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SHORT COMMUNICATION

Mutagenic Consequences of the Incorporation of 6-Thioguanine into DNA

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ABSTRACT. 6-Thioguanine (S^6G) has been used in the treatment of acute leukemias because of its cytotoxic effect on proliferating leukemic cells. The cytotoxicity of S^6G is thought to derive from its incorporation into DNA in place of guanine. The deoxyribonucleoside triphosphate of S^6G, SdGTP, is a good substrate for bacterial and human DNA polymerases (Ling *et al.*, Mol Pharmacol **40:** 508–514, 1991). Since SdGTP was observed to misincorporate in place of adenine at a greater frequency than did dGTP, it appeared plausible that this analog could produce more subtle effects (mutations) due to mispairing with thymine. To assess whether such mutations occur, SdGTP was incorporated into the *lacI* gene of phage M13lacISaXb in reactions that omitted dGTP (−G) or dATP (−A). *LacI* mutation frequency was determined by β-galactosidase colorimetric staining (inactivation of the lac repressor results in blue plaques in the absence of inducer). When a high concentration of SdGTP (24 μM) was used in the DNA polymerase reaction, phage infectivity was inhibited. When a relatively low concentration (2.4 nM) was added to the −G and −A reactions, mutagenic effects were observed. DNA sequencing of mutant progeny arising from the −G + S^6G reaction revealed C-to-T base transitions and some C-to-A transversions. Similarly, the presence of SdGTP in the −A reactions led to mutants with T-to-C transitions. No insertions or deletions were observed. These data indicate that mispairing of S^6G with thymine leads to mutagenic effects in this assay. BIOCHEM PHARMACOL **54**;3:419–424, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. 6-thioguanine; DNA; mutation; *lacI*; *E. coli*

S^6G** has been proven to have activity against acute myelogenous leukemia, and MP is widely used in the maintenance therapy of childhood acute lymphocytic leukemia [1, 2]. Although the mechanism of the antileukemic effects is unknown, incorporation of both analogs into DNA as S^6G nucleotide has clearly been established to play a role, if not the major role, in their cytotoxicity [3–6]. Previously, we reported that SdGTP is a good alternative substrate for dGTP in DNA polymerase reactions catalyzed by *Escherichia coli* and human DNA polymerases [7, 8]. In synchronized Chinese hamster ovary (CHO) cells treated with S^6G, DNA synthesis and elongation proceeded normally during the incorporation of the analog; however, elongation was slowed and DNA damage became apparent in daughter cells [9]. This observation is consistent with reports of delayed cytotoxicity [4] and unilateral chromatid damage [10] in S^6G-treated cells. Accordingly, Ling *et al.* [6] observed slowed elongation of primers hybridized to DNA templates containing S^6G. Ling *et al.* [7, 8], using a gel electrophoretic assay of *in vitro* misincorporation, also reported that SdGTP had a propensity to misincorporate in place of adenine in DNA synthesis reactions catalyzed by both bacterial and human DNA polymerases. To assess whether this misincorporation or substitution of the analog for guanine might lead to mutations, a genetic assay of mutations arising *in vivo* after incorporation of S^6G into M13-lacI DNA *in vitro* was performed [11, 12]. In this assay, *in vitro* primer elongation is carried out in a region of the *lacI* gene that is highly susceptible to mutational inactivation of the lac repressor. The DNA products are then analyzed in an *in vivo* transfection assay that detects mutant phage progeny through the production of blue plaques [12]. SdGTP was incorporated into the *lacI* gene during *in vitro* DNA synthesis of the M13lacISaXb bacteriophage genome;

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** *Abbreviations:* S^6G, 6-thioguanine; SdGTP, 2′-deoxy-6-thioguanosine triphosphate; dNTP, 2′-deoxyribonucleoside 5′-triphosphate; ddNTP, 2′,3′-dideoxyribonucleoside 5′-triphosphate; U-ssDNA, uracil-containing single-strand DNA; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; IPTG, isopropyl-β-D-thiogalactoside; and MP, 6-mercaptopurine.

