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Studies on the mechanism of 3-deazaguanine cytotoxicity in L1210-sensitive and -resistant cell lines*, **

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Summary. 3-Deazaguanine (3-DG), a purine analogue, has unusual antitumor activity against experimental mammary tumor models and a number of other solid tumors. Others have shown that mutant CHO cells deficient in hypoxanthine guanine phosphoribosyl transferase (HGPRTase) or adenine phosphoribosyl transferase (APRTase) are resistant to 3-DG. We developed a L1210 cell line resistant to 3-DG, L1210/3-DG, by subculturing the parent L1210/0 cells in the presence of increasing concentrations of 3-DG. The IC_{50} was 3.5 μM and 620 μM for L1210/0 and L1210/3-DG, respectively. Cytotoxicity studies proved the resistance to be stable. Examination of the baseline-specific activity of HGPRTase and APRTase showed that the former was 118-fold lower in L1210/3-DG than in L1210/0, and the latter demonstrated no difference. A 4-h treatment of the cell lines at IC_{50} doses showed 48% and 23% reductions in IMP dehydrogenase in L1210/0 and L1210/3-DG, respectively. The rate of de novo purine biosynthesis was studied by using [^{14}C]formic acid. Formate flux increased 2-fold in L1210/3DG in concert with the observed deficiency of HGPRTase in the cell line. 3-DG uptake was studied with [^{14}C]-labelled compound. The total radioactivity was 9-fold higher in L1210/0 than in L1210/3-DG at 2 h. Subsequent chromatographic separation of radioactivity showed the 3-DG and 3-deazaguanosine pools of the drug to be equal in both lines. However, 3-DG nucleotide pools at 1 min and 2 h were 2.5-fold and 16-fold lower, respectively, in L1210/3-DG than in L1210/0. 3-DG incorporation studies with radiolabelled drug demonstrated that 3-deazaguanine is incorporated in the acid-insoluble fraction of the cell. These studies conclude that HGPRTase, and not APRTase, is required for the activation of drug. Inhibition of IMP dehydrogenase is partially responsible for antitumor activity of the drug. The incorporation of drug into nucleic acids may be a major mechanism for its antitumor activity. Further studies using a cloned cDNA probe for hypoxanthine guanine phosphoribosyltransferase (HGPRT) demonstrated no change in the DNA arrangements of the L1210/3-DG cell line, and Northern blot analysis showed approximately equal expression of mRNA in both cell lines.

Introduction

3-Deazaguanine, a purine analogue, has been shown to possess unusual antitumor [9–11], antibacterial [14, 15], and antiviral [1, 4] activity. The metabolism of the drug has been studied by several groups. Streeter and Koyama [20] studied the enzymes of purine metabolism in intact Ehrlich ascitic cells and found that 3-deazaguanine, its nucleoside, and its nucleotide showed a similar pattern of inhibition. Hypoxanthine phosphoribosyl transferase and inosine monophosphate dehydrogenase activities were reduced by all three compounds. Saunders et al. [19], using variant lines of Chinese hamster ovary cells, showed that cells deficient in HGPRTase activity were resistant to 3-deazaguanine but not to its nucleoside. This implies that formation of nucleotides is necessary for cytotoxicity of the drug. Khwaja et al. [8] showed that 3-deazaguanine is converted to its corresponding nucleotides and is incorporated into the nucleic acids.

The mechanism of cytotoxicity of 3-deazaguanine appears to be suppression of DNA synthesis. Schwartz et al. [21] reported that in L1210 cells 12-h exposure of the drug at 10^{-4} M concentration caused 70% suppression of DNA synthesis. This effect could be due either to the incorporation of the drug into the nucleic acids or to inhibition of enzymes involved in the synthesis and/or regulation of nucleotides. As far as synthesis of RNA and proteins is concerned, Saunders et al. [18], studying the incorporation of radiolabelled uridine, thymidine, and leucine, observed no effect on RNA and protein synthesis in Chinese hamster ovary cells, whereas Rivert et al. [17], employing L1210 cells, showed that both DNA and protein syntheses were inhibited at 50 μM drug concentration.

