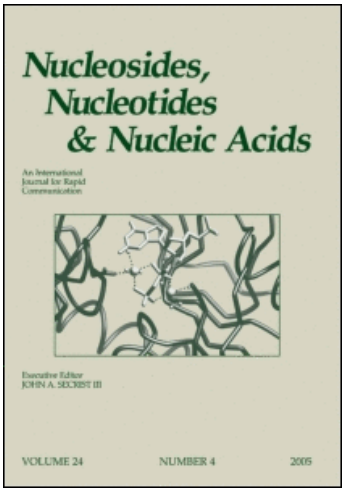


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# Utilization of 2'-Deoxy-6-thioguanosine 5'-Triphosphate In DNA Synthesis Catalyzed by DNA Polymerase I Klenow Fragment of Escherichia Coli

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# UTILIZATION OF 2'-DEOXY-6-THIOGUANOSINE 5'-TRIPHOSPHATE IN DNA SYNTHESIS CATALYZED BY DNA POLYMERASE I KLENOW FRAGMENT OF ESCHERICHIA COLI[1]

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## ABSTRACT

The substrate kinetics of 2'-deoxy-6-thioguanosine 5'-triphosphate for DNA polymerase I (Klenow fragment) of Escherichia coli were investigated. The analog nucleotide was readily incorporated in place of dGTP and it was misincorporated in place of dATP at low but detectable frequency.

## INTRODUCTION

$S^6G$[2], an analog of guanine, has antineoplastic properties in experimental animal tumor models and in humans[3-6]. It has been proposed that a key component in the antitumor activity of $S^6G$ is its ability to be incorporated into RNA and DNA[7-9]. Several investigators have suggested that incorporation of $S^6G$ into DNA may cause DNA strand breaks, chromosomal damage and abnormalities that result in death of tumor cells[10-12]. Recently, we have found that $S^6G$ causes DNA single strand breaks in daughter cells presumably following its incorporation during DNA replication[13].

As a continuation of our studies of the biochemical action of this agent, we have chemically synthesized $S^6$dGTP and measured its substrate properties during in vitro DNA synthesis catalyzed by Kf Pol.

The experimental tool used in this investigation was a gel electrophoretic assay of primer elongation and misincorporation that can be used to directly examine the base pairing specificity of chemically modified dNTPs during DNA synthesis catalyzed by purified DNA polymerases[14-18]. A synthetic primer was 5'-[$^{32}$P]-labeled and annealed to the single-stranded circular DNA of bacteriophage M13. The elongation of [$^{32}$P]primer by Klenow enzyme in a series of "minus" reactions (containing 3 dNTPs) in the presence or absence of $S^6$dGTP was monitored by polyacrylamide gel electrophoresis, followed by autoradiography. In this assay the incorporation of the analog is seen by the stimulation of primer elongation in a "minus" reaction by the presence of $S^6$dGTP.

Our results demonstrate that $S^6$dGTP is a good substrate for Kf Pol, in place of dGTP, and is misincorporated at low but readily detectable efficiency in place of dATP. A preliminary report of this work has appeared[19].



## MATERIALS AND METHODS

**Chemicals.** dNTPs and ddNTPs, ultrapure grade, were purchased from Pharmacia LKB Biotechnology. [$\gamma^{32}$P]ATP (3000 Ci/mmol) and [$\alpha$-$^{32}$P]dNTPs (3000 Ci/mmol) were purchased from ICN. Polynucleotide kinase was obtained from New England Biolabs. Kf Pol was purchased from Pharmacia LKB Biotechnology and U.S. Biochemicals. $S^6$dGTP was prepared and purified essentially as described by Ruth and Cheng[20].

**Preparation of primer-templates.** Synthetic universal 17mer M13 primer (New England Biolabs) was 5'-labeled using [$\gamma$-$^{32}$P]ATP and polynucleotide kinase as described by Maniatis et al.[21]. M13mp9 and M13mp18 single-stranded circular DNA were prepared as described by Messing[22]. The [$^{32}$P]primer was annealed to M13 template DNA (20 pmol) in 250 µl 500 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 at 65° C for 8 min, followed by slow cooling to room temperature. Primer-templates were passed through a Sepharose 2B column (0.7 cm x 20 cm) in TE buffer, and the fractions comprising the first peak of [$^{32}$P] (containing primer-template) were pooled, precipitated with ethanol, dissolved in 20 µl TE buffer and stored at -20° C until used.