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the DNA was then used in a transfection assay of mutation and viability, and phenotypic mutant progeny phage were characterized by DNA sequencing. The results indicate that misincorporation of S^6G in place of A or misreading of S^6G in DNA by T accounts for the mutagenic effects of this analog in this assay.

MATERIALS AND METHODS

Reagents and Chemicals

T4 polynucleotide kinase, T4 DNA ligase, the four dNTPs and ddNTPs, *E. coli* DNA polymerase I Klenow fragment, X-Gal, IPTG, and Sequenase kits were obtained from the United States Biochemical Co. (Cleveland, OH). [γ-^{32}P]ATP was from ICN Radiochemicals (Irvine, CA). Synthetic oligonucleotides were obtained from Genosys Biotechnologies, Inc. (The Woodlands, TX). NACS Prepac was supplied by Gibco-BRL (Grand Island, NY). SdGTP was synthesized as previously reported [7].

Bacterial Strains

E. coli strain CJ236 *dut-1, ung-1, thi-1, relA 1/pCJ105 (F'Cm^r)* was supplied by Dr. C. Joyce (Yale University, New Haven, CT). *E. coli* strain JM103 *Δ(lac-pro), supE, thi, strA, sbc-15, endA, hspR4 F' (traD36, proAB, lacIqZΔM15)* was provided by Dr. J. Messing (Waksman Institute, Newark, NJ). *E. coli* strain SBQ2 ara, *Δ(lac-pro), strA, thi, Φ80-DlacI, lacZDM15, endA$^-$, Δ(srl1-recA), F' (pro$^+$, lacI$^-$, lacZΔM15)* was constructed previously [11].

Bacteriophage

The M13lacISaXb phage strain derived from M13lacI was used. This recombinant phage contains the complete *lacI* gene, which is expressed under phage control when hosted in *E. coli* with a *ΔlacI* genotype. Its construction and properties were described previously, and it has been used to test the mutagenic capability of 1-N^6-ethenodeoxyadenosine [11].

Template-Primers for Genetic and Electrophoretic Assay

Uracil-containing single-strand M13lacISaXb DNA (U-ssDNA) was obtained by growing the phage in *E. coli* CJ236 in the presence of uridine as described by Kunkel *et al.* [13]. The U-ssDNA was purified by precipitation with polyethylene glycol as described by Maniatis *et al.* [14], followed by NACS Prepac purification as recommended by the supplier. Oligonucleotide primers were phosphorylated with T4 polynucleotide kinase using 0.3 mM ATP or [γ-^{32}P]ATP (1 μL, 62.5 mCi/mL). U-ssDNA (30 pmol) was annealed with a mixture of radioactive (60 pmol) and nonlabeled (120 pmol) phosphorylated primer for 1 hr at 55° in 0.5 M NaCl, followed by slow cooling to room temperature. Template-primer was then purified by gel filtration through a Sepharose 2B column previously equilibrated with TE buffer, pH 7.6 (10 mM Tris–HCl, 1.0 mM EDTA). Fractions (150 μL each) were collected, and the first radioactive peak was used for the extension reactions.