This study was designed to examine the enzymatic and genetic mechanisms of resistance to 3-deazaguanine in L1210 cell line. The metabolism of the drug in the parent L1210 cell line and the one resistant to 3-deazaguanine differed to a significant extent, indicating that the drug must be metabolized to its nucleotides before it is cytotoxic. Our

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Abbreviations used: APRTase: adenine phosphoribosyltransferase; HGPRTase: Hypoxanthine guanine phosphoribosyltransferase; IMPD: Inosine mono-phosphate dehydrogenase; PRPP: 5-Phosphorylribose-1-pyrophosphate; AOPCP: α, β-Methyleneadenosine 5'-diphosphate; NAD: Nicotineamide dinucleotide; EDTA: Ethylenediamine tetra acetic acid

genetic studies indicate that mRNA production for HGPRTase is equal in both lines and that the resistance in the L1210/3-DG is due to a mutation that renders the HGPRTase inactive.

Materials and methods

3-Deazaguanine (mesylate salt) and the [^{14}C]-radiolabelled compound CI-908 [^{14}C]mesylate (9.15 mCi/mol), were gifts from Dr Black at Warner Lambert Company (Ann Arbor, Mich). [U-^{14}C]Adenine (276 mCi/mol) and [8-^{14}C]inosine-monophosphate, ammonium salt (54 mCi/mol) were purchased from Amersham/Searle (Arlington Heights, Ill). [8-^{14}C]Hypoxanthine (52.8 mCi/mol) was obtained from New England Nuclear (Boston, Mass). Specific activities of [U-^{14}C]adenine and [8-^{14}C]hypoxanthine were diluted to 17.25 mCi/mmol and 48.93 mCi/mmol, respectively, with the corresponding cold compounds prior to use. Sodium [^{14}C]formate (57 mCi/mmol) was provided by Moravek Biochemicals (Brea, Calif). All other biological chemicals were obtained from Sigma Chemicals, St Louis, Mo. The HGPRT probe pHPT 5 was a gift from Dr C. Thomas Caskey, Howard Hughes Medical Institute, Houston, Tex. The restriction enzymes *Pst1, Ecori* and *Hind*III were purchased from Boehringer Mannheim (Indianapolis, Ind). Nytron (0.45-μm nylon membrane) was purchased from Schleicher and Schuell (Keene, NH).

Development of L1210 leukemia resistant to 3-deazaguanine. Male DBA_2 mice received i.p. implants of L1210/0 leukemia (10^6 cells) and were treated with 3-DG at 20 mg/kg per day i.p. on days 1–7. On day 7 tumor cells were aspirated and implanted i.p. into the new hosts, which were similarly treated. The dose of 3-DG was gradually increased to 80 mg/kg per day between the 3rd and 11th transplant generations, resulting in the resistant cell line, L1210/3-DG. Female BDF_1 mice bearing i.p. implants of the tumor L1210/0 or L1210/3-DG (10^5 cells) were treated with either 0.9% NaCl solution or 3-deazaguanine (80 mg/kg) i.p. on days 1 to 9 inclusive. Weight and mortality were recorded daily.

Cell growth and cytotoxicity. Cells were grown in suspension in RPMI-1640 medium supplemented with 10% horse serum, containing 10 m*M* HEPES, penicillin G 100 units/ml, and streptomycin 0.1 mg/ml at 37° C in an atmosphere of 5% CO_2. The L1210 cell line resistant to 3-deazaguanine was developed by subculturing the parent cell line with increasing concentrations of the drug for approximately 15 passages. Subsequently, the resistant cell line was carried in the presence (100 μ*M*) and absence of the drug. Cells (10^5 per 60 × 15 mm plate) were seeded, with 5 ml medium for performance of cytotoxicity studies with 3-deazaguanine. At 48 h after drug treatment, cells were counted in an FN Coulter counter.

Enzyme assays. Cells 1×10^6 were incubated for 4 h at 37° C with IC_{50} doses of the drug. Cells were harvested by centrifugation at 200 *g* for 5 min. The cell pellet was lysed by freezing and thawing the cells three times in 3 volumes of 20 m*M* Tris-HCl, 60 m*M* potassium phosphate buffer (pH:7.2) containing 1 m*M* EDTA, and 0.5 m*M* dithiothreitol. Homogenates were centrifuged at 12000 *g* for 15 min at 4° C, and the cytosol fraction was used for various enzymes assays.

Then, 5 μl [^{14}C]adenine (7 nmol) or 5 μl [^{14}C]hypoxanthine (5 nmol) for APRTase or HGPRTase enzyme assays, respectively, were mixed with 5 μl 10 m*M* PRPP in 10 m*M* $MgCl_2$. The reaction was started by the addition of 5 μl of the cytosol fraction (approx. 100 μg protein) in an Eppendorf tube, and incubation at 37° C for 10 min followed. The reaction was stopped by mixing 10 μl 2 *N* HCl to the reaction mixture and then freezing on dry ice. Tubes were centrifuged at 12000 *g* for 5 min, and 5 μl supernate was spotted on to Whatman-3M paper. Adenosine 5'-monophosphate or inosine 5'-monophosphate was co-spotted to permit UV detection of products. The paper was developed in 95% ethanol:1 *M* ammonium acetate (pH 5) 70:30; v/v containing 3.3 m*M* EDTA. Spots were marked under UV light, cut, transferred to scintillation vials containing 10 ml betafluor, and counted in a Tri-carb 360 liquid scintillation counter.

For the inosine-monophosphate dehydrogenase assay [16] 5 μl enzyme preparation was mixed with 5 μl [^{14}C]inosine-monophosphate (1.5 nmol) and 5 μl 100 m*M* Tris-HCl (pH 8) containing 100 m*M* KCl, 3 m*M* EDTA, 0.2 m*M* AOPCP, 0.2 m*M* allopurinol, and 0.25 m*M* NAD in an Eppendorf tube, and the reaction mixture was incubated at 37° C for 10 min. The reaction was stopped by heating the tube to 95° C and chilling on ice. Tubes were centrifuged at 12000 *g* for 5 min, and the supernate was chromatographed in the same way as for the APRTase and HGPRTase enzyme assays. Xanthosine 5'-monophosphate was used as the marker. The protein assay was done by the Bio-rad method [3].

Formate flux. Rates of de novo purine biosynthesis were studied by suspending 2×10^6 cells per ml in RPMI-1640 supplemented with 10% horse serum. Cells were incubated with 100 μ*M* sodium [^{14}C]formate. Incubation was terminated after various times by chilling the tubes on ice. Cells were pelleted by centrifugation at 200 *g* for 3 min. Medium was replaced with 2 ml 0.4 *M* perchloric acid. Samples were heated to 100° C in a water bath for 1 h and then chilled on ice. Cell debris was removed by centrifugation at 800 *g* for 15 min. The supernate was applied to Dowex 1 and 50 W (size 4.5 × 16 mm) columns. Each column was washed with 25 ml 0.1 *N* HCl, and then the bases were eluted with 6 *N* HCl. Elute was collected in 5, 5-ml fractions, and 0.5 ml of each fraction was counted in 9 ml fluorodyne to determine the rats of de novo labelling of total intracellular purines.

Uptake and metabolism of 3-DG in leukemia cell lines. Cells (4×10^6/ml) in RPMI-1640 containing 10% horse serum were incubated with 50 μ*M* [^{14}C]3-deazaguanine. Incubations at various time intervals were terminated by using versilube F50 silicone oil [7]. The pellet containing 9×10^6 cells was transferred to scintillation vials containing 10 ml fluorosol and counted in an LS9000 β-counter. To study the metabolism of the drug, the cell pellet was extracted with 50 μl 5% perchloric acid for acid-soluble and -insoluble fractions. The mixture was centrifuged at 12000 *g* for 10 min. All the steps were performed at 4° C. The supernate was transferred to a fresh tube. The acid-insoluble precipitate was washed with PBS, and then dissolved in 25 μl 20% KOH by heating at 60° C for 10 min. The solution was transferred to a scintillation vial containing 10 ml fluorosol and counted in LS-9000 β-counter. The acid-soluble

fraction was neutralized with 1 *M* potassium phosphate and 20% KOH and an aliquot (20–30 µl) was spotted on Whatman-3M paper, air-dried and co-spotted with 5 µl 10 m*M* 3-deazaguanine, 5 µl 10 m*M* guanosine, and 10 µl of solution containing 5 m*M* each of guanosine 5'-monophosphate, guanosine 5'-diphosphate, and guanosine 5'-triphosphate. The paper was developed overnight in an 85% saturated solution of ammonium bicarbonate (16 *g*/100 ml). The Rf values for 3-deazaguanine, 3-deazaguanosine and 3-deazaguanine nucleotides were 0.44, 0.67, and 0.88 respectively. Spots were marked under UV light, cut, transferred to scintillation vials containing 10 ml betafluor, and counted in an LS-9000 β-counter.