**Polymerase reactions and measurement of primer elongation.** In a total volume of 100 µl, 0.5 pmol primer-template was incubated with 0.1

unit Kf Pol, 20 μM each dNTP, 8 mM $MgCl_2$ and 30 mM Tris-HCl (pH 7.5). After 30 min at 30° C the reaction was terminated by addition of 4 μl 250 mM EDTA and the samples were precipitated in ethanol overnight at -20° C. DNA was dissolved in 5 μl TE buffer and mixed with 5 μl 80% formamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, and 0.1% each of xylene cyanol FF and bromophenol blue (Eastman Kodak Co., Rochester, NY). Samples were heated 5 min at 80° C, placed on ice, loaded onto a 8% polyacrylamide, 8 M urea gel (230 x 375 x 0.8 mm) and electrophoresed at 1,200 V for 3 hr. The position of [$^{32}$P]primer within the gel was visualized by autoradiography using Kodak XR-1 film during exposure at -70° C overnight[15,17]. The precise extent of primer elongation was determined by including the corresponding "dideoxy" sequencing lane adjacent to each "minus" reaction lane[23].

**Determination of DNA polymerase kinetic parameters.** The kinetics of primer elongation catalyzed by Kf Pol in "minus" polymerization reactions with or without $S^6$dGTP was measured by the method of Bryant *et al.*[24] with slight modification. Briefly, the 100 μl polymerase reaction mixture contained 0.5 pmol primer-M13mp9 template, 8 mM $MgCl_2$, 30 mM Tris-HCl, pH 7.5, 20 μM each of three dNTPs, 2 μM [$\alpha$-$^{32}$P]dCTP (about 1000 cpm/pmol), various concentrations of the fourth dNTP or $S^6$dGTP, and 1 unit of Kf Pol. After incubation at 30° C for appropriate times, 10-μl aliquots were placed onto DE-81 filter disks (Whatman). The disks were washed twice with washing solution (5% $Na_2HPO_4$) for 5 min, and washed once with 95% ethanol. Disks were dried and counted in a Beckman LS 8100 liquid scintillation counter.

## RESULTS

**Substitution of $S^6$dGTP for dNTPs during DNA synthesis.** To examine the utilization of $S^6$dGTP as substrate in place of normal dNTPs during polymerization catalyzed by Kf Pol we utilized the gel electropohoretic assay of Hillebrand *et al.*[15]. In this assay the primer-template is incubated with DNA polymerase in the presence of only 3 of the 4 dNTPs. Primer elongation pauses opposite each template position complementary to the "missing" dNTP, until a misincorporation occurs, and the extent of primer elongation is monitored by gel electrophoresis and autoradiography. For each "minus" reaction a corresponding "dideoxy" sequencing reaction is conducted, to provide marker bands corresponding

to primers elongated to each successive template residue complementary to the missing dNTP. If a dNTP analog is present which can be incorporated (or misincorporated) in place of the missing dNTP, stimulation of primer elongation in the "minus" reaction occurs. The results of such an experiment with $S^6$dGTP are seen in Fig.1. Lanes 1,4,7 and 10 display the products of the dideoxy termination reactions, ddA, ddG, ddC and ddT, respectively. Following each dideoxy lane is a pair of lanes representing products of the corresponding "minus" reaction, conducted in the absence and presence of $S^6$dGTP, respectively. The set of bands in the lower part of the autoradiograph representing the "minus" reactions corresponds to primers that have undergone a few misincorporation events during primer elongation. Note that the extent of primer elongation in the "-A" reaction was moderately stimulated by the presence of $S^6$dGTP (compare lanes 2 and 3), indicating that the analog can be misincorporated in place of A. The stimulation of elongation by the presence of $S^6$dGTP was substantial in the "-G" reaction (compare lanes 5 and 6), indicating that the analog can be readily incorporated in place of G (as expected from its structure). There was no evidence of misincorporation of $S^6$G in place of C (lanes 8 and 9) or T (lanes 11 and 12). In the absence of both dATP and dGTP there was practically no primer elongation in the absence of $S^6$dGTP (lane 13), while in the presence of the analog (lane 14) the extent of primer elongation was similar to that seen in the "-A + $S^6$dGTP reaction (lane 3), as expected if the analog could readily replace G and be misincorporated to a limited degree in place of A. These data indicate that incorporation of $S^6$G in place of G does not stimulate the subsequent misincorporation of the analog in place of A. Finally, the results shown in lanes 15 and 16 ("complete" reactions conducted in the absence and presence of $S^6$dGTP, respectively) indicate that $S^6$dGTP does not significantly inhibit the activity of Kf Pol.

