Human RNase H-Mediated RNA Cleavage from DNA-RNA Duplexes Is Inhibited by 6-Deoxythioguanosine Incorporation into DNA

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ABSTRACT

Mercaptopurine and thioguanine are anticancer and immunosuppressive agents that exert their primary cytotoxic effects via incorporation of deoxythioguanosine (dG^s) into DNA, but the precise mechanism(s) by which this causes cytotoxicity remains unknown. We initially determined that the level of dG^s incorporation into DNA of human T- and B-lineage leukemia cell lines did not correlate significantly with the extent of cytotoxicity (IC_{50}), except that there was no cytotoxicity in the absence of dG^s incorporation. To elucidate biological processes perturbed by dG^s incorporation into DNA, we chemically synthesized oligodeoxyribonucleotides containing a single dG^s (11 mer and 19 mer), which decreased the melting temperature (T_m) of DNA-DNA duplexes without major structural changes, as evidenced by circular dichroism spectra. Using nuclear extracts from human lymphoblastic leukemia cells (CCRF-CEM, NALM6, and Molt4), we documented that dG^s incorporation into the DNA strand of DNA-RNA heteroduplexes significantly inhibited human RNase H-catalyzed RNA cleavage (80–90% inhibition) and that a similar inhibition was evident with bacterial RNase H. These data provide the first evidence that thiopurines inhibit the function of RNase H, indicating that their mechanism of cytotoxicity may involve interference with this component of the replication machinery.

Mercaptopurine (MP) is an important antileukemic agent used in the majority of modern treatment regimens for acute lymphoblastic leukemia (ALL) (Elion, 1989). Whereas current therapy can cure >75% of children with ALL (Pui and Evans, 1998), chemotherapy resistance leads to relapse in a substantial number of patients. In contrast to some human leukemia cell lines, in which resistance to thiopurines is acquired via inactivation of hypoxanthine-guanine phosphoribosyl transferase (HPRT), no clear relation has been found between HPRT activity and in vitro resistance to thiopurines in primary leukemia cells from patients with ALL (Pieters et al., 1992). New insights into mechanism(s) of thiopurine cytotoxicity and resistance are therefore needed to facilitate further improvements in the use of these medications.

Cytotoxic effects of MP and 6-thioguanine (TG) are achieved primarily through 6-deoxythioguanosine (dG^s) incorporation into DNA (LePage, 1963), an essential feature of the delayed cytotoxicity produced by these agents. Inhibition of dG^s incorporation (e.g., with mycophenolic acid, hydroxyurea, 1-β-D-arabinofuranosyl cytosine, or 5-fluorodeoxyuridine) protects cells against the delayed lethal effects of MP (Tidd and Paterson, 1974a,b; Lee and Sartorelli, 1981). A number of anticancer drugs other than thiopurines induce

delayed cytotoxic effects; a common property of these agents is their potential to alter DNA structure (Maybaum and Mandel, 1983).

Treatment of cells with TG produces severely disrupted chromatin during G_2 of the cell cycle, as visualized by premature chromosome condensation (Maybaum and Mandel, 1981, 1983). Chromatid damage is a dose-related effect, appearing as sharp curling or "kinking" of chromatid at lower TG concentrations and as unilateral chromatid damage and gross chromosome disruption at higher TG concentrations (Maybaum and Mandel, 1983).

During replication, different components of the replication complex (synthesome) interact with single-stranded DNA, double-stranded DNA, and with hybrid DNA-RNA duplexes. It is postulated that dG^s incorporated into DNA may alter these interactions and thereby inhibit normal DNA replication. Previous studies have demonstrated that dG^s incorporation into DNA partially inhibits the activity of DNA polymerases and DNA ligase (Ling et al., 1992). In the present study, we provide the first evidence that dG^s incorporation into DNA significantly inhibits the activity of RNase H, another critical enzyme participating in DNA replication.

ABBREVIATIONS: MP, 6-mercaptopurine; TG, 6-thioguanine; dG^s, 6-deoxythioguanosine; RNase H, ribonuclease H; HPRT, hypoxanthine-guanine phosphoribosyl transferase; TPMT, thiopurine methyltransferase; DTT, dithiothreitol (treo-1,4-dimercapto-2,3-butanediol); PAAG, polyacrylamide gel; AMCA, *N*-[6-(7-amino-4-methylcoumarin-3-acetamido)hexyl]-3′-(2′-pyridyldithio)-propionamide.

Materials and Methods

Chemicals and Reagents. Dulbecco's PBS, tris(hydroxymethyl) aminomethane (Tris-HCl), ammonium phosphate, MP, *Escherichia coli* RNase H, bacterial alkaline phosphatase, proteinase K, and phosphodiesterase I were obtained from Sigma Chemical Company (St. Louis, MO). RNase-free DNase I from bovine pancreas (6000 activity units/25 μl) was purchased from Stratagene (La Jolla, CA). dG^s was the generous gift of J. J. Johnson, Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. *N*-[6,7-Amino-4-methylcoumarin-3-acetamidohexyl]-3′-(2′-pyridylthio)propil-amide (AMCA) was purchased from Pierce Chemical Co. (Rockford, IL). S6-Dinitrophenol-dG-cyanoethyl phosphoroamidite was obtained from Glen Research (Sterling, VA).

The human T cell leukemia cell lines CCRF-CEM and Molt4 and human B-lineage WIL2-NS (B lymphoblast) and WIL2-S (B lymphoblast) cells were obtained from American Type Culture Collection (Rockville, MD). The human T cell leukemia P12, CCRF-CEM with inactivated HPRT (HPRT-deficient CEM), pre- B cell leukemia 697, and B-lineage NALM6 cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Cell number and viability were determined in duplicate in a Burker-Turk chamber using trypan blue exclusion. Cells in logarithmic phase (doubling time about 24 h) were suspended in fresh medium with 10% fetal bovine serum (Biowhittaker, Walkersville, MD). All experiments were started with an initial concentration of 0.25×10^6 cells/ml before addition of MP, which was dissolved in medium and added as a single dose to achieve a final concentration of 10 μM drug in the culture medium (determined by optical density at 320 nm). Cytotoxicity was expressed as a ratio of viable cells incubated with MP (i.e., cell count × % of viable cells) to the number of viable cells (cell count × % of viable cells) in the control (without MP).