DNA analysis. DNA (5 µg) from L1210/0 and L1210/3-DG cells prepared by lysing the cells in a 1.0% sodium dodecyl sulfate solution and extracting with chloroform and phenol [13]. DNA was digested with the appropriate restriction endonuclease under conditions recommended by the manufacturer (Boehringer Mannheim) and electrophoresed in a 1.0% agarose gel. The gel was denatured in 0.5 *M* NaCl, then neutralized in 1 *M* Tris (pH 7.7) and 1.5 *M* NaCl. DNA was capillary-blotted onto Nytron in 20× standard saline citrate [22].

Southern hybridization. The nylon membrane filter was pretreated for 3 h at 42° C in a buffer containing 1.0% Denhardt's 5× standard saline citrate, 50 m*M* sodium phosphate (pH 6.8), and 0.1% sodium dodecyl sulfate, and prehybridized overnight at 42° C in a solution containing 50% formamide, 5× standard saline citrate, 1.0× Denhardt's, 20 m*M* sodium phosphate (pH 6.8), 0.1% sodium dodecyl sulfate, and 80 µg/ml denatured salmon sperm DNA. The filter was hybridized with ^{32}P-labelled cDNA probe in 50% formamide, 5× standard saline citrate, 1.0× Denhardt's 20 m*M* sodium phosphate (pH 6.8), 0.1% sodium dodecyl sulfate, 0.1% dextran sulfate, and 80 µg/ml denatured salmon sperm DNA at 42° C for 48 h. Extensive washing was performed initially in 3× standard saline citrate with 0.1% sodium dodecyl sulfate at room temperature, next in 1× standard saline citrate with 0.1% sodium dodecyl sulfate at 55° C, and lastly in 0.3× standard saline citrate with 0.1% sodium dodecyl sulfate at 55° C using an Omniblot system (American Bionautics). Hybridization was detected by autoradiography. Kodak films XAR and XBP were exposed for 1–4 days at −80° C using Kronax Lightning Plus intensifying screens.

RNA analysis. RNA from wild-type and 3-DG-resistant L1210 and L1210/3-DG cells were prepared by lysing the cells with a hypotonic buffer containing NP-40 followed by removal of the nuclei by centrifugation [6]. RNA was deproteinized with two phenol chloroform extractions, followed by three chloroform extractions. The purified RNA was precipitated with absolute ethanol. RNA (10 µg and 20 µg) was electrophoresed into a 1% formaldehyde agarose gel and blotted onto a Nytron filter. The Northern hybridization was performed as described above for the Southern blot hybdridization.

Probe preparation. The pHPT5 probe in *E. coli* RR1 cells transformed with pBR322 was generously donated by Dr C. Thomas Caskey [12]. Plasmid DNA was isolated using the alkaline lysis procedure [2] following an overnight amplification in the presence of chloramphenicol. The circular plasmid was purified by banding in a CsCl gradient containing ethidium bromide. The pHPT5 probe was excised with a *Pst*1 restriction digest and electrophoresed into a 1.3% low-melt agarose gel. The 1.3-kilobase probe was labelled in a Klenow reaction [5].

Results

In vivo

Sensitivity or resistance to 3-deazaguanine. The native murine L1210 leukemia (L1210/0) is sensitive to 3-deazaguanine therapy, whereas the resistant variant selected under in vivo conditions is nonresponsive to 3-deazaguanine treatment. The treatment of mice bearing L1210/0 i.p.-implanted tumor with 3-deazaguanine produced an increase in the life span (ILS) of 151%, while no such increase was observed in mice bearing L1210/3-DG tumor (Table 1).

In vitro

Cell growth. The effect of a 2-day in vitro treatment with 3-deazaguanine on L1210/0 and L1210/3-DG in culture is shown in Table 2. The IC_{50} concentrations of 3-deazaguanine were 3.5 and 620 µ*M* in L1210/0 and L1210/3-DG, respectively.

Enzymes participating in the anabolism of 3-deazaguanine. Specific activities of HGPRTase, APRTase and IMP dehydrogenase were determined at baseline and following 4 h 3-deazaguanine treatment at its IC_{50} dose. Baseline

Table 1. Effect of treatment with 3-deazaguanine on the survival of mice bearing i.p.-transplanted L1210/0 and L1210/3-DG tumors

3-Deazaguanine dose (mg/kg i.p.)	L1210/0		L1210/3-DG	
	Medium survival time (days)	ILS[a]	Medium survival time (days)	ILS
0	8.75		12.0	
80[b]	13.25	151	12.5	104

Groups of four female BDF_1 mice weighing 20–25 g were given i.p. injections of 1×10^5 cells of L1210/0 or L1210/3-DG tumor. Twenty-four hours later treatment was started. Animals were fed with rodent blox and water ad libitum. Weight and mortality of each were recorded daily

[a] Increased life span

[b] 3-DG was administered on days 1–9

Table 2. IC_{50} doses of 3-deazaguanine in L1210/0 and L1210/3-DG

Cell line	3-Deazaguanine
L1210/0	3.5 µ*M*
L1210/3-DG	620 µ*M*

10^5 cells were seeded in 60 × 15 mm plates containing RPMI-1640 supplemented with 10% horse serum and the chemotherapeutic agent at various concentrations. Cells were counted in FN Coulter counter 48 h later

Table 3. Effect of 3-deazaguanine on APRTase, HGPRTase and IMP dehydrogenase enzyme activities in L1210/0 and L1210/3-DG after 4-h exposure to IC_{50} doses of the drug

Enzyme	L1201/0		L1210/3-DG	
	Control	Treated[b]	Control	Treated
APRT	120.3±2.6[a]	123.6±1.4	128.0±1.5	122.3±2.6
HGPRT	94.3±0.3	110.0±3.0	0.8±0.2	0.7±0.1*
IMPD	30.1±0.5	15.8±0.5	20.5±0.4	15.9±1.4

[a] (nmol/h per mg protein)
[b] L1210/0 cells were incubated with 3.4 μ*M* of drug and L1210/3-DG with 620 μ*M* of the drug for 4 h
* Significant

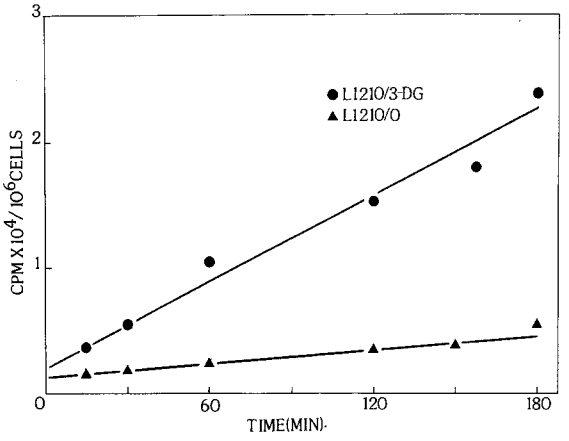

Fig. 1. Rate of de novo purine biosynthesis in L1210/0 and L1210/3-DG. Cells (2×10^6/ml) in RPMI-1640 containing 10% horse serum were incubated with 100 μm sodium [^{14}C]formate (57 mCi/mmol) at 37° C. Various times, incubation was terminated by chilling the tube on ice and cells were pelleted by centrifugation at 200 *g* for 3 min. The details of the procedures are described in the *Materials and methods*

HGPRTase activity was 94.3±0.3 and 0.8±0.2 nmol/h per mg protein in the sensitive and resistant cells, respectively. Baseline APRTase activity in the sensitive and the resistant cells was approximately 120 nmol/h per mg protein. IMP dehydrogenase activity was 30.1 and 20.5 nmol/h per mg protein in the sensitive and resistant cells, respectively. The 4-h treatment with the IC_{50} dose of 3-deazaguanine resulted in no alteration in the APRTase and HGPRTase activities; however, IMP-dehydrogenase activity was inhibited by 48% and 23% in L1210/0 and L1210/3-DG, respectively (Table 3).

Rate of de novo purine biosynthesis. Since the activity of HGPRTase is substantially lower in the L1210/3-DG cell line, it was no surprise to detect enhancement of formate flux in the resistant variant. At 2 h, incorporation of sodium [^{14}C]formate in the L1210/3-DG was 5-fold higher than in the sensitive cell line (Fig. 1).

Uptake and metabolism studies. Since uptake and the subsequent metabolism of 3-deazaguanine is the key to sensitivity and resistance of the cells to the drug, we examined the fate of 3-deazaguanine in the sensitive and resistant cell lines. As shown in Table 4, following incubation of the cells with 3-deazaguanine, the parent drug was rapidly internalized (less than 1 min). Furthermore, the sensitive and the resistant cell lines did not differ significantly in the intracellular concentrations of the parent drug or its nucleoside. Conversely, the concentration of 3-deazaguanine nucleotides was 2.5-fold higher at 1 min and 16-fold higher at 2 h in the sensitive than in the resistant variant. Therefore, 3-deazaguanine nucleotide pools constitute the active moieties of the drug.

3-Deazaguanine incorporation in the acid-insoluble fraction. Following incubation of the cells with radiolabelled drug, there is a linear relationship between the incubation time and the amount of labelled drug incorporated into the acid-insoluble fraction of the sensitive cell line; however, incorporation into the resistant cell line is substantially

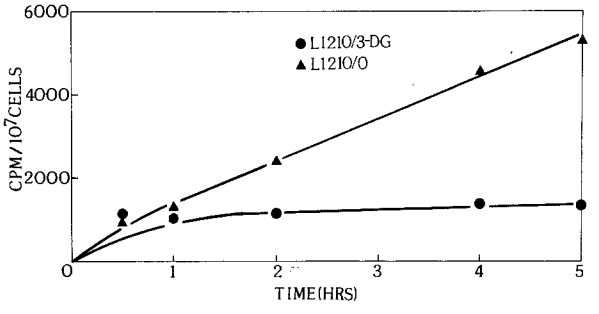

Fig. 2. 3DG Incorporation in acid-insoluble fraction of L1210/0 and L1210/3-DG. Cells (4×10^6/ml) in RPMI-1640 medium containing 10% horse serum were incubated with 50 μm [^{14}C]deazaguanine (9.15 mCi/mmol). At various times, incubation was were terminated by spinning the sample at 12000 *g* for 2 min. The details of the procedures are outlined in *Materials and methods*

Table 4. Comparison of 3-deazaguanine, 3-deazaguanosine and 3-deazaguanine nucleotide pools in L1210/0 and L1210/3-DG cell lines

Time (min)	3-Deazaguanine (pmol/10^6 cells)		3-Deazaguanosine (pmol/10^6 cells)		3-DG-Nucleotides (pmol/10^6 cells)	
	L1210/0	L1210/3-DG	L1210/0	L1210/3-DG	L1210/0	L1210/3-DG
1	149.4	209.3	46.8	31.2	72.9	28.7
10	152.7	209.6	60.9	39.5	259.4	31.6
30	170.9	196.7	37.3	35.4	386.9	43.0
60	155.7	213.3	40.9	36.4	795.5	52.6
120	176.7	225.8	47.1	33.4	1103.0	67.9

Cells (4×10^6/ml) RPMI-1640 supplemented with 10% horse serum were incubated with 50 μm ^{14}C radiolabelled drug. At various times, incubation were terminated by pelleting the cells in an Eppendorf tube containing versilube F50 silicon oil. Cell pellets were prepared as described in *Materials and methods*

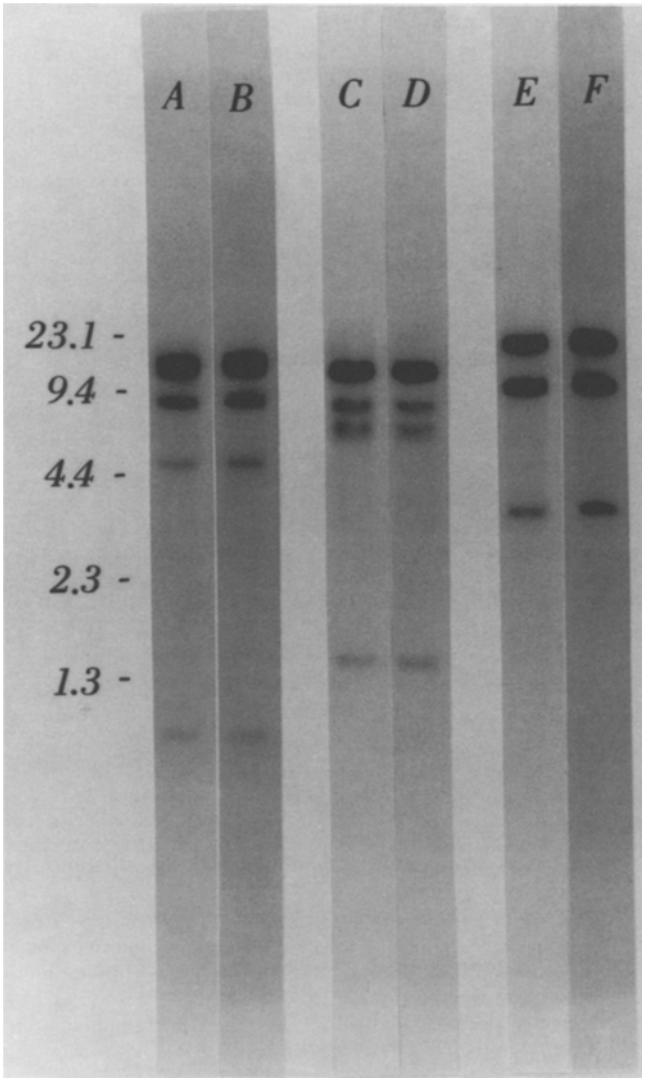

Fig. 3. Southern blot of *Pst*1, *Eco*RI and *Hind*III, digests of L1210/0 and L1210/3-DG DNA. Southern blotting of PST I, *Hind*III and *Eco*RI digested DNA from L1210/0 and L1210/3-DG cells. Filters were hybridized with HGPRT probe to establish that the two cell lines have similar genomic equivalents of DNA. The position of the migration and sizes of markers are indicated. Lane A, 5 μg *Pst*I digested L1210/0 DNA; lane B, 5 μg *Pst*I digested L1210/3-DG DNA; lane C, 5 μg *Eco*RI digested L1210/0 DNA; lane D, 5 μg *Eco*RI digested L1210/3-DG DNA; lane E, 5 μg *Hind*III digested L1210/0 DNA; lane F, 5 μg *Hind*III digested L1210/3-DG DNA

reduced. The significantly lower incorporation of drug into nucleic acids in the resistant line indicates that 3-deazaguanine incorporation into nucleic acids may be responsible for its antitumor activity.

HGPRT Gene in L1210/0 and L1210/3-DG. To further elucidate the mechanism of L1210/3-DG resistance to 3-deazaguanine, we examined HGPRTase gene in the HGPRT-deficient cell line. We conducted blot hybridization between restrictive fragments of high-molecular-weight DNA from the sensitive and the resistant cell lines and the cDNA probe from the recombinant plasmid pHPT5. Figure 3, a Southern blot, illustrates the results obtained with cellular DNAs digested to completion with

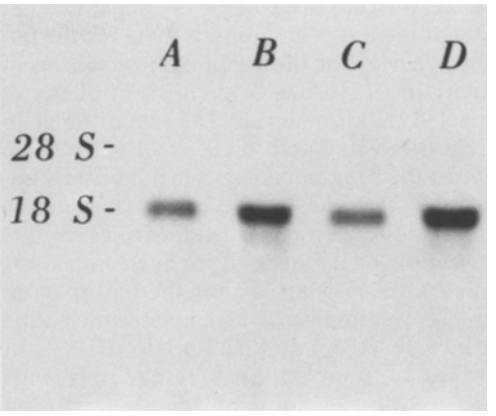

Fig. 4. Northern blot of L1210/0 and L1210/3-DG mRNA. Northern hybridization of L1210/0 and L1210-3-DG mRNA with HGPRT. Lane A, 10 μg L1210/0 mRNA; lane B, 20 μg L1210/0 mRNA; lane C, 10 μg L1210/2-DG mRNA; lane D, 20 μg L1210/3-DG mRNA. Positions of 28S and 18S markers are indicated

*PST*I, *Eco*RI and *Hind*III restriction enzymes. The number and size of *Pst*I (lanes A, B) *Eco*RI (lanes C, D) and *Hind*III (lanes E, F) restriction fragments detected by the probe were the same for the DNA from L1210/0 and L1210/3-DG. DNA deletions, insertions or rearrangements were not detected in the L1210/3-DG cell line.

HGPRT mRNA in L1210/0 and L1210/3-DG. We conducted Northern blot hybridization experiments to determine the levels of HGPRT mRNA in the sensitive and the resistant cell lines. As shown in Fig. 4, cDNA probe prepared from pHPT5 identified a single species of HGPRT mRNA in the preparation of polyA$^+$ RNA from the sensitive parent sensitive cell line, L1210/0 (lanes A, B). The same pattern was observed in the lanes containing equivalent amounts of mRNA from L1210/3DG, the resistant cell line (lanes C, D).

Discussion

Earlier studies have established that 3-deazaguanine is an inhibitor of HGPRTase and IMP dehydrogenase [20]. In the present study we have conducted a series of biochemical and genetic studies to reveal the mechanism of resistance of the L1210/3-DG cell line. According to our enzymatic data, HGPRTase is the key enzyme involved in the activation of 3-deazaguanine. In the resistant cell line the specific activity of HGPRTase was near zero; it was 118-fold lower than in the sensitive line. APRTase activities were not significantly different in the sensitive and resistant cell lines. A 4-h treatment at the IC_{50} dose of 3-deazaguanine inhibited IMP-dehydrogenase in both lines, but inhibition was 2-fold lower in the resistant line than in the sensitive line. Hence, on the basis of these data we believe reduction of HGPRTase activity is the major mechanism leading to resistance in L1210/3-DG cell line. Subsequently, we conducted a series of in vitro studies to substantiate our findings. Purine requirements of cells in vitro are met by a combination of de novo synthesis and utilization of hypoxanthine and guanine bases available in medium. In a cell line deficient in HGPRTase, utilization of bases is not

possible and, hence, the cells are completely dependent on de novo synthesis of inosine 5'-monophosphate for the purine requirements. In view of the significant reduction of HGPRTase activity in L1210/3-DG, de novo synthesis of purines was expected to be enhanced. The rate of de novo biosynthesis of purines was 2-fold higher in L1210/3-DG than in L1210/0. In the drug uptake and metabolism studies, we demonstrated that the uptake of 3-deazaguanine was not inhibited in the resistant cell line. However, the subsequent metabolism of the drug to its nucleotides was substantially lower in the resistant than in the sensitive cell line. The difference was maximal at 2 h, at which time 3-DG nucleotides were 1103.0 and 67.9 pmol/10^6 cells in the sensitive and the resistant cell lines, respectively. Furthermore, increased 3-deazaguanine nucleotide formation in the sensitive cell line was consistent with increased 3-deazaguanine incorporation in the acid-insoluble fraction. Hence, a significant reduction of HGPRTase activity in the resistant cell line leads to lower conversion of 3-DG to its nucleotides, lower IMP dehydrogenase inhibition, and less incorporation of the drug into nucleic acids.

Further studies were performed to extend our understanding of the molecular basis of HGPRTase deficiency in the resistant cell line. A cloned cDNA probe for HGPRTase was used to analyze HGPRT gene and mRNA in an HGPRT-deficient variant and its HGPRT-positive, L1210/0, parent cell line. Southern blot analysis with *Pst*I, *Eco*RI and *Hind*III demonstrated no change in the DNA arrangement of the L1210/3-DG cell line. Further study with S-1 nuclease mapping or sequencing is needed to demonstrate the nature and location of a mutation. Northern blot analysis demonstrated that L1210/3-DG cells continue to produce normal levels of HGPRT mRNA comparable to those in L1210/0 cells. Therefore, the resistant variant produces an altered form of the enzyme, and L1210/3-DG carries a missense mutation that causes a substantial reduction of functional HGPRTase in the resistant cells.

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