The greater propensity of $S^6$G than G to misincorporate for A is illustrated in Figure 2. Specifically, concentrations of dGTP as high as 20 μM in the illustrated experiment failed to replace dATP in the reaction (lanes 2 to 5 in Figure 2). On the other hand, misincorporation of $S^6$G for dATP was apparent at 0.02 to 0.2 μM (lanes 8 and 9 in Figure 2).

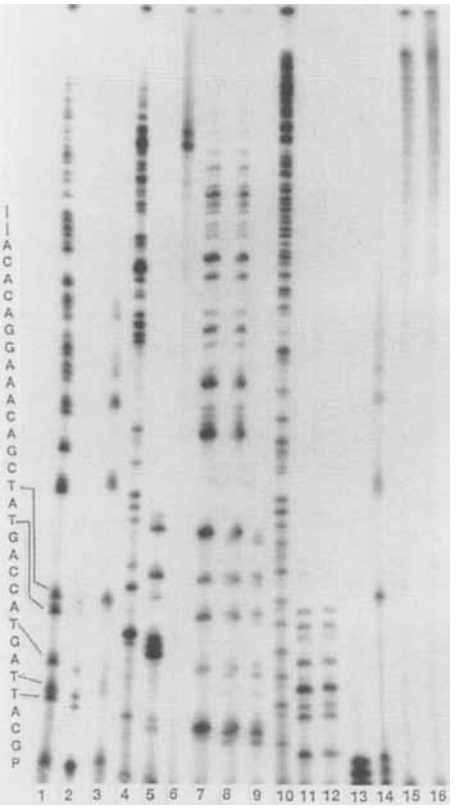


**FIGURE 1. Electrophoretic analysis of the specificity of incorporation of $S^6$dGTP during DNA synthesis.** DNA synthesis reactions (100 µl total volume) contained 20 µM of each dNTP, 0.5 pmol [5′-$^{32}$P]primer-template, 30 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$ and 0.1 unit E. coli DNA polymerase I (Klenow fragment). After 30 min incubation at 30° C, the reaction was terminated by addition of 5 µl of 0.25 M EDTA. The DNA was precipitated with ethanol and then subjected to electrophoretic analysis in a 8% polyacrylamide, 7M urea slab gel. The extent of elongation of [5′-$^{32}$P]primer was visualized by autoradiography. Lanes 1, 4, 7, and 10 display the products of dideoxy-A (ddA), ddG, ddC and ddT sequencing reactions, respectively. Lanes 2 and 3, represent polymerization in the absence of dATP ("-A" reaction) without and with addition of 20 µM $S^6$dGTP, respectively. Lanes 5 and 6 represent "-G" reactions conducted in the absence and presence of 20 µM $S^6$dGTP, respectively. Lanes 8 and 9, "-C" reaction conducted in the absence and presence of 20 µM $S^6$dGTP, respectively. Lanes 11 and 12 represent "-T" reactions conducted in the absence and presence of 20 µM $S^6$dGT, respectively. Lanes 13 and 14, ("-A, -G") reaction conducted in the absence and presence of 20 µM $S^6$dGTP, respectively. Lanes 15 and 16 represent "complete" reaction (containing all four canonical dNTPs) conducted in the absence and presence of 20 µM $S^6$dGTP, respectively. The nucleotide sequence of the template is displayed on the left (5′ to 3′, top to bottom) with lines connecting T residues with the corresponding "dideoxy-A" bands in lane 1. The template position complementary to the 3′-terminal primer residue is denoted "P".