Isolation of dG^s-Containing Genomic DNA. Genomic DNA was isolated from 5×10^6 cells after 0 to 48 h of incubation of cells with 10 μM MP, using the QIAGEN cell culture DNA kit (QIAGEN, Chatsworth, CA), according to the manufacturer's instructions. In brief, nuclei were isolated by centrifugation after lysis of cell membranes. The nuclear pellets were then lysed by sonication (30 s) and treated with proteinase K for 50 min at 50°C. Genomic DNA was isolated by adsorption to the QIAGEN column. DNA solution was treated with DNase I (30 min at 37°C) and then with a mixture of phosphodiesterase I and bacterial alkaline phosphatase (16 h at 50°C).

Quantitation of dG^s in Genomic DNA by UV Absorbance. After enzymatic hydrolysis of DNA, reaction mixtures were injected onto a Supelcosil LC-$_{18}$C column (250 × 4.6 mm; Supelco Inc., Bellefonte, PA) and separated using a gradient of methanol (3–20%) in 3 mM ammonium phosphate, pH 3.7 (Gehrke et al., 1983). The eluate composition was monitored by UV detection at 340 nm. Extinction coefficient 24.8×10^3 at 56 $_{max}$ 340 nm was used to determine dG^s concentrations (Merck & Co., Inc., 1989). The amount of dG^s was calculated using a calibration curve with dG^s (7.5–75 pmol) as a reference standard.

Fluorometric Quantitation of dG^s in Genomic DNA. The resulting mixture of nucleosides, after completion of hydrolysis, was treated with AMCA as described (Warren et al., 1995). The derivatized products were injected onto the Supelcosil LC-8 (150 × 4.6 mm) column (Supelco Inc.) and separated using isocratic elution with 0.2 M sodium formate buffer, pH 4.0, methanol (63:37, v/v) for 20 min. The column was then washed with sodium formate/methanol (20:80, v/v) for 12 min. The dG^s-AMCA derivative was detected using the fluorometer at an excitation wavelength of 345 nm and emission of 450 nm. Quantification was achieved by using a calibration curve with dG^s (0.1–10 pmol) as a reference standard.

HPRT Activity. HPRT activity in cell lysates was estimated by formation of [^{14}C]inosine monophosphate from [^{14}C]hypoxanthine

according to the following protocol: 20 μl cell lysate, 10 μl water, and 70 μl cocktail (20 μl 0.5 M glycine buffer, 10 μl 10 mM 5-phosphoribosyl-1-pyrophosphate, 10 μl 50 mM MgCl$_2$, 10 μl 1.5 mM [^{14}C]hypoxanthine, and 20 μl water) were mixed and incubated for 15 min at room temperature. The reaction was stopped by placing mixtures on ice and adding 5 μl of 0.25 M EDTA. Ten microliters of sample mixture or standard (containing the known amount of [^{14}C]inosine monophosphate) were spotted on polyethyleneimine cellulose paper, dried, and washed in 1 mM NH$_4$HCO$_3$ and water. The bound radioactivity was counted in 20 ml of Scint A-X-F (Packard, Meriden, CT).

Thiopurine Methyltransferase (TPMT) Activity. TPMT activity in cell lysates was determined by the nonchelated radiochemical assay of Weinshilboum et al. (1978), as previously reported by our lab (McLeod et al., 1994).

Modified and Nonmodified Oligodeoxyribonucleotides. Oligodeoxyribonucleotides were synthesized using standard protocols with an automatic synthesizer (Applied Biosystems, Foster City, CA). The syntheses of modified oligodeoxyribonucleotides 5′-d(AC-CACCG^{s}CGCT) and 5′-d(TTGCCTTTAAGG^{s}AAAGTAT) containing dG^s were accomplished using standard phosphoroamidite chemistry with S6-dinitrophenol-dG-cyanoethyl phosphoroamidite (Glen Research) and isolated by HPLC (Altex Electronics, San Antonio, TX; Xu et al., 1992). Purity of the oligonucleotides was estimated to be 99.5% by ion-pair HPLC (Waters, Milford, MA) using an Armsorb C16 (7.5 μm) column (4 × 250 mm), eluted with 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM tetrabutylammonium phosphate with a logarithmic acetonitrile concentration gradient from 5 to 40% over 40 min (flow rate, 1 ml/min) at 47°C.

Oligonucleotide concentrations were determined spectrophotometrically. The following molar extinction coefficients (ϵ_{260}) were used for nucleotides: pA, 15,400; pT, 9,300; pU, 8,800; pC, 7,300; and pG, 11,700. The molar extinction coefficient (ϵ_{340}) 24,800 was used for thioguanosine (Merck & Co., Inc., 1989). Duplex substrates were prepared by annealing a 5-fold molar excess (for cleavage reaction) of the unlabeled strand to the appropriate labeled strand or by mixing equimolar amounts of the each strand in 10 mM Tris-HCl, pH 8.0, and 10 mM MgCl$_2$. Oligonucleotides were heated to 70°C and cooled slowly to room temperature.

Physicochemical Characterization of G^s-Containing Duplexes. Stability of dG^s-modified duplexes (I–IV) was assessed by the temperature dependence of the UV absorption of heteroduplexes, with continuous increase of the temperature at a rate of 0.5°C/min. Changes in optical density of the solution were monitored with a Hitachi model 150–20 spectrophotometer (Tokyo, Japan), using thermostatically controlled quartz cuvettes with a path length of 10 mm. The increase in UV absorbance at 260 nm (A$_t$–A$_0$) was monitored, and A$_0$ immediately after enzyme addition was adjusted to zero.

Circular dichroism spectra from 320 to 220 nm were recorded at 21°C, with a dichrograph Roussel-Jouan III (Marcel, France). The cell compartment was continuously purged with dry N$_2$. Digitized data obtained at every nanometer of wavelength were corrected for baseline and smoothed by a least-squares polynomial fit up to the third order. CD spectra per mole of monomer were plotted as ϵ_L–ϵ_R in units of liter × mole^{-1} × centimeter^{-1}.

