$V_{max} \pm$ S. E. (nmoles/min)
Guanine	20 ± 2·7	2·8 ± 0·1
Hypoxanthine	59 ± 5·5	10·9 ± 0·6
8-Azaguanine	91 ± 23·3	1·9 ± 0·3
6-Thioguanine	42 ± 13·2	10·2 ± 1·9
6-Selenoguanine	16 ± 1·0	2·9 ± 0·1

* One ml of the reaction mixture contained PNPase activity 2·8 nmolar units (extract containing PNPase activity described in Materials and Methods section); HEPES–NaOH (*N*-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid–NaOH) buffer, pH 6·5, 50 mM; ribose-1-phosphate, 1 mM and the substrate 10–100 μM. K_m and V_{max} values were calculated by using Cleland's computor program.[13]

amount of the enzyme that will catalyze the phosphorylation of 1 μmole of GMP/min under the conditions of the standard assay.

Measurement of activity of 6-selenoguanosine-5'-phosphate and 6-thioguanosine-5'-phosphate as substrates for guanylate kinase. SeGMP was prepared chemically as described above and TGMP was synthesized enzymatically according to Miech *et al.*[9]

The reaction mixture for this assay was a modification of that described by Miech *et al.*[9] and contained: Tris–acetate, pH 7·5, 100 mM; $MgCl_2$, 10 mM; KCl, 100 mM; analog nucleotide, 0·2 mM; ATP, 1 mM; PEP, 3 mM; GMP kinase, 5 units; pyruvate kinase, 5 units. The final volume was 1 ml. The reaction mixture was incubated at 30°.

Aliquots (100 μl) were removed from the reaction mixture at zero time and after 30 min of incubation. Each aliquot was treated with equal volume of 10% TCA. The precipitated proteins were removed by centrifugation and the supernatant fluid was neutralized with 20 μl of 2 M Tris. The neutralized extracts were stored at −80° for further study.

An enzymic peak shift experiment was employed to identify the analog triphosphate nucleotide peaks. Fifty μl of the neutralized TCA extract, obtained after 30 min of incubation from the above experiment, was added to 50 μl of a mixture containing: Tris–acetate, pH 7·5, 100 mM; $MgCl_2$, 20 mM; KCl, 100 mM; glucose, 4 mM; hexokinase (sp. act. 245 units/mg of protein) 4 units; and erythrocytic NDP kinase (sp. act. 200 units/mg of protein), 4–5 units. After incubation for 1 hr, a neutralized TCA extract was obtained as described above, and stored at −80° until chromatographed.

The LCS-1000 high pressure liquid chromatographic system was employed for separation of nucleotides as described by Brown.[15] The flow rate was increased to twice of that normally used. The starting volume was 35 ml with a flow rate of 24 ml/hr for the column and 12 ml/hr for the gradient. Twenty μl of the neutralized extracts was applied and the u.v. output was either 0·02 or 0·08 absorbance units.

RESULTS

Antitumor activity of purine analog bases and ribonucleosides. The effects of 6-SeG, 6-SeGR, 6-TG and 6-TGR on the survival time of mice bearing S-180 ascites cells are shown in Table 1. 6-SeGR was found to be the most effective agent with a greater response at lower doses and with only slight toxicity at the highest dose tested. The data indicate that the 6-seleno analogs are less toxic than the 6-thio analogs and are as effective or somewhat more effective than the related thio analogs in inhibiting the growth of Sarcoma 180. This confirms the earlier results of Mautner *et al.*[1]

Metabolism of 6-selenoguanine in vivo. The sole detectable metabolite of 6-SeG found in acid extracts of S-180 cells exposed to 6-SeG for 1 hr is seen in Fig. 1. This has been identified as SeGMP on the basis of its migration on the Dowex-1-formate column. This 370-nm absorbing metabolite was eluted after GMP and overlapped with the GDP peak and contained more than 90 per cent of the 370 nm O.D. units in the diluted extracts. When chemically synthesized SeGMP was chromatographed on the same Dowex-1-formate column, the peak of 370 nm absorbance emerged in the same fraction as was observed for the 6-SeG metabolite. On the basis of the molar absorbancy value of 6-SeGR[6] at pH 1, the concentrations of SeGMP in the cells were estimated to be about 50 μM (8 mg/kg dose) and 100 μM (16 mg/kg dose). In this estimation it is assumed that SeGMP is uniformly distributed through cellular water which constitutes about 80 per cent of cellular volume. It is significant that no evidence was obtained of 370 nm absorbing material eluting in the positions expected for the di- and triphosphate nucleotides of 6-SeG, i.e. just before and after the GTP peak. This is consistent with the finding that SeGMP is a very poor substrate for guanylate kinase (see below).

Characterization of enzymatically prepared 6-selenoguanosine-5′-phosphate. SeGMP, synthesized as described above by the reaction of 6-SeG with PRPP in the presence of HGPRT from S-180 cells, chromatographed on DEAE–HCO_3 columns in an identical fashion to chemically synthesized SeGMP. The peak of activity emerged between 0·1–0·13 M TEAB. The u.v. spectrum of the isolated compound was essentially identical with that of authentic 6-SeGR. When the enzymatically prepared SeGMP was incubated with 5′-nucleotidase, partially purified from snake venom (Sigma product), a 1·1 : 1 ratio of 6-SeGR to P_i released was obtained. The nucleoside released after 5′-nucleotidase treatment migrated on PEI–cellulose (water-solvent) identically with authentic 6-SeGR (R_f 0·40).

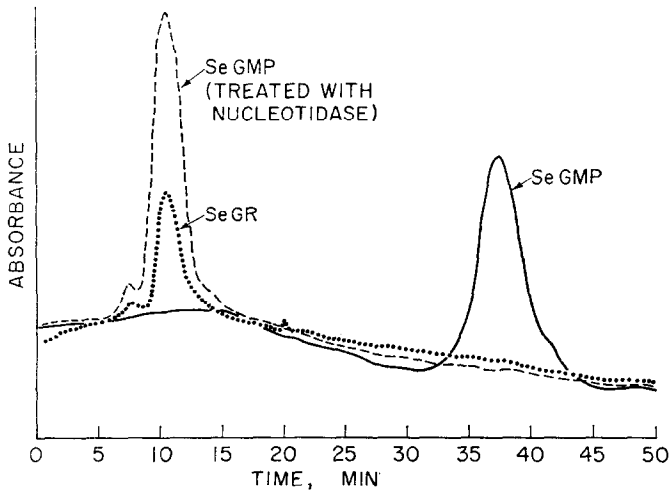

FIG. 4. High pressure liquid chromatographs of 6-SeGR, SeGMP and 5′-nucleotidase-treated SeGMP. 6-SeGR and 6-SeGMP, before and after treatment with 5′-nucleotidase, were subjected to high pressure liquid chromatography on a Varian LCS-1000. (· · ·) authentic 6-SeGR; (———) 6-SeGMP untreated; and (– – –) the reaction products of 6-SeGMP after treatment with 5′-nucleotidase.

Figure 4 shows the behavior of SeGMP on high pressure liquid chromatography on a Varian LCS-1000 under the conditions described by Brown.[15] A single peak with a retention time of 37 min was obtained. When the SeGMP was treated with 5′-nucleotidase and chromatographed on the LCS-1000, a peak shift was observed and the retention time of the new peak coincided with that of authentic 6-SeGR. In all chromatographic and enzymatic studies, chemically and enzymatically synthesized SeGMP behaved identically.

Inhibition of guanylate kinase by SeGMP. Under the conditions used to test chemically synthesized SeGMP as an inhibitor of erythrocytic GMP kinase, substrate activity was not seen. Rather, SeGMP behaved as a classical competitive inhibitor with GMP (Fig. 5) with a K_i value of $1{\cdot}1 \times 10^{-4}$ M. A similar K_i value for SeGMP was obtained with enzymatically prepared analog nucleotide.

Kinetic parameters of PNPase with guanine, hypoxanthine and guanine analogs. It was found that all of the analogs of guanine examined can serve as substrates for S-180 PNPase. 6-TG and 6-SeG were similar to guanine and hypoxanthine in their ability

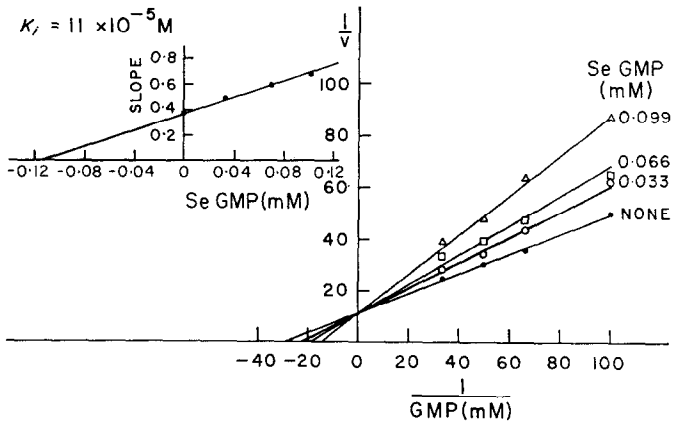

FIG. 5. Competitive inhibition of erythrocytic GMP kinase by 6-seleno-GMP. The reciprocal of the initial velocities ($1/A_{340}$ min^{-1}) is plotted against the reciprocal of the varying concentrations of GMP in the presence of a fixed concentration of 6-SeGMP. The insert is a plot of the slopes against the concentration of 6-SeGMP. From this a K_i value of 1·1 × 10^{-4} M was calculated.

to react with this enzyme as indicated by their similar K_m values (Table 3), and 8-AzaG (highest K_m, Table 3) appears to be the poorest substrate of the guanine analogs tested. V_{max} values with these substrates vary over about a 5-fold range. 6-TG and hypoxanthine have greater V_{max} values under these conditions than guanine, 8-AzaG, or 6-SeG. The kinetic parameters for crystalline erythrocytic PNPase with guanine, hypoxanthine and 6-SeG as substrates are similar to those observed with the S-180 enzyme (Table 3).

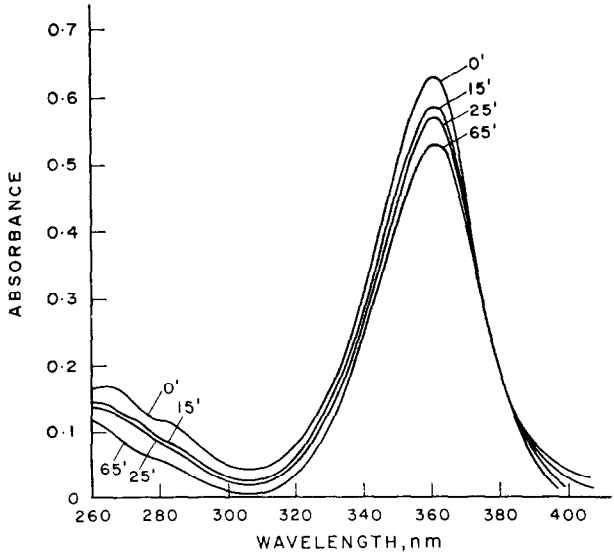

FIG. 6. Conversion of 6-selenoguanosine to 6-selenoguanine by erythrocytic PNPase. The reaction mixture contained in 1 ml: PNPase, 10 nmolar units (sp. act. 80 μmolar units/mg of protein); potassium phosphate buffer (pH 6·5), 50 mM; 6-SeGR, 0·026 mM; and DTT, 1 mM. The spectra were recorded as described in Fig. 2. No further change in the spectrum occurred after 65 min.

It was also observed that either crystalline PNPase from human erythrocytes or S-180 cells can cleave 6-SeGR and 6-TGR to their respective bases. Figure 6 represents the change in u.v. spectral patterns when 6-SeGR is incubated with P_i and human erythrocytic PNPase.

Kinetic parameters of HGPRT with guanine analogs. Guanine, 6-TG and 6-SeG were found similar in their interaction with HGPRT on the basis of K_m and V_{max} values. However, 8-AzaG is a significantly less effective substrate since the V_{max} is only one half and the K_m about 30-fold greater than the values measured with guanine. Krenitsky *et al.*[16] have observed that guanine and 6-TG are both potent inhibitors

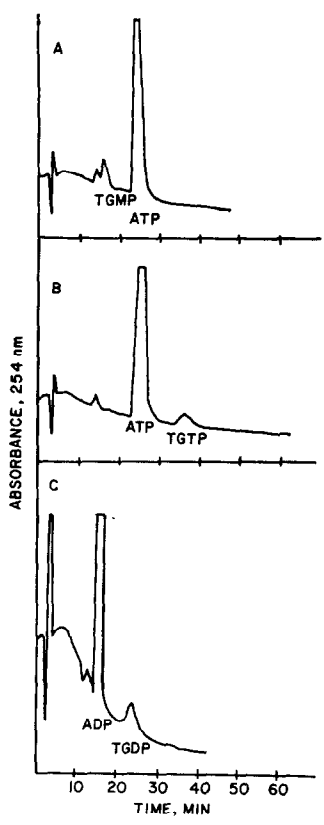

FIG. 7

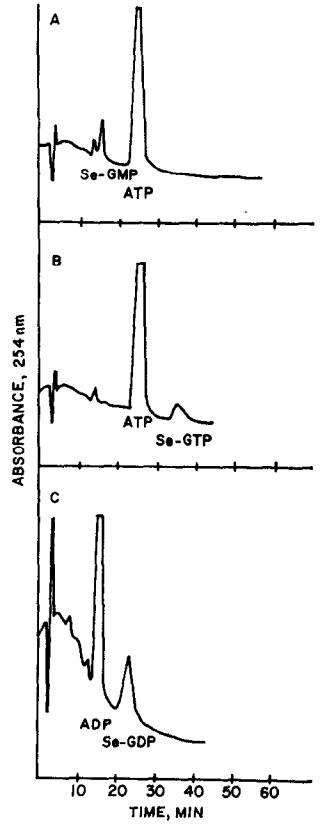

FIG. 8

FIG. 7. Weak substrate activity of 6-thioGMP with guanylate kinase. The nucleotides in the neutralized TCA extracts were subjected to high pressure liquid chromatography. A and B show the nucleotide patterns obtained from neutralized TCA extracts of reaction mixtures initially containing 6-thioGMP and ATP at 0 and 30 min. incubation, of respectively, as described in Materials and Methods. C shows the enzymic peak shift for characterization of 6-thioGTP. Extract of pattern B was incubated with NDP kinase and hexokinase as described in Materials and Methods.

FIG. 8. Weak substrate activity of 6-seleno GMP with guanylate kinase. The nucleotides in the neutralized TCA extracts were subjected to high pressure liquid chromatography. A and B show the nucleotide patterns obtained from neutralized TCA extracts of reaction mixtures initially containing 6-seleno GMP and ATP at 0 and 30 min of incubation, respectively, as described in Materials and Methods. C shows the enzymic peak shift for characterization of 6-seleno GTP. Extract of pattern B was incubated with NDP kinase and hexokinase as described in Materials and Methods.

(K_i, 1·8 and 2·4 μM, respectively) of erythrocytic HGPRT with hypoxanthine as the substrate whereas 8-AzaG was a poor inhibitor (K_i, 280 μM). These observations are consistent with the above findings that 6-TG is as good as a substrate as guanine for HGPRT whereas 8-AzaG is a relatively poor one and indicate that Krenitsky *et al.*[16] were observing an alternative substrate effect rather than a true inhibition.

Substrate activity of SeGMP and TGMP with erythrocytic guanylate kinase. Miech *et al.*[9] demonstrated the ability of a high concentration of hog brain GMP kinase to convert TGMP to the triphosphate nucleotide in the presence of pyruvate kinase and the necessary substrates and cofactors. In a similar system with erythrocytic GMP kinase, the formation of the analog triphosphate nucleotide from either TGMP or SeGMP occurs within 30 min as shown in Figs. 7A, B and 8A, B respectively. Both of these analog nucleotide triphosphates eluted after GTP (26 min). This observation agrees with other observations from this laboratory that 6-SeG and 6-TG containing nucleotides elute after the corresponding guanine nucleotides using either a weak (DEAE) or strong (DOWEX-1) anion exchanger.[11]

A further chromatographic test was carried out to confirm the formation of the analog triphosphate nucleotides. It was reasoned that the analog triphosphate in the presence of excess ADP and NDP kinase may act as a phosphate donor for ADP. This reaction would convert the analog triphosphate nucleotide to the diphosphate nucleotide which should elute between GDP and GTP. Since the TCA-extracted and neutralized solutions containing the analog triphosphate nucleotides already contained excess ATP, hexokinase was used to convert ATP to ADP in the presence of necessary substrates and cofactors. Figures 7C and 8C clearly show that, in the presence of the above enzymes with supplementary substrates and cofactors, the analog triphosphate nucleotide peaks disappeared and new peaks appeared eluting with retention times placing these peaks between GDP (16 min) and GTP (26 min) as predicted. It should be noted that a more rapid elution system was used with the LCS-1000 than is usually employed by this laboratory.[15] The relatively long retention times of SeGTP and TGTP made this desirable.

DISCUSSION

The increased survival time of mice bearing S-180 after treatment with thio and seleno analogs of guanine and guanosine is in essential agreement with the earlier studies of Mautner *et al.*[1] In both studies somewhat greater increases in survival were achieved with the selenium analogs. When compared on a molar basis, the ribonucleoside, 6-SeGR, appeared superior to the base, 6-SeG. This is somewhat surprising since 6-SeGR is a good substrate for purine nucleoside phosphorylase and should be readily degraded to 6-SeG and ribose-1-phosphate by the plentiful erythrocytic PNPase. Also, there is no evidence for a guanosine kinase capable of synthesizing SeGMP from 6-SeGR. It is possible that the greater solubility of 6-SeGR is responsible for its somewhat greater antitumor activity.

Ross[4] has found that 6-SeG and 6-TG are unreactive with S-180 and murine hepatic guanase. The kinetic parameters of 6-SeG with PNPase and HGPRT suggest that it is an excellent substrate for both enzymes. This is in agreement with studies *in vivo* that demonstrate that the main metabolite of 6-SeG metabolism by S-180 ascites cells is SeGMP. The intracellular accumulation of SeGMP probably results from its good activity with HGPRT and the fact that it is a very poor substrate for

GMP kinase. Very similar results are found with 6-SeG and 6-TG and their nucleotides with all enzymes examined, i.e. PNPase, HGPRT and GMP kinase.

Miech *et al.*[17] have proposed that GMP kinase inhibition by TGMP may play a role in the inhibitory mechanism of 6-TG. Here, SeGMP inhibited S-180 GMP kinase and its K_i (100 μM) was close to that reported for TGMP inhibition of this same enzyme.[9] The marked similarities between 6-TG and 6-SeG metabolism and enzymatic interaction suggest that the mechanism of action of 6-SeG against this tumor line may be similar if not identical to that of 6-TG.

For both 8-AzaG and 6-TG, conversion to 5'-monophosphate nucleotides is a necessary step for inhibition of tumor growth.[18,19] Although S-180 is sensitive to both 6-TG and 6-SeG,[1,7,20] this tumor is resistant to 8-AzaG.[21] These results may be explained by the relative substrate activity of these analogs with HGPRT (Table 2) since 6-TG and 6-SeG are excellent substrates for this enzyme while 8-AzaG is a very poor substrate. In addition, S-180 guanine deaminase (guanase) readily converts 8-AzaG to the inactive 8-azaxanthine whereas 6-TG and 6-SeG have neither substrate nor inhibitory activity with S-180 guanase.[4]

Although these studies demonstrate the remarkable similarity of 6-TG and 6-SeG and their respective nucleosides and nucleotides in their reactions in S-180 cells and with isolated enzymes of purine metabolism, they do not offer evidence on the somewhat superior therapeutic index of the selenium analogs. Detailed examination of the metabolic reactions of various normal tissues will probably be required to obtain a satisfactory explanation. It is possible that further examination of these selenium analogs will aid in explaining the mechanism of action of this class of guanine analogs. Of particular interest will be studies of the incorporation of 6-SeG into DNA since LePage[20] has postulated that incorporation of 6-TG into DNA is the cause of the cytolytic action of this compound. However, these studies must await the availability of a suitable radioactive form of 6-SeG.

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