Misincorporation Reactions

The primer extension reactions with 0.2 pmol of template-primer were performed under the following conditions: (1) the complete reaction contained all four dNTPs (dATP, dGTP, dCTP, and dTTP; 50 μM each) in a mixture supplemented with 20 mM Tris–HCl, pH 7.5, 2.6 mM $MgCl_2$, and 1 U of Klenow fragment DNA polymerase in a final volume of 15 μL; and (2) the "minus" reactions were similar to the complete reaction except that one or two dNTPs were omitted, i.e. the "minus A" reaction (−A) lacked dATP and the "minus G" reaction (−G) lacked dGTP. Primer extension reactions were conducted in the presence or absence of SdGTP. All the mixtures were incubated for 30 min at room temperature. Aliquots (3 μL each) were saved for electrophoretic analysis of primer extension. To permit full synthesis and ligation of the complementary strand, the corresponding missing nucleotides (50 μM each) were first added to each "minus reaction;" then, to all samples were added 2 μL ligase buffer, 10 mM ATP, 1 U T4 DNA ligase, and 1 U Klenow fragment DNA polymerase in a final volume of 20 μL. The mixtures were incubated for 16 hr at 16°. Aliquots (3 μL each) were again saved for electrophoretic analysis. The products obtained were used in the genetic (transfection) assay described below. Primer extension and misincorporation were analyzed by electrophoresis in 6% denaturing polyacrylamide gel and revealed by autoradiography. Primers P2.1 (5′-ACGCCAATCAGCAACGACTG-3′) and F1 (5′-CGCTTCCACTTTTTCTCGAGTTTTCG CAGA-3′) that anneal to the nucleotide sequence 206–225 and 119–147 in the *lacI* gene, respectively, were used for the elongation reactions reported herein. Primer P2.0 (5′-CAACGCCAATCAGCAACGAC-3′) that anneals to the nucleotide sequence 208–227 was used for sequence determinations.

Genetic Assay

Ten microliters of product obtained from each extension reaction was used to transfect 250 μL *E. coli* SBQ2-competent cells obtained by treatment with $CaCl_2$ as described in Maniatis *et al.* [14]. Transfected cells (serving as infective centers for plaque formation) were first mixed with 400 μL of *E. coli* SBQ2 in logarithmic phase ($OD_{540\ nm}$ = 0.4) and 24 μL of 2% X-Gal in 3 mL of soft agar. They were then placed on Petri dishes with solid 2xYT medium. After 16–24 hr of incubation at 37°, the transfection efficiencies and i^- mutation frequencies were determined.

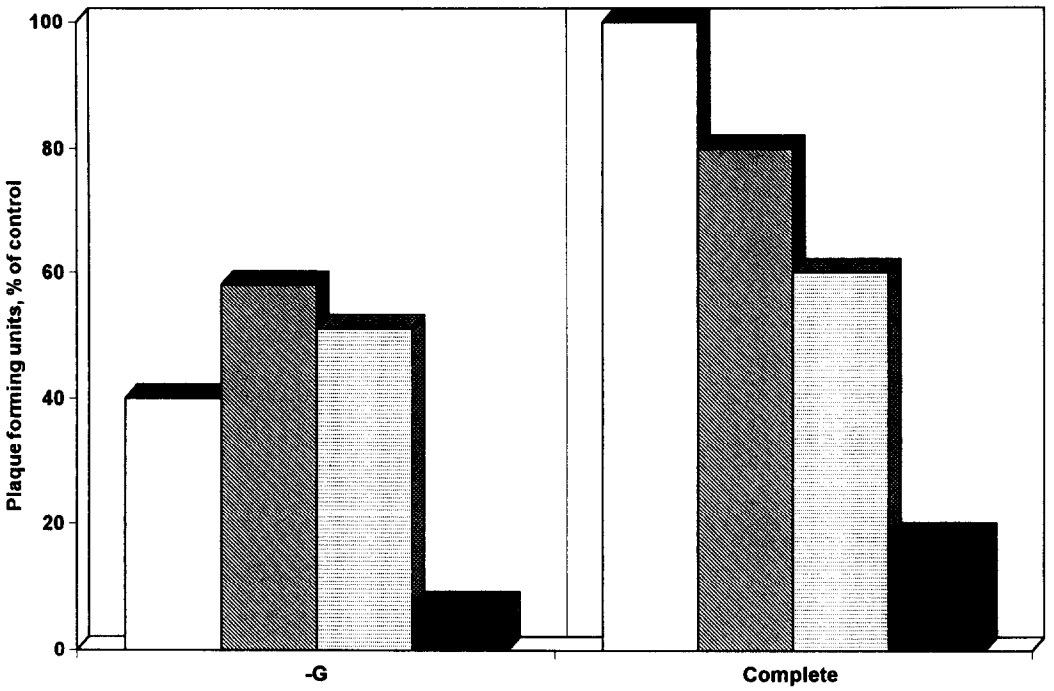

FIG. 1. Effect of SdGTP concentration on the transfecting activity of DNAs synthesized in the minus and complete reactions. The complete reaction without SdGTP corresponds to 100%. Key: no SdGTP (□); 2.4 nM SdGTP (▨); 24 nM SdGTP (▤); and 24 μM SdGTP (■).

Dideoxy Sequencing of Progeny Phage

Each phage plaque was placed in a culture tube with 3 mL 2xYT broth, inoculated with 0.3 mL *E. coli* JM103 in log phase, and incubated for 7 hr at 37° and 300 rpm in an orbital shaker. Single-stranded DNA was purified from the supernatant using the small-scale preparation protocol described by Maniatis *et al.* [14]. The DNA was then annealed with a [5′-^{32}P]-labeled primer and sequenced using the dideoxynucleotide termination method [15].

RESULTS AND DISCUSSION

Misincorporation and Lethal Effects of SdGTP in DNA

Misincorporation of SdGTP during *in vitro* DNA synthesis with primer P2.1 or F1 was evaluated by electrophoresis and autoradiography as described by Ling *et al.* [7]. For this analysis, SdGTP is used for the primer extension in place of each of the normal, endogenous dNTPs. The results obtained were identical to those published previously and are summarized briefly as follows. SdGTP was observed to substitute effectively for guanine and to be misincorporated in the place of adenine at a greater frequency than was guanine (Fig. 1 in Ref. 7). Misincorporation in the −A reaction was evident at loci opposite T in the template, but polymerization stopped abruptly when two or more consecutive Ts were in the template. The extension in the complete reaction, which contained the four dNTPs, was very efficient, and no difference in the size of the products with and without the analog was observed, indicating that the analog does not inhibit the DNA polymerase. Also, the analog was added at three different concentrations in both the −G and complete conditions. The electrophoretic results showed a correlation between the concentration of SdGTP and the rate of incorporation in the −G reaction (data not shown). The potential lethal effect of S^{6}G incorporation was tested at three different concentrations of SdGTP (2.4 nM, 24 nM, and 24 μM) added to the complete or −G mixtures. As seen in Fig. 1, the recovery of phage in the transfection assay was reduced substantially when the highest concentration of SdGTP was added to the −G and complete reactions. The yield of recovered phage plaques at the higher concentration of SdGTP, in the −G + SdGTP and in the complete + SdGTP reactions, was about 6 and 20%, respectively, compared with the yield from the complete reaction in the absence of SdGTP. At this concentration (24 μM), mainly clear phage plaques were recovered. The best concentration for recovering mutants (blue phage plaques) in the absence of lethal effects, therefore, was observed when 2.4 nM SdGTP was added to the −G reaction.

SdGTP Mutagenic Effect

The potential for SdGTP to produce mutagenic effects was evaluated at an SdGTP concentration of 2.4 nM for reasons described above (Fig. 1). When primer 2.1 was used in the initial elongation reaction in the absence of dGTP and the presence of limited SdGTP (−G + SdGTP), the mutation frequency was increased relative to that observed in the absence of SdGTP (complete reaction) from 0.082 to

TABLE 1. Mutations (i^-) induced in the *lacI* gene by incorporation of SdGTP during *in vitro* primer elongation, followed by *in vivo* replication in the transfected host

Reaction condition*	Total plaques counted	Blue plaques observed	Mutation frequency (Blue/Total)
Primer 2.1			
Complete	368	30	0.082
Complete + SdGTP	303	26	0.086
−G + SdGTP	207	58	0.280
Primer F1			
Complete	1679	6	0.004
Complete + SdGTP	840	18	0.021
−G + SdGTP	484	32	0.066
−A	428	18	0.042
−A + SdGTP	468	89	0.190

* Primer elongation within the *lacI* gene was performed using the primers shown in the absence or presence of 2.4 nM SdGTP as described in Materials and Methods.

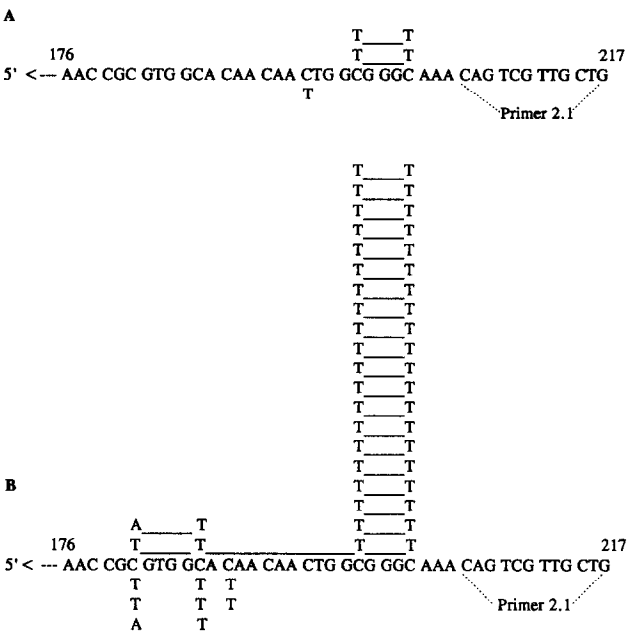

FIG. 2. Mutations in the *lacI* gene arising from *in vitro* synthesis. The synthesis reactions were performed with 1 U Klenow fragment and the primer P2.1. (A) Mutants obtained from −G reactions where dGTP was absent in the initial elongation of the primer. (B) Mutants obtained from −G + SdGTP reactions with 2.4 nM SdGTP added during the initial elongation of the primer. The original sequence of the template is displayed [18], and the substitution is indicated by the respective base. Multiple base substitutions in the same molecule are connected by horizontal lines. The numbers indicate the respective nucleotide in the original *lacI* gene. The primer P2.0 was used for sequencing reactions.

0.280 (Table 1). Inclusion of dGTP and the other dNTPs appeared to prevent this mutagenic effect, i.e the frequency was 0.082 and 0.086 in complete and complete + SdGTP reactions, respectively. This apparent reversal is presumably due to the competing substrate activities of dGTP and SdGTP for the polymerase [7]. When primer F1 was used, SdGTP again appeared to produce a mutagenic effect when incorporated in place of dGTP (−G + SdGTP), i.e. the mutation frequency in that reaction was 0.066 vs 0.004 in the complete reaction. Again, the presence of dGTP in the complete + SdGTP reaction appeared to reduce this mutagenic effect of SdGTP from 0.066 to 0.021. The −A reaction was mutagenic (frequency increased from 0.004 to 0.042) due to the misincorporation of G in place of A as previously described [11, 16]. When SdGTP was present in the absence of A (−A + SdGTP), the frequency of mutation was further enhanced to 0.190, probably because S^6G miscodes for A at a greater frequency than does G [7]. The spontaneous mutation rate using primer F1 appeared to be lower than that observed using primer 2.1 (0.004 vs 0.082; Table 1). Although we do not know the mechanism for this difference, primer F1 is somewhat closer to the N-terminal region of the gene, which suggests that fewer mutants are possible or that there is sequence specificity within these regions with regard to mutability [17].

The base changes [18] produced in mutants analyzed in −G and −G + SdGTP reactions are illustrated in Fig. 2. Of seventeen phenotypic mutants analyzed in the control (−G) reaction, only three represented base changes in the analyzed region (Sequence A in Figure 2). These three corresponded to C-to-T base transitions: two double mutants and one single-base substitution. These results agree with a previous report regarding −G reactions during *in vitro* DNA synthesis due to misincorporation of dATP [17]. In contrast, 29 of 32 phenotypic mutants from the −G + SdGTP reaction demonstrated single- or multiple-base transition mutations in the analyzed region (Sequence B in Fig. 2). We do not know the basis for the apparent higher frequency of C-to-T transitions at the first two C-sites. It may be due to the limited incorporation of TG at the low concentration of SdGTP used (2.4 nM), i.e. the probability that misincorporation occurs would be greatest at sites near the start of replication. Also, some substitutions may not lead to a change in function of the repressor so that "blue plaques" would not be observed. For example, the first C site codes for glycine, and a single C-to-T at this site would not alter the protein sequence. Although SdGTP was present in high molar excess over dGTP, the polymerase may discriminate against the analog more efficiently at some positions than at others. Second, during *in vivo* replication of SG-containing phage template, misincorporation (of T) opposite certain SG residues in the template may occur more frequently than opposite other SG residues. This may be the main reason for the lack of C->T mutations at some positions near the primer. In these samples, we did not detect insertions or deletions; however, two mutants with single C-to-A base transversions were recovered. The sequence analyses of blue phage plaques obtained from the −A reaction, in the presence or absence of the analog, showed single or multiple T-to-C base transitions as previously described by Maldonado-Rodriguez *et al.* [12] (data not shown). Thus, two major changes in the distribution of mutations due to SdGTP were observed in

this system, i.e. an increase in the frequency of C-to-T base substitutions and the presence of additional sites for this mutation type in the region analyzed.

The results suggest that the mutations induced by S^6G are formed as follows. First, the SdGTP is incorporated at almost the same efficiency as dGTP during *in vitro* DNA synthesis to produce strand 1 with S^6G (**S-1**):

SdGTP, Klenow fragment

3′-A-A-A-C-G-G-G-C-G-G-T-5′

3′-A-A-A-C-G-G-G-C-G-G-T-5′

5′-T-T-OH-3′

→ 5′-T-T-T-$\underline{S^6G}$-C-C-C-$\underline{S^6G}$-C-C-A-3′

S-1

During subsequent *in vivo* replication, strand 1 (**S-1**) is used as a template to synthesize strand 2 (**S-2**) such that S^6G will misread, leading to the incorporation of T at some sites:

S-1 5′-T-T-T$\underline{S^6G}$-C-C-C-$\underline{S^6G}$-C-C-A-3′

→ **S-2** 3′-A-A-A-T-G-G-G-T-G-G-T-5′

During the third round of replication, the mutations will be fixed in strand 3 (**S-3**):

3rd round **S-2** 3′-A-A-A-T-G-G-G-T-G-G-T-5′

→ **S-3** 5′-T-T-T-A-C-C-C-A-C-C-A-3′

The genetic assay also revealed S^6G-induced mutations arising from *in vitro* misincorporation of the analog in place of dATP (mutation frequency increased from 0.042 to 0.190 upon addition of the analog to the −A reaction) (Table 1). This result agrees with the misincorporation of S^6G in the −A reaction as seen in the gel electrophoretic assay (this study; data not shown) [6–8].

These data confirm that mispairing of S^6G with T occurs and accounts for the mutagenic effects of the analog. According to CPK models, S^6G (in the thiol form) can pair with T in a manner that is sterically more favorable than a G-T base pair (brought to our attention by Dr. Allen Ansevin, University of Texas M.D. Anderson Cancer Center). The mispairing of G with T [19] and S^6G with T [20] has been studied both theoretically and experimentally. The lethal effect of S^6G due to its incorporation into DNA in place of G may contribute to its therapeutic effects in rapidly replicating leukemic cells. However, MP is used clinically in maintenance chemotherapy of acute lymphocytic leukemia (ALL) in a setting of infrequent, chronic dosing. It is interesting, therefore, to speculate whether the observed mutagenic effects of S^6G incorporation into DNA may also participate in the favorable therapeutic response to the 6-thiopurines. We have observed previously that human DNA polymerases also misincorporate S^6G for A as shown for *E. coli* DNA polymerase herein [8]. Thus, it is likely that the C-to-T transitions also occur in the cells of humans treated with S^6G or MP (MP is incorporated into DNA following its conversion to SdGTP [1, 4]). Mutagenic activity of S^6G and MP has been difficult to establish. This is true, in part, due to the inherent toxicity of these compounds toward the cells used to evaluate this property. MP has been shown to have activity in the dominant lethal assay [21]; however, there is no strong evidence among cured ALL patients that secondary malignancies or other signs of mutagenesis occur at a frequency higher than that in a normal population [22].

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