Nuclear Extracts from Cell Lines. Nuclear extracts were prepared according to Dignam et al. (1983). Briefly, approximately 1×10^8 cells were collected by centrifugation (700g, 10 min at 4°C), washed in 10 ml of PBS, and resuspended in 700 μl of buffer A [10 mM HEPES, pH 7.9 (4°C), 1.5 mM MgCl$_2$, 10 mM KCl, and 0.5 mM dithiothreitol (DTT)]. After incubation on ice for 10 min, cells were collected by centrifugation, suspended in 280 μl of buffer A, and lysed by 10 to 20 strokes of a Kontes all-glass Dounce homogenizer (B type pestle, 1 ml capacity). The lysate was centrifuged for 20 min at 25,000g (Ti70.1 rotor) at 4°C. The pellet of crude nuclei was resuspended in 300 μl buffer C [20 mM HEPES, pH 7.9 (4°C), 1.5 mM MgCl$_2$, 0.42 M KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride), resuspended by 10 to 20 strokes of a Kontes all-glass Dounce homogenizer (B type pestle, 1 ml

capacity), and the resulting suspension was extracted by gentle stirring for 30 min on ice with a magnet stirrer. The resulting suspension was centrifuged for 30 min at 25,000g (Ti70.1 rotor) at 4°C, and the clear supernatant was dialyzed against buffer D [20 mM HEPES, pH 7.9 (4°C), 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride) for 5 h. The preparation was aliquoted, snap-frozen in liquid nitrogen, and kept at −70°C. Protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA).

RNase H Catalyzed Cleavage of RNA Strand of Hybrid Duplexes. Hydrolysis of the heteroduplexes by ribonuclease H (RNase H) was carried out in 10 μl 0.02 M Tris-HCl buffer, pH 7.9, containing 0.15 M NaCl, 11 mM MgCl$_2$, 0.5 mM DTT, 1 mM EDTA using a 5′- or 3′- [^{32}P]RNA strand (Schmidt et al., 1992). The reaction was initiated by adding 2 μl *E. coli* RNase H (final concentration, 2.5 nM) or 2 μl of nuclear extract (4–8 μg protein). The reaction was carried out at 20°C for 10 to 60 min. The reaction was terminated by adding 5 μl of 3 M sodium acetate, 1 μl of tRNA (5 μg/μl), and 100 μl of cold ethanol. The mixture was incubated at −70°C overnight and the precipitate was collected by centrifugation. Cleavage products were analyzed by electrophoresis in 12 or 20% polyacrylamide gel (PAAG) and quantitated by a Molecular Dynamics (Sunnyvale, CA) PhosphorImager system.

Statistical Analysis. Cell viability and dG^s incorporation among different cell lines were compared using ANOVA, followed by the Tukey multiple comparison test. Spearman rank order correlation was used to assess the relation between dG^s incorporation and cytotoxicity. ANOVA was used to compare differences in RNase H-mediated RNA hydrolysis within heteroduplexes that did versus did not contain a dG^s inserts. Computations were performed with STATISTICA, version 5.1 (StatSoft Inc., Tulsa, OK), and $p < .05$ was considered statistically significant.

Results

Cell Viability. The effects of 10 μM MP incubation on cell viability of human T-lineage lymphoblastic leukemia cells (CEM, HPRT-deficient CEM, Molt4, and P12) and human B-lineage lymphoblastic leukemia (NALM6, WIL2S, and WIL2NS, 697) are shown in Fig. 1A. There were significant differences in cytotoxicity among these cell lines after exposure to 10 μM MP for 48 and 72 h (ANOVA, $p < .03$), but little or no difference after 24 h of exposure to MP (Fig. 1). As anticipated, the HPRT-deficient cell lines (HPRT-deficient CEM and WIL2S) used as negative controls in these experiments, were not sensitive to MP treatment.

dG^s Incorporation into Genomic DNA. Incorporation of dG^s into genomic DNA was quantitated as the number of dG^s residues per 100 residues of dG or as pmoles of dG^s per 10 micrograms of DNA. Nucleoside analysis of DNA hydrolysate by dual-wavelength HPLC is shown in Fig. 2. Results of HPLC quantification of dG^s in genomic DNA after 24 h of exposure to 10 μM MP are summarized in Table 1 and depicted Fig. 1B. Despite higher sensitivity of the fluorometric method, direct UV monitoring of thioguanosine at 340 nm was found to be accurate and straightforward, thus this method was routinely used for our experiments with DNA from cultured cells. As shown in Table 1, dG^s incorporation into DNA after 24 h incubation with 10 μM MP varied more than 50-fold (range 0.045–2.8 dG^s residues/100 dG residues) among these cell lines ($p < .02$). However, among cells with HPRT activity, there was not a significant correlation between the level of dG^s incorporation into DNA and the extent of cytotoxicity at 72 h (Fig. 1, A and B; Spearman rank order $r = 0.2$; $p = .7$).

Table 1 summarizes activities of the two major enzymes catalyzing phosphoribosylation (HPRT) and methylation (TPMT) of MP and the extent of dG^s incorporation into DNA. Consistent with previous findings, cells without HPRT activity (HPRT-deficient CEM, WIL2S) were resistant to MP, whereas HPRT activity in MP-sensitive cells varied 3- to 4-fold. In contrast, TPMT activity differed as much as 10-fold in these cell lines (Table 1); among the B-lineage cells, TPMT activity was highest in cells that were most sensitive to MP (NALM6) and lowest in the most resistant cell line (697), whereas no such association was observed in the four T-lineage cell lines.

Synthetic dG^s-Containing Duplexes. The presence of dG^s in oligodeoxyribonucleotides was confirmed by spectral analysis (λ_{max} 260 and 340 nm) and enzymatic hydrolysis to nucleosides followed by HPLC separation of the reaction mixture under conditions similar to that for dG^s quantification in genomic DNA (see Fig. 2).

The stability of dG^s-deoxy-deoxy duplexes (Table 2) was estimated by hypochromicity effect during thermal denaturation. G^s-containing oligodeoxynucleotides were able to anneal with their complementary strand and all duplexes, including those modified by dG^s incorporation, were stable at 20–37°C (i.e., physiological conditions). However, the melting temperatures of G^s-modified duplexes were notably lower compared with the natural duplex with the same nucleotide sequence; with the melting temperature (T_m) decreasing by
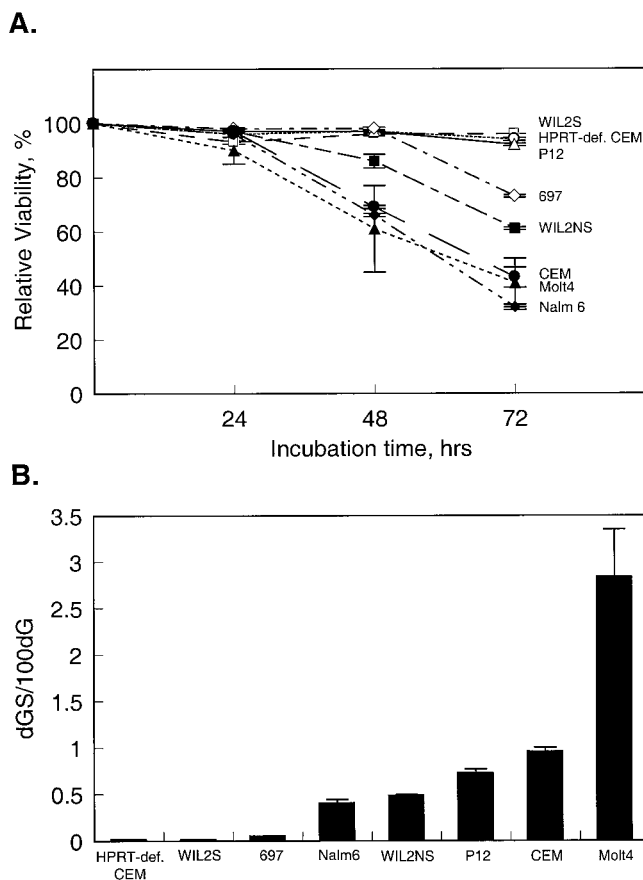
A.

B.

Fig. 1. A, viability of human B and T lymphoblast cells after 10 μM MP treatment. B, dG^s incorporation into DNA of these cell lines after 24 h incubation 10 μM MP. Each point was calculated from data of four to six independent experiments and presents mean ± S.E.

7–10°C due to incorporation of dG[s] residues into the oligonucleotide duplex (Table 2). Furthermore, substitution of deoxyguanosine for dG[s] at position 7 of 5′-d(ACCACCG[s]CGCT), as well as a similar substitution at position 12 of 5′-d(TTGC-CTTTAAGG[s]AAAGTAT), led to local structural distortion of B-form DNA, with a decrease of the positive circular dichroism amplitudes for both modified G[s]-duplexes II and IV and a change of the *x*-axis intercept (Fig. 3).

Inhibition of RNase H-Mediated Hydrolysis of dG[s]-Containing DNA-RNA Duplexes. The influence of dG[s] incorporation into the deoxy strand of hybrid (DNA-RNA) duplexes on the activity of RNase H was assessed using nonmodified and modified substrates (structures in Table 3). *E. coli* 5S ribosomal RNA or its 13-mer fragment and complementary oligodeoxyribonucleotide (natural and dG[s]-containing) heteroduplexes were obtained by annealing (hybrid duplexes V–VIII in Table 3). Nuclear extracts were prepared from the cell lines (CEM, P12, NALM6, Molt4, and HeLa) used in the previous experiments on MP sensitivity and dG[s] incorporation into DNA. Human RNase H present in the
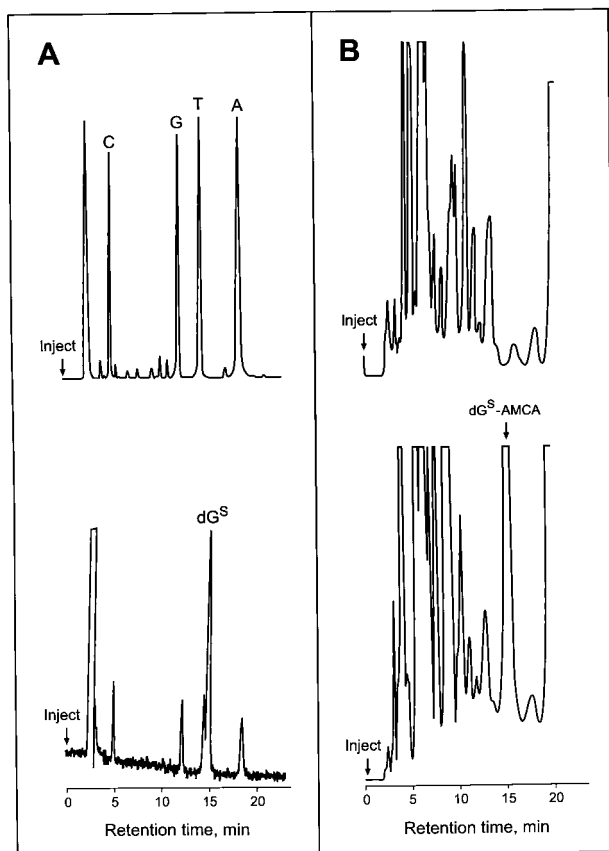

Fig. 2. dG[s] incorporation into DNA of CEM cells. A, HPLC nucleoside analysis of enzymatic digestion of genomic DNA after 24 h incubation with 10 μM MP with UV detection at 260 nm (top) and at 340 nm (bottom). B, fluorometric quantification of dG[s]-AMCA after enzymatic digestion of the genomic DNA before (top) and after (bottom) incubation with 10 μM MP.

TABLE 1
HPRT activity, TPMT activity, and dG[s] modification of genomic DNA extracted from human leukemia cells after 24 h of exposure to 10 μM MP

Human Leukemia Cell Lines	HPRT	TPMT	UV Detection
	$nmol/h/10^6 cells$	$U/10^9 cells$	$pmol\ dG^s/100\ pmol\ dG$
T-lineage			
HPRT-deficient CEM	UD	4.5 ± 0.6	
CEM	9.4 ± 1.9	5.6 ± 1.3	0.95 ± 0.05
P12	9.3 ± 0.6	21.6 ± 3.0	0.71 ± 0.5
Molt4	12.1 ± 0.6	2.6 ± 0.4	2.8 ± 0.5
B-lineage			
WIL2S	UD	1.3 ± 0.3	
WIL2NS	9.4 ± 2.3	10.2 ± 2.1	0.48 ± 0.02
697	3.1 ± 0.3	8.8 ± 2.1	0.045 ± 0.006
NALM6	5.6 ± 0.5	43.6 ± 2.4	0.40 ± 0.045

Data represent average of three independent experiments, mean ± SE; UD, undetectable.

TABLE 2
Effect of G-thiodeoxyguanosine incorporation on stability of oligodeoxyribonucleotide duplexes

Duplex[a]	Structure[b]	T_m	Hypo-chromicity
		°C	h, %
I	5′-ACCACCGCGCT-3′ 3′-CCTGGTGGCGCGATGC-5′	60	15
II	5′-ACCACC**X**CGCT-3′ 3′-CCTGGTGGCGCGATGC-5′	53	17
III	5′-TTGCCTTTAAGGAAAGTAT-3′ 3′-TGAGAACGGAAATTCCTTTCATAGATTT-5′	63	19
IV	5′-TTGCCTTTAAG**X**AAAGTAT-3′ 3′-TGAGAACGGAAATTCCTTTCATAGATTT-5′	53	20

[a] C_O, 0.2×10^{-3} M/monomer in a buffer 0.01 M Tris-HCl, pH 7.9; 0.15 M NaCl; 0.011 M MgCl$_2$; 1 mM EDTA; and 0.5 mM DTT.
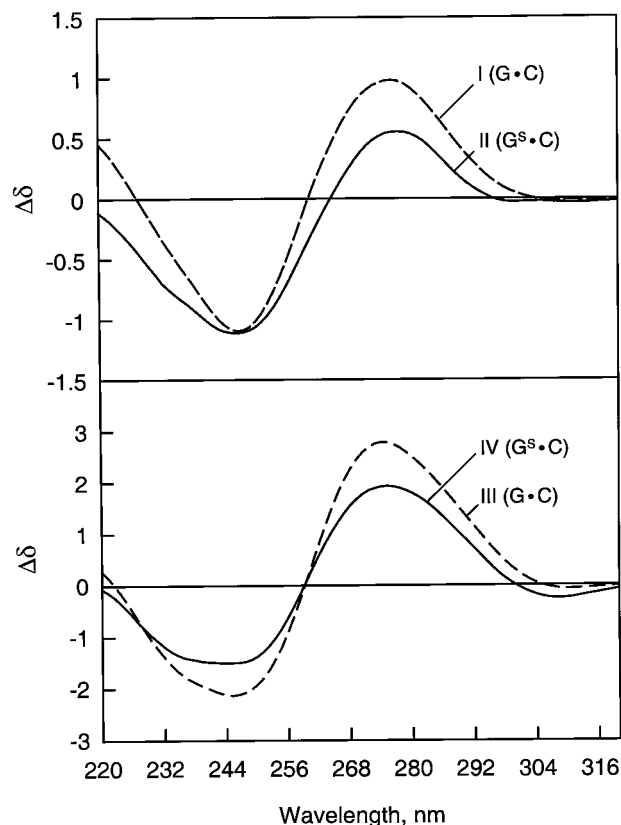[b] **X**, dG[s].

Fig. 3. CD spectra of dG[s] modified and nonmodified DNA-DNA duplexes I–IV in buffer containing 0.01 M Tris-HCl, pH 7.9; 0.15 M NaCl; 0.011 M MgCl$_2$; 1 mM EDTA; and 0.5 mM DTT. C$_o$, 0.2×10^{-2} M per monomer. See Table 2 for structure of DNA duplexes (II and IV contain one G[s] · C base pair; I and III are identical except that the dG[s] is replaced by dG in the G-C base pair).

nuclear extracts in Mg^{2+}-containing buffer (Frank et al., 1994) and bacterial RNase H produced the same cleavage pattern of RNA from the DNA-RNA heteroduplexes (Fig. 4). The products of the RNase H-mediated cleavage of 5S rRNA and its 13-mer fragment were analyzed by PAAG electrophoresis (Figs. 4 and 5). *E. coli* RNase H cleavage of 5S rRNA was used as a control, and its hydrolysis efficiency was used to normalize the hydrolysis by cellular nuclear extracts. Results of quantification of the hydrolysis of RNA by nuclear extracts of human lymphoblastoid cell lines within the dG^s-DNA-RNA heteroduplex are summarized in Table 3, revealing significant reduction of RNase H cleavage of 5S RNA (duplex 6) when dG^s was incorporated in the DNA strand ($p < .00002$, ANOVA).

For the short duplexes VII and VIII, there were also significant changes in total RNA cleavage ($p < .012$, ANOVA). Moreover, this experimental system allowed estimation of the efficiency of phosphodiester bond hydrolysis around the modified G^S-C pair. Figure 6 depicts the ratio of hydrolysis of specific phosphodiester bonds in the duplex 7 relative to hydrolysis at the same positions in the duplex 8. As depicted in Fig. 6, there was a notable decrease of hydrolysis in the vicinity of the modified G^S-C pair (positions -1, $+1$, and $+2$), an effect evident with nuclear extracts from all cell lines tested.

Discussion

Thiopurines are inactive prodrugs, requiring metabolism to nucleotides to exert cytotoxicity (Elion, 1989), with the principal mechanism of cytotoxicity requiring dG^s incorporation into DNA (Krynetski et al., 1996; Krynetski and Evans, 1999). dG^s-5'-triphosphate (dG^s TP), a final metabolite of MP and TG, is a good substrate for human DNA polymerases α, δ, and γ with K_ms similar to those of natural substrates (Ling et al., 1992). Incorporation of thioguanosine into DNA leads to substantial morphological changes of chromatid and is probably important for a number of MP and TG effects, including delayed cytotoxicity (Maybaum and Mandel, 1981, 1983).

We initially investigated the relationship between cytotoxic effects of mercaptopurine and the level of dG^s incorporation into DNA of human B- and T-lineage leukemic cells. These studies revealed substantial differences in the extent of dG^s incorporation into DNA, with B-lineage cells incorporating less dG^s than T-lineage cells (Table 1). Among all cell

lines evaluated ($n = 8$), there was significant correlation between HPRT activity and the level of dG^s incorporation into DNA after 24 h exposure to 10 μM MP (Spearman rank order $r = 0.68$; $p = .0005$). However, there was no relation between the level of dG^s incorporation into DNA and the extent of cytotoxicity at 48 or 72 h after exposure to 10 μM MP ($p > .7$).

With the exception of HPRT-deficient cells that are resistant because they do not form deoxythioguanosine nucleotides, the viability of cells after 48 h did not correlate significantly with the activity of two enzymes catalyzing the major anabolic (phosphoribosylation by HPRT, $p = .44$) and catabolic (methylation by TPMT, $p = .8$) pathways of MP. As evidenced by our and other results (Maybaum and Mandel, 1983), cytotoxic effects of thiopurines become evident after the second round of cell division (Fig. 1). This indicates that initial incorporation of dG^s into DNA, quite notable after 24 h of incubation with MP (Table 1), does not lead to immediate cell death and therefore is not the sole mechanism for immediate cytotoxicity. After 48 h, cytotoxicity is prominent, suggesting that attempted replication of the thioguanylated template is important for cytotoxicity (Glaab et al., 1998). To further elucidate the mechanisms(s) by which thiopurines alter DNA replication and exert cytotoxicity, we investigated the effect of thiopurine incorporation on RNase H function, a key enzyme in DNA replication. Because human B- and T-lineage leukemia cells manifest different sensitivity to antileukemia agents, we used nuclear extracts from both human B- and T-lineage lymphoblasts in our in vitro experiments to determine the effects of dG^s incorporation on RNase H interaction with dG^S-DNA-RNA heteroduplex.

Three types of thioguanylated substrates participate in the replication process: double-stranded DNA, single-stranded DNA, and DNA-RNA heteroduplexes (Bambara et al., 1997). To determine the effects of dG^s incorporated into DNA, we synthesized oligodeoxyribonucleotides containing dG^s incorporated into specific positions and prepared two types of oligonucleotide duplexes (dG^s-deoxy-deoxy and dG^s-deoxy-ribo duplexes) as models for G^S-DNA and G^S-DNA-RNA heteroduplexes formed in cells following thiopurine treatment. These model systems permitted assessment of changes in stability, structure, and substrate properties of such modified nucleic acids. By determining the thermal dependence of hypochromicity, we demonstrated that incorporation of deoxy-6-thioguanosine (dG^S) into oligodeoxyribonucleotides de-

TABLE 3

Structure of DNA-RNA hybrid duplex formed by *E. coli* 5S ribosomal RNA or its fragments and complementary undecadeoxyribonucleotides and efficacy of RNase H-mediated RNA cleavage

Duplex[a]	Structure	Nuclear Extract	Relative RNase H Cleavage (%, 15 min)	
			X = dG	X = dG^s
V (X = dG)	15 25 5'..UGGCGGCCGUAGCGCGGUGGUCCCACCUGA..3' 3'-TCGC**X**CCACCA-5'	*E. coli* RNase H	100	13 ± 1
		Molt4	76 ± 2	8 ± 1
VI (X = dG^S)		CEM	72 ± 4	6 ± 1
		NALM6	58 ± 2	6 ± 1
		HeLa	89 ± 1	19 ± 2
VII (X = dG)	5'..UAGCGCGGUGGUC..3' 3'-TCGC**X**CCACCA-5'	*E. coli* RNase H	100	54 ± 6
		CEM	81 ± 2	69 ± 2
VIII (X = dG^S)		NALM6	81 ± 2	69 ± 3
		P12	83 ± 1	66 ± 2
		HeLa	74 ± 4	74 ± 3

[a] In all experiments duplex concentration C_O, 0.2×10^{-5} M.

creases the T_m by approximately 10°C (compare duplexes I-II and III-IV in Table 2). Circular dichroism spectra of undecamers and nonadecamers (Fig. 3) revealed typical B-DNA spectra with a positive peak at 278 nm and a negative peak at 250 nm (Ivanov et al., 1973). However, there was a decrease in intensity of the positive band in the CD spectrum of duplex II, and a decrease in both the positive and negative bands for duplex IV. These spectral changes are consistent with a decrease in rigidity of the duplex molecule due to distortion away from B-DNA structure because G^s is unable to form normal Watson-Crick base pair with C residues on the complementary strand. Evidence for a distorted B-type helix is present for both modified duplexes II or IV. We hypothesize that modification of DNA by incorporation of dG^s, and formation of a G^s-C pair instead of a G-C pair, causes local structural distortions of B-form DNA. It was previously shown that pair T^{4S}-A slightly changes the CD

spectrum, but oligos containing T^{2S} instead of T do not have a B-DNA like spectrum (Connolly and Newman, 1989). The presence of a sulfur atom in the sixth position changes the geometry of the G^s-C pair so that one hydrogen bond is $0.6\times$ longer, resulting in weakening of the other two hydrogen bonds or a distinct propeller-like distortion (Saenger, 1984). The exact nature of distortions introduced by dG^s to the double helix structure remains to be determined through direct structural studies. Nonetheless, such subtle structural changes can dramatically change nucleotide-protein interactions, consistent with the inhibition of RNase H activity that we observed.

To determine whether incorporation of dG^s impairs RNase H functions in normal replication of DNA along the modified template, we used a chemically modified oligodeoxyribonucleotide strand in hybrid DNA-RNA duplexes. The enzyme RNase H catalyzes the hydrolysis of RNA from DNA-RNA heteroduplexes; this enzyme is uniformly present in various organisms and plays an important role in DNA replication (Crouch, 1990). RNase H from *E. coli* and retroviral origin have been extensively characterized (Wintersberger, 1990), including crystallographic structure (Yang et al., 1990), but little is known about the structure and properties of two classes of RNase H in higher eucaryotes (Frank et al., 1994). RNase H activity was previously demonstrated in nuclear extracts from the human acute lymphoblastic leukemia Molt4 cell line (Giles et al., 1995) and in human erythroleukemia K562 cells (Eder and Walder, 1991). It was also previously shown that modified oligodeoxyribonucleotides with inserted 2'-O-methylcytidine, 2'-deoxy-2'-fluoronucleosides, ribonucleosides, or α-oligonucleotides change the geometry of hybrid duplexes, dramatically decreasing the hydrolytic efficiency of *E. coli* RNase H, and that the oligonucleotide structure influences the position of cleavage within the phosphodiester chain of RNA (Krynetskaya et al., 1991; Schmidt et al., 1992; Alekseev et al., 1996). Previously, we characterized *E. coli* RNase H-catalyzed hydrolysis of *E. coli* 5S rRNA in the presence of complementary oligodeoxyribonucleotides (Schmidt et al., 1992). In the present work, we used the same well-established system to characterize RNase H-catalyzed cleavage
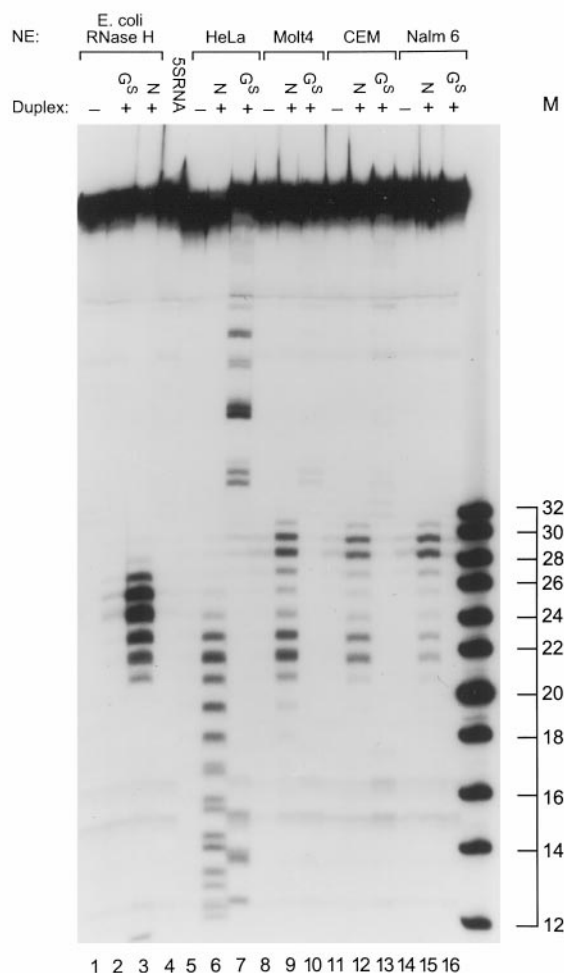
Fig. 4. Electrophoretic analysis in 12% PAAG of the reaction mixtures after RNase H-catalyzed hydrolysis of the 5'-labeled *E. coli* 5S rRNA by RNase H from *E. coli* (lanes 1–3) or nuclear extracts (NE) from HeLa (lanes 5–7), Molt4 (lanes 8–10), CEM (lanes 11–13), and NALM6 (lanes 14–16) in the presence of modified complementary d(ACCACCG^{s}CGCT) (duplex: + G^s) or nonmodified complementary d(ACCACCGCGCT) (duplex: + N). Duplex: (−), no complementary oligodeoxyribonucleotide added. 5'-labeled *E. coli* 5S rRNA without any RNase H and complementary oligodeoxyribonucleotide (lane 4) was used as a blank control. M, length markers.
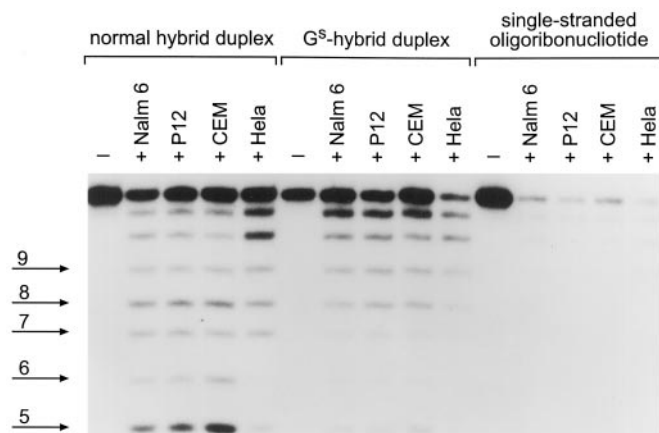

Fig. 5. Electrophoretic analysis in 20% PAAG of the reaction mixtures after RNase H-catalyzed hydrolysis of the 5'-^{32}P-labeled 13-mer oligoribonucleotide (14–26 fragment of 5S rRNA) in normal hybrid duplex VII, in G^S-hybrid duplex VIII or single-stranded 5'-^{32}P-labeled oligoribonucleotide r(UAGCGCGGUGGUC) by nuclear extracts (+) from NALM6, P12, CEM, and HeLa. (−), no nuclear extracts added. Arrows indicate size of oligoribonucleotides.

of 5S rRNA or its fragment in dG^s-modified and nonmodified hybrid DNA-RNA duplexes.

As depicted in Fig. 4, RNase H from different sources (bacterial and human) hydrolyzed 5S rRNA in the presence of oligonucleotides 5′-d(ACCACCGCGCT) and 5′-d(ACCACCG-^{s}CGCT). Both oligonucleotides are complementary to the 15–25 fragment of 5S rRNA, and RNase H cleaves the RNA strand within the region involved in heteroduplex formation. The nonmodified duplex V results in multiple scission points with nuclear extracts from all cell lines tested. The cleavage products formed in the course of the reaction with RNase H from the nuclear extracts of HeLa, Molt4, CEM, and NALM6 are similar to those formed by *E. coli* RNase H. However, when dG^s was incorporated in the deoxy strand of the heteroduplex VI, efficiency of RNase H-catalyzed cleavage was decreased 10-fold (Table 3). This may reflect more efficient formation of the complementary duplex with nonmodified oligodeoxyribonucleotide compared with its dG^s counterpart. Surprisingly, an additional nonspecific activity resulting in formation of additional cleavage sites beyond the heteroduplex was detected in cellular nuclear extracts and was most pronounced in HeLa cells in the presence of dG^s-undecanucleotide (Fig. 4).

Interestingly, in experiments with a short heteroduplex (13-mer fragment of 5S rRNA and complementary undecadeoxyribonucleotides), we also detected inhibition of RNase H-mediated RNA cleavage in the presence of dG^s-containing oligodeoxyribonucleotide, but inhibition was not as dramatic as that observed in the presence of 5S RNA with a high content of helical structures (Table 3, duplex VII versus duplex VIII). In addition, the reaction with this short heteroduplex revealed differences in the cleavage pattern (Fig. 5). The number of cleavage fragments was changed, with the inhibition of RNA phosphodiester bond cleavage detected in the vicinity of the G^s · C base pair. The notable degradation of the single stranded 13-mer by nuclear extracts, in contrast to relatively stable 120-mer 5S rRNA (Figs. 4 and 5) is evidently due to lack of secondary structure in the former. Figure 6 depicts the level of RNase H-catalyzed cleavage of RNA within duplex VIII, relative to the nonmodified substrate 7. Maximum inhibition of RNA cleavage was detected in two phosphodiester bonds adjacent to the G^s · C base pair (-1, $+1$) and at position $+2$ (Fig. 6). Enhanced hydrolysis at more distant phosphodiester bonds $+5$ and $+6$ may be attributed to predominant interaction of RNase H with this part of the duplex, with no other substrate available. This held true both for the human nuclear extracts from NALM6, P12, and CEM, and the purified bacterial enzyme. We postulate that the enzyme interacts with a limited region of the heteroduplex outside the G^s · C base pair.

Taking into consideration the structural data suggesting interaction of bacterial RNase H with the minor groove of the heteroduplex (Yang et al., 1990), we hypothesize 1) that incorporation of dG^s into the deoxy strand of the modified
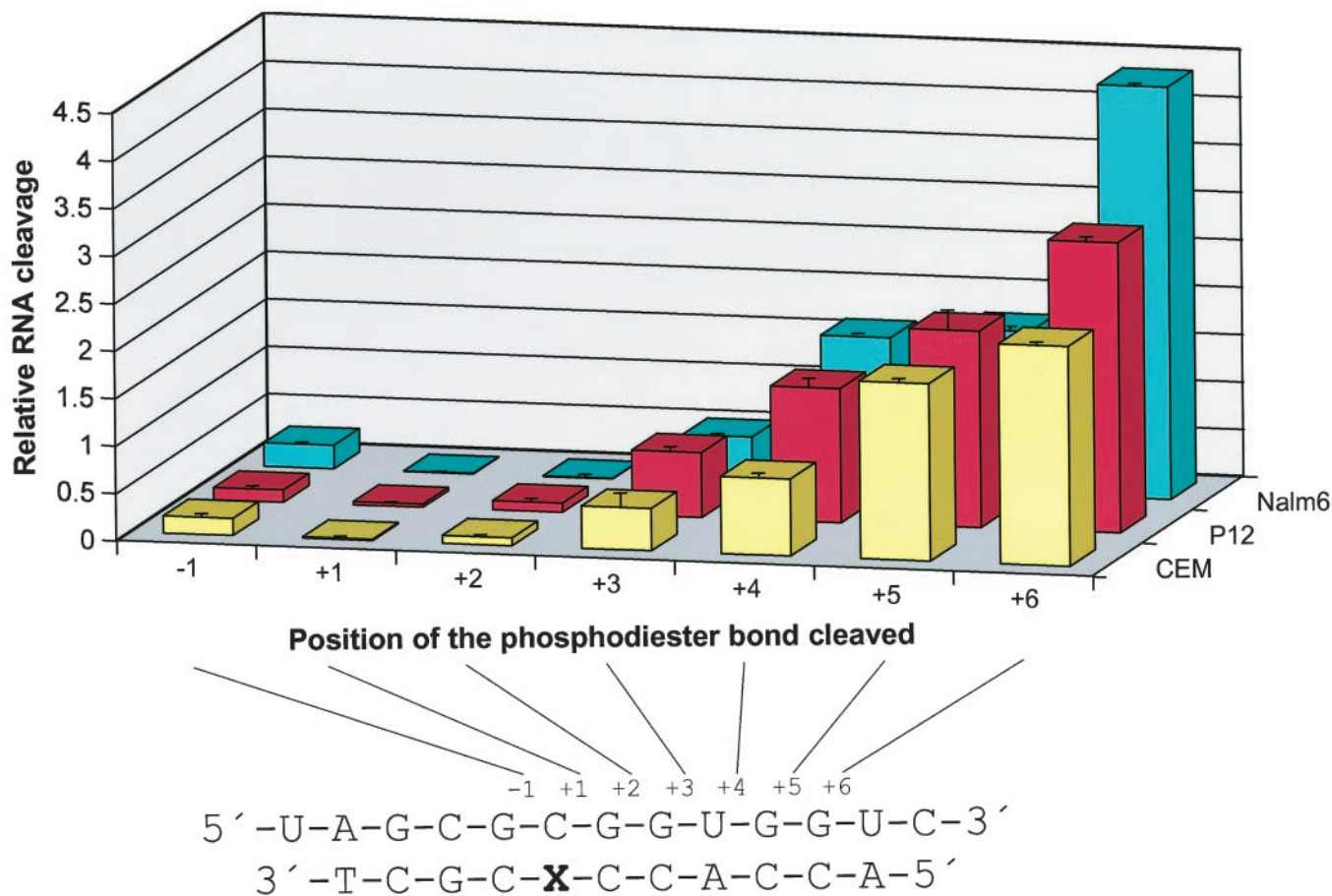

Fig. 6. Effect of G^S position in the DNA strand on RNA cleavage mediated by human RNase H from nuclear extracts. Relative cleavage of phosphodiester bonds in dG^s-containing duplex VIII versus the corresponding cleavage within the nonmodified duplex VII was determined (value 1.0 on the *y*-axis means that cleavage level does not change). Data represent the average of three independent experiments.

heteroduplex alters the minor groove of the DNA-RNA heteroduplex, and 2) that human RNase H also binds to the heteroduplex similarly to the bacterial enzyme, i.e., with the minor groove of the heteroduplex. Our finding that dG[s] incorporation into DNA causes significant impairment of RNase H cleavage of the DNA-RNA duplex, is consistent with the hypothesis that impaired DNA replication across thioguanylated DNA is the basis for delayed cytotoxic effects of thiopurines. Because replication is a multistep process, and the replication machinery encompasses many components, elucidation of specific targets affected by thioguanosine incorporation into DNA is a crucial step toward fully elucidating the mechanism(s) by which thiopurines produce their antileukemic effects. The finding that thiopurine incorporation into DNA inhibits RNase H function, raises the intriguing possibility that these agents may perturb retroviral replication, because these viruses require RNase H activity to replicate. Incorporation of dG[s] into the first strand of cDNA could inhibit subsequent hydrolysis of RNA in the DNA-RNA hybrid and therefore arrest the early stages of viral infection. Thus, the current studies have revealed a new pharmacological effect for thiopurines, providing new insights to their mechanism of cytotoxicity and suggesting other potential therapeutic effects.

Acknowledgments

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