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INHIBITION OF PURINE BIOSYNTHESIS *DE NOVO* IN SARCOMA 180 ASCITES CELLS BY MERCAPTO AND SELENO PURINE ANALOGS*

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Abstract—6-Methylmercaptopurine ribonucleoside (MeMPR)† is the most potent inhibitor known of PRPP amidotransferase, the first step in purine biosynthesis *de novo*. We have examined other 6-methylmercapto- and 6-methylselenopurine ribonucleosides for their ability to act as feedback inhibitors of this pathway as well as for their ability to form analog nucleotides. Methylmercaptopurine ribonucleoside (MeMPR), methylselenopurine ribonucleoside (MeSePR), and methylmercaptopyrazolo pyrimidine ribonucleoside (MeMPPR) were potent inhibitors (90 per cent), methylselenopyrazolo pyrimidine ribonucleoside (MeSePPR) was less potent (75 per cent), and methylselenoguanosine (MeSeGR) did not significantly inhibit this pathway. Large amounts of the corresponding 5′-monophosphate nucleosides were formed from MeMPR and MeMPPR, lesser amounts from MeSePPR and MeSePR and none from MeSeGR. The ability of 6-selenoguanine, its ribonucleoside and the α- and β-anomers of the deoxyribonucleoside were also examined as possible inhibitors of purine synthesis *de novo*. Further investigation is needed to determine if these compounds have significant activity as antineoplastic agents, but in any case they may lead to structural modifications which will produce active compounds.

STUDIES on the feedback inhibition of purine biosynthesis *de novo* by purines and purine analogs in various types of mammalian tissues, including tumors, have indicated that the adenosine analog 6-methylmercaptopurine ribonucleoside (MeMPR) is one of the most potent inhibitors of this process known.[1] MeMPR is known to be an effective antitumor agent against several animal neoplasms especially when used in combination with 6-mercaptopurine (6-MP) or 6-thioguanine.[2–5] These properties have led us to examine several compounds structurally related to MeMPR for their ability to act as inhibitors of purine biosynthesis *de novo*. The compounds tested were 6-methylselenopurine ribonucleoside (MeSePR), 6-methylselenoguanosine (MeSeGR), 7-methylmercapto-3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidine (MeMPPR), 7-methylseleno-3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidine (MeSePPR). In a sepa-

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† Abbreviations used: 6-MP, 6-mercaptopurine; MeMPR, 6-methylmercaptopurine ribonucleoside; MeSeGR, 6-methylselenoguanosine; MeSePR, 6-methylselenopurine ribonucleoside; MeMPPR, 7-methylmercaptopyrazolo-[4,3-d]pyrimidine; MeSePPR, 7-methylselenopyrazolo-[4,3-d]pyrimidine; PRPP, 5-phosphoribosyl-1-pyrophosphate; SeG, 6-selenoguanine; SeGR, 6-selenoguanosine; α-SeGdR and β-SeGdR, α- and β-selenodeoxyguanosine; FGAR amidotransferase, formylglycineamide ribonucleotide amidotransferase.

rate but related study, the compounds 6-selenoguanine (SeG), 6-selenoguanosine (SeGR) and the α- and β-anomers of 2′-deoxy-6-selenoguanosine (α- and β-SeGdR) were also tested. The structural formulae of these compounds are given in Fig. 1A and B.

It is generally believed that for compounds such as MeMPR to exert their *de novo* inhibitory and antitumor activities they must first be converted to the corresponding 5′-nucleotides which are the active forms of these compounds.[6,7] Accordingly, we have examined the ability of the analogs used in this study to form nucleotides.

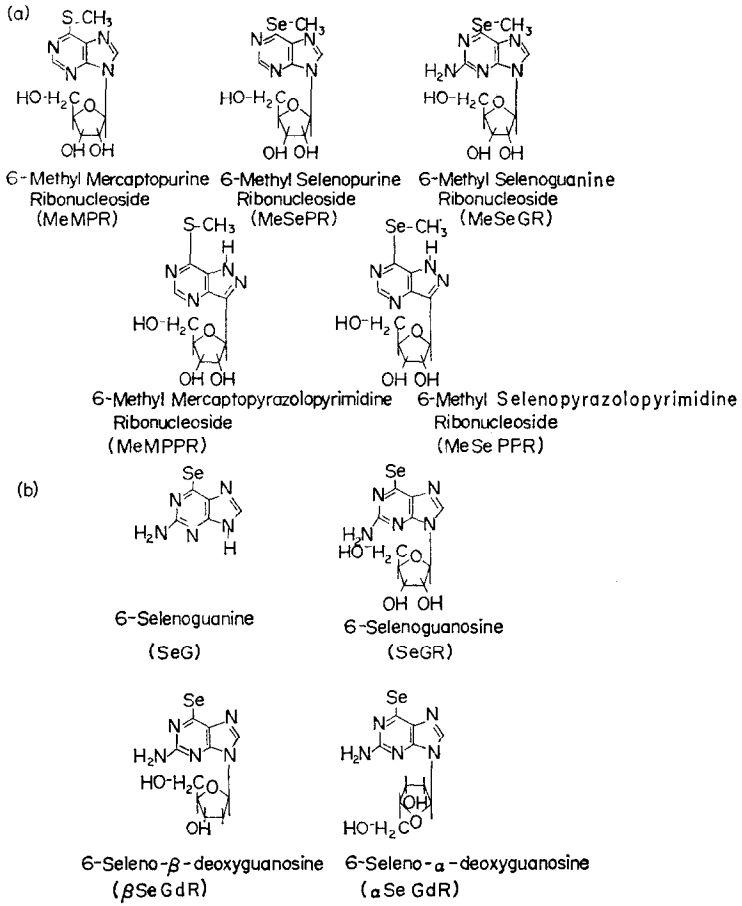

FIG. 1. Structures of mercapto and seleno purine analogs used in the present study. A. Methylmercapto and methylseleno analogs. B. Analogs of selenoguanine and selenoguanosine.

MATERIALS AND METHODS

L-Glutamine and glycine-2-^{14}C were purchased from Schwarz/Mann. The latter compound, as obtained from the supplier, contains a small amount of impurity. This impurity tends to be retained on the ion-exchange columns after the 0·5 M formic acid wash resulting in high background values for 5′-phosphosibosyl-formylglycine-

amide (FGAR). However, this impurity is easily removed as follows: 5 ml of the glycine-2-^{14}C (40 mM; sp. act. = 39 μCi/μmole) are placed on a 2·5 × 12·0 cm column of Bio-Rad AG 1-x-10 (50–100 mesh; formate form). The column is eluted with a linear gradient of 0–4 M formic acid. Five-ml fractions are collected. Glycine-2-^{14}C elutes from the column in fractions 5–13; the impurity elutes between fractions 21 and 40. Fractions 5–13 are pooled, evaporated to dryness, and the resulting residue is dissolved in 5 ml of water. This solution which contains about 95 per cent of the original radioactivity is used for the experiments.

Azaserine (*O*-diazoacetyl-L-serine) was obtained from CalBiochem. Methylmercaptopurine ribonucleoside was supplied by the Aldrich Chemical Company. Snake venom, 5′-nucleotidase (*Crotalus adamanteus* venom, Grade II, 18 units/mg protein) was obtained from the Sigma Chemical Company. Bio-Rad AG 1-x-10 resin was purchased from Bio-Rad Labs. All other chemicals used were of the highest purity available.

Eastman Kodak Chromagram sheets were obtained from Eastman Kodak Company. MeMPPR was a gift from Dr. L. B. Townsend, University of Utah.

Chemical syntheses. MeSePPR, MeSeGR, MeSePR, SeG, SeGR and α- and β-SeGdR were synthesized by Dr. S.-H. Chu of this laboratory.[8,9]

Isolation of tumor cells and conditions for incubation. Five or 6 days after implantation of 2·5 × 10^6 cells into the peritoneal cavity of female CD1 mice, cells were removed and washed three or four times with incubation medium (see below) containing 5·5 mM glucose. Erythrocytes were removed by differential centrifugation and all samples that contained more than a trace of blood were discarded. Incubations were carried out at 37° in a water bath with shaking (80 oscillations/min) with air as the gas phase.

Measurement of inhibition of purine biosynthesis de novo. Purine biosynthesis *de novo* was studied by using the technique of Henderson with a slight modification.[10] This technique involves studying the accumulation of FGAR in cells in which the reaction catalyzed by formylglycineamide ribonucleotide amidotransferase (FGAR amidotransferase) has been inhibited by the glutamine antagonist, azaserine. When glycine-2-^{14}C is added as a precursor, the extent of ^{14}C-FGAR accumulation is determined which gives a measure of the activity of the first portion of the *de novo* pathway for purine biosynthesis. A decrease in FGAR formation in the presence of various analogs may thus be ascribed to "pseudo-feedback" inhibition at the site of the first committed step in the *de novo* pathway, i.e. the reaction catalyzed by PRPP amidotransferase [ribosylamine-5-phosphate:pyrophosphate phosphoribosyltransferase (glutamate-amidating), EC 2.4.2.14] (Fig. 2). The incubation medium (2·0 ml) contained sarcoma 180 cells (approx. 5 × 10^6 cells), azaserine (2 μg/ml; 6·4 μM), glucose (5·5 mM), glycine-2-^{14}C (2 mM; sp. act. 39 μCi/μmole), glutamine (2 mM) and analogs (1 mM) as desired. The analogs were dissolved in 0·01 M NaOH and added immediately in 0·1-ml portions; equivalent amounts of 0·01 M NaOH were added to control samples. Cells were preincubated with azaserine, glucose, and purine, nucleoside analog for 5 min before glycine-2-^{14}C and glutamine were added.

The incubations were carried out for 60 min after addition of glycine-2-^{14}C, and cells were inactivated and extracted by the addition of 0·5 ml of 2·6 M perchloric acid. The extracts were neutralized to pH 7–8 with potassium hydroxide, and the potassium perchlorate precipitates were removed by centrifugation after chilling.

For the isolation of FGAR, the neutralized extracts were placed on 1 × 5 cm columns of Bio-Rad AG 1-x-10 (200–400 mesh; formate form). After adsorption, residual glycine-2-^{14}C and the small amount of glycineamide ribonucleotide which might have accumulated were removed by washing the columns with 30 ml of 0·5 M formic acid; these washes were discarded. FGAR was eluted by passing 25 ml of 4 M formic acid through the columns. The eluates were evaporated to dryness, redissolved in 1 ml of water and then counted in 9·0 ml of Bray's solution.[11]

Formation of analog nucleotides. Sarcoma 180 cells (approx. 1 × 10^8) were incubated in air for 1 hr in 5 ml of the incubation medium described above containing the purine nucleoside analog (1 mM). Cells were extracted by addition of cold perchloric

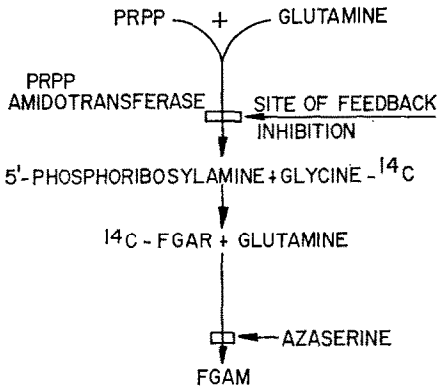

FIG. 2. Schematic diagram of the site of feedback inhibition of purine biosynthesis in S-180 ascites tumor cells.

acid to a final concentration of 0·4 M. After removal of denatured protein by centrifugation, extracts were neutralized with potassium hydroxide, and potassium perchlorate was removed by centrifugation. The neutralized extracts were placed on 1 × 10 cm columns of Bio-Rad AG 1-x-8 (200–400 mesh, formate form). Separation of analog nucleoside from the corresponding nucleotide was achieved by using the ammonium formate–formic acid gradient system of Moore and LePage.[12] Five-ml fractions were collected from the columns, and elution of the nucleotides was followed by monitoring the fractions at either 321 nm (MeSeGR), 331 nm (MeSePPR), 293 nm (MeMPR), 305 nm (MeSePR) or 317 nm (MeMPPR). Since authentic nucleotides were not available for this study, the wavelengths and extinction coefficients used here are those of the chemically synthesized nucleosides. Figure 3 shows the elution profile of an extract after incubation of MeSePR with sarcoma 180 cells. The large peak in fractions 12 through 50 is unreacted MeSePR while the peak in fractions 96–108 contains MeSePR 5′-nucleotide. This elution profile is very similar to those obtained with the other analogs.

To establish that the materials called "nucleotides" above were indeed nucleotides and not nucleosides or bases, the following methods were used: a. Twenty-five μl samples of the extracts were spotted on Eastman Kodak Chromagram sheets for thin-layer chromatography, with cellulose as adsorbant, adjacent to authentic nucleosides. The chromatograms were developed for 3·5 hr with *n*-butanol–methanol–water–ammonia (60:20:20:1), dried, and spots were visualized with u.v. light at 254 nm.

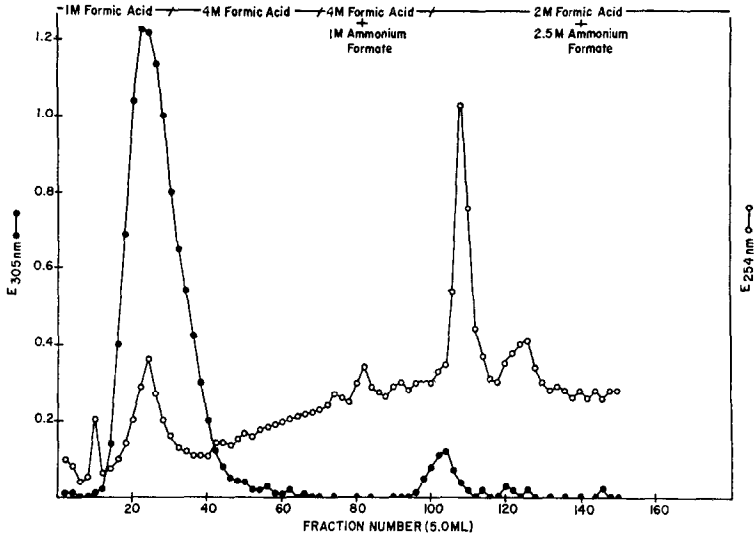

FIG. 3. Elution profile of neutralized extracts of S-180 cells after incubation with methylselenopurine ribonucleoside. Approximately 1×10^8 S-180 ascitic cells were incubated for 1 hr with 0·1 mM MeSePR *in vitro* in a Krebs–Ringer phosphate medium. Neutralized PCA extracts of the cells were chromatographed on 1×10 cm AG1-x-8 (200–400 mesh, formate form) columns as described in Materials and Methods. Fractions were monitored at 305 nm (●——●) and 254 nm (○——○); 5-ml fractions were collected off the column.

In no case was any u.v.-absorbing spot observed in the extracts corresponding to free nucleoside, whereas in all cases u.v.-absorbing material was observed in the area of the chromatogram where nucleotides might be expected to occur. In the solvent used in these studies, the free nucleosides migrate up the plate (all the nucleosides used had $R_f = 0{\cdot}6$), whereas the nucleotides stay near the origin ($R_f = 0{\cdot}3$). (b). Hydrolysis by snake venom (*C. adamanteus*) 5′-nucleotidase to the nucleoside and subsequent identification of the nucleoside by thin-layer chromatography. The enzymic hydrolysis with 5′-nucleotidase was conducted for 30 min at 37° in a solution containing Tris–acetate buffer, pH 8·6, 0·13 M; $MgCl_2$, 0·001 M; 1 mg of enzyme, and nucleotide substrate.[13]

RESULTS AND DISCUSSION

The ability of the analogs structurally related to MeMPR to inhibit purine biosynthesis *de novo* is shown in Table 1. This table also includes results obtained with 6-selenoguanine and three of its derivatives. MeMPR was included in these studies as a positive control because of its well-documented potent pseudo-feedback inhibition of this pathway. The extent of inhibition produced by MeMPR (Table 1) compares favorably with that reported by other workers.[14]

With respect to the methylmercapto- and methylselenopurine ribonucleosides, MeMPR, MeMPPR and MeSePR are all very potent inhibitors (about 90 per cent) of purine biosynthesis *de novo*. MeSePPR was less potent (75 per cent) whereas MeSeGR was not significantly inhibitory.

Among the selenoguanine derivatives, SeG itself was most active as a feedback inhibitor (47 per cent). Although SeGR and β-SeGdR had small amounts of activity

(18 and 13 per cent, respectively), α-SeGdR was completely devoid of feedback inhibitory action. The small but apparently significant inhibition exerted by SeGR on purine biosynthesis *de novo* was intriguing since the presence of a kinase capable of phosphorylating guanosine or guanosine analogs is a subject of controversy at the present time. Until fairly recently no direct evidence has been found in mammalian tissue for enzymes capable of phosphorylating inosine or guanosine; however, Pierre and LePage[15] showed apparent inosine and guanosine kinase activity in a

TABLE 1. PER CENT INHIBITION OF BIOSYNTHESIS *de novo* BY MERCAPTO AND SELENO PURINE ANALOGS*

Analog	Inhibition (%)
MeMPR	88·8
MeSePR	90·4
MeSeGR	0
MeMPPR	89·4
MeSePPR	76·1
SeG	47·3
SeGR	18·1
β-SeGdR	12·6
α-SeGdR	0

* Tumor cells, 5×10^6, were preincubated in Krebs–Ringer phosphate medium, pH 7·4, in air for 5 min at 37° with $5{\cdot}5 \times 10^{-3}$ M glucose, $6{\cdot}4 \times 10^{-6}$ M azaserine, and 10^{-3} M analog before 2×10^3 M 2-^{14}C-glycine and 2×10^{-3} M glutamine were added. After a 60-min incubation, ^{14}C-FGAR was determined on neutralized PCA extracts as described in "Materials and Methods". Each figure is the average of results from six flasks in two experiments.

subline of Ehrlich ascites carcinoma, and Payne *et al.*[16] believe that inosine kinase exists in leucocytes of a patient with Lesch–Nyhan disease. However, since SeGR can be cleaved to SeG and ribose-1-phosphate by the enzyme purine nucleoside phosphorylase (purine nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1),[17] the feedback inhibition shown with SeGR might simply be due to the SeG produced in this reaction.

Formycin B [7-hydroxy-3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidine] is known to be a competitive inhibitor of purine nucleoside phosphorylase.[18] Accordingly, an experiment was carried out in which the extent of inhibition of purine biosynthesis *de novo* by SeG and SeGR alone was compared with that produced by SeG plus Formycin B or SeGR plus Formycin B. The results shown in Table 2 show that SeG

exerts the same inhibitory activity with or without addition of formycin B. However, when formycin B is incubated along with SeGR, the inhibition is decreased below that observed with SeGR alone. These observations indicate that the small inhibitory activity observed with SeGR is probably due to its cleavage to SeG.

TABLE 2. EFFECT OF FORMYCIN B ON INHIBITION OF PURINE SYNTHESIS *de novo* BY SeG AND SeGR*

Analog	Inhibition (%)
MeMPR	89·0
Formycin B	0
SeG	44·8
SeGR	29·2
SeG + formycin B	48·8
SeGR + formycin B	17·3

* Experimental conditions are given in Table 1.

As discussed above, for any of the analogs used in this study to be effective antitumor agents, it is assumed that they must first be converted to the corresponding 5′-nucleotides which are believed to be active forms. For example, MeMPR forms the analog nucleotide 6-methylmercaptopurine ribonucleoside 5′-monophosphate

TABLE 3. SYNTHESIS OF ANALOG NUCLEOTIDES*

Analog	Nucleotide formed (μmoles/g of cells)
MeMPR	1·37
MeSeGR	0
MeMPPR	1·14
MeSePPR	0·50
MeSePR	0·74

* Analogs were incubated with approx. 1×10^8 sarcoma 180 ascites cells in Krebs–Ringer phosphate medium, pH 7·4, for 1 hr with 0·1 mM of the indicated analog. Aliquots of neutralized PCA extracts of the cells were then assayed for analog nucleotide formation by chromatographing on 1 × 10 cm AG 1-x-8 (200–400 mesh, formate form) columns as described in Materials and Methods.

(MeMPR-P) in several tumor lines and in erythrocytes.[4,19,20] The abilities of the compounds used in the present study to form the corresponding nucleotides are given in Table 3. Four of the five compounds examined form significant amounts. MeSeGR apparently did not form any nucleotide. The amount of MeMPR-P produced in these studies compares favorably to the amount obtained by other workers.[4,5,21]

Since MeMPR is phosphorylated to MeMPR-P by the enzyme adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20),[19,21] this same enzyme may utilize (as substrate) all of the compounds tested with the exception of MeSeGR, a guanosine analog. Apparently neither replacement of the methylthio group on the 6-position of the purine ring by a methylseleno group nor replacement of the N—C glycosydic bond by a C—C linkage eliminates the ability of these compounds to act as substrates for adenosine kinase. However, detailed kinetic studies with purified adenosine kinase will be required to draw further conclusions about the relative substrate activities of these analogs.

Large amounts of nucleotides are formed from MeMPR and MeMPPR, and these compounds are potent inhibitors of purine biosynthesis *de novo*. Although smaller but significant amounts of nucleotides are formed from MeSePR and MeSePPR, these are potent feedback inhibitors whereas MeSeGR has no activity in this regard. Presumably, the lack of inhibition *de novo* by MeSeGR is due to its inability to form the 5′-monophosphate derivative.

From the elution profiles of the analogs observed in this study it appears that none of the analog nucleotides are phosphorylated to the di- or triphosphate forms. This observation is consistent with the concept that none of these analogs are substrates for 5-monophosphate nucleotide kinases.

The precise site of inhibition of purine biosynthesis *de novo* cannot be ascertained by the above experiments. It is possible that the analogs inhibit at steps other than that catalyzed by the enzyme PRPP amidotransferase. One possibility is that these compounds inhibit the formation of 5-phosphoribosyl-1-pyrophosphate (PRPP), itself, a compound essential for purine biosynthesis *de novo*. Investigations to answer these questions are in progress.

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