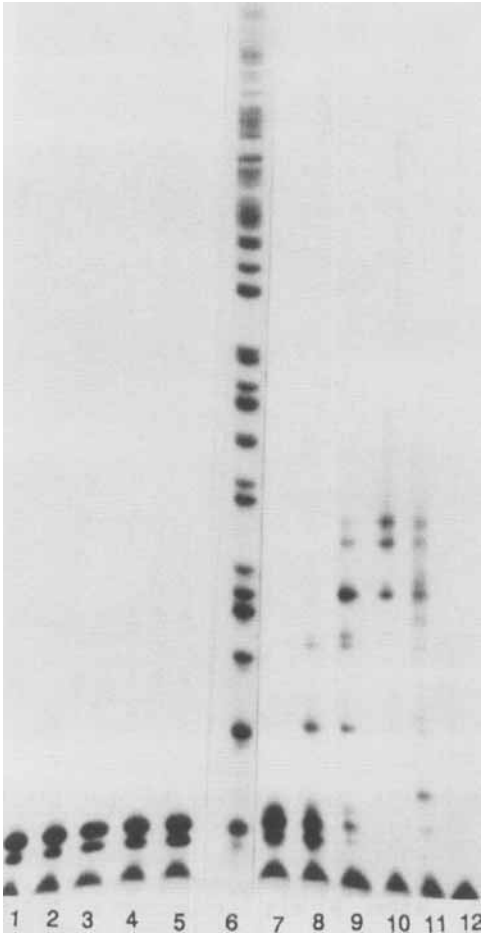


**FIGURE 2. Misincorporation of $S^6G$ for A in DNA synthesis catalyzed by Kf Pol.** Experimental conditions were essentially as described in the Legend for Figure 1. Reactions were performed in the absence of dATP at various concentrations of dGTP or $S^6$dGTP as follows. Lanes 1-5, dGTP at 0, 0.02 , 0.2, 2 and 20 μM; Lane 6, ddATP sequence; Lanes 7-11, $S^6$dGTP at 0.002, 0.02, 0.2, 2 and 20 μM; Lane 12, primer.

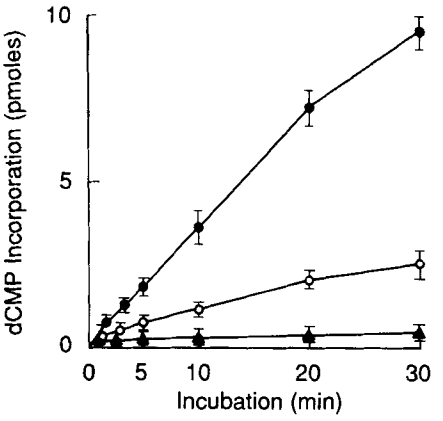


**FIGURE 3.** **Effect of substitution of $S^6$dGTP for dGTP on DNA synthesis catalyzed by E. coli DNA polymerase I (Klenow fragment).** Polymerase reactions contained 30 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 0.5 pmol unlabeled primer-template, 20 μM dATP, dTTP, 2 μM [α-$^{32}$P-α]dCTP ($10^3$ cpm/pmol) and 0.1 μM dGTP (●-●) or $S^6$dGTP (o-o) or no additional dNTP (▲-▲). Reactions were initiated by addition of 0.1 unit Klenow enzyme. At the indicated times, samples were removed and placed onto DE81 filters. Filters were washed twice with 5% (w/v) $Na_2HPO_4$ and incorporation was measured with a Beckman LS8100 scintillation counter. All points represent the average of three separate experiments, ± SD.



**Kinetic studies of $S^6$dGTP utilization by Kf Pol.** Although the gel electrophoretic assay provides a very sensitive indication of rare misincorporation events, a more quantitative measurement of the efficiency of $S^6$dGTP utilization in place of dNTPs was acquired by use of the DE81 filter assay of incorporation, in which reactions were conducted with [α-$^{32}$P]dCTP and nonlabeled primer-M13mp9 template in the presence and absence of $S^6$dGTP. The results of the time course for polymerization in the presence of $S^6$dGTP are displayed in Fig. 3. In this experiment, unlabeled primer-template was incubated with Kf Pol in the presence of 20 μM each of dATP and dTTP and 2 μM [α-$^{32}$P]dCTP, and the effect of adding 0.1 μM dGTP or $S^6$dGTP on incorporation of dCMP was quantitated over the course of 30 minutes.

As shown in Table 1, $S^6$dGTP substituted for dGTP in polymerization catalyzed by Kf Pol and the rate of primer elongation in the presence of

TABLE 1
UTILIZATION OF $S^6$dGTP IN PLACE OF THE CANONICAL NUCLEOTIDES BY E. COLI DNA POLYMERASE I (KLENOW FRAGMENT)[a]

| Conditions | pmol[$\alpha$-$^{32}$P]dCMP Incorporation[b] | Relative Polymerase Activity (%) |
|---|---|---|
| Complete | 57.3 ± 6.8 | 100 |
| Complete + $S^6$dGTP | 58.7 ± 2.7 | 102 |
| Minus dATP | 0.82 ± 0.19 | 1.4 |
| Minus dATP + $S^6$dGTP | 1.85 ± 0.51 | 3.2 |
| Minus dGTP | 1.55 ± 0.24 | 2.7 |
| Minus dGTP + $S^6$dGTP | 18.8 ± 4.1 | 33 |
| Minus (dATP, dGTP) | 0.44 ± 0.11 | 0.8 |
| Minus (dATP, dGTP) + $S^6$dGTP | 1.30 ± 0.56 | 2.3 |
| Minus dTTP | 2.04 ± 0.93 | 3.6 |
| Minus dTTP + S6dGTP | 2.10 ± 1.34 | 3.7 |

[a]DNA polymerase activity was measuring using the DE81 filter assay described in Methods.
[b]Values are the average of triplicate determinations ± S.D.



0.1 μM $S^6$dGTP was 1/4-1/3 that seen at the same concentration of dGTP. In a "-A" polymerization, the analog gave a low but detectable stimulation of polymerization (about 1/10 of the stimulation seen in the "-G" reaction, in agreement with the gel electrophoretic evidence for misincorporation of $S^6$G for A.

The DE81 filter assays conducted under steady state conditions over a range of dNTP concentrations yielded the kinetic parameters shown in Table 2, calculated by double reciprocal plots as described by Lineweaver and Burk[25]. The apparent Km values were identical for dGTP and $S^6$dGTP (0.34 μM for both), whereas the Vmax for the analog was only 0.19 that for dGTP. Thus, it appears that the binding affinity of Kf Pol for dGTP and $S^6$dGTP are similar but that the catalytic efficiency is lower for incorporation of the analog. The apparent Km value for $S^6$dGTP (12 μM) was about forty times that for dATP (0.28 μM) and the relative Vmax for the analog was only 0.05 that for dATP. Thus, in the misincorporation of $S^6$dGTP in place of dATP the analog is bound weakly to the enzyme (compared with dATP) and the rate of phosphodiester bond formation is greatly reduced. The relative frequency of incorporation

TABLE 2
KINETIC PARAMETERS FOR dGTP, dATP AND $S^6$dGTP ULILIZATION IN PRIMER ELONGATION BY E. COLI DNA POLYMERASE I (KLENOW FRAGMENT)

| Condition | Substrate | Km[a] | Relative Vmax[a,b] | Vmax/Km | $f$[c] |
|---|---|---|---|---|---|
| minus dGTP | dGTP | 0.34 μM | 1 | 2.94 | 1 |
| minus dGTP | $S^6$dGTP | 0.34 μM | 0.19 | 0.56 | $1.90 \times 10^{-1}$ |
| minus dATP | dATP | 0.28 μM | 1 | 3.57 | 1 |
| minus dATP | $S^6$dGTP | 12 μM | 0.05 | 0.004 | $1.35 \times 10^{-3}$ |

[a]Apparent Km and Vmax were evaluated from double reciprocal plots (1/v versus 1/[S]).
[b]Vmax for dGTP and dATP are 1600 and 1160 pmol/unit enzyme/hr, respectively.
[c]The relative frequency, $f$, of incorporation of analog compared with unmodified dNTP is the ratio of Vmax/Km for the analog divided by that for unmodified dNTP.



of the analog in place of the unmodified dNTP, $f$, is given by the ratio of Vmax/Km for the analog divided by that for the unmodified dNTP, as described by Fersht[26]. The $f$ value for incorporation of $S^6$G in place of G ($1.90 \times 10^{-1}$) is about 140 times that for misincorporation of $S^6$G in place of A ($1.35 \times 10^{-3}$).

## DISCUSSION

$S^6$G is an effective antileukemia agent, used alone or in combination with other drugs. However, the precise mechanism of action for this agent is not yet completely understood. LePage[7] first proposed that the cytotoxic effect of $S^6$G may be associated with its incorporation into nucleic acids *in vivo* following its conversion to analog nucleotides. A number of investigators have provided evidence in support of this hypothesis. Yoshida *et al.*[27] showed that $S^6$dGTP can serve as a substrate for purified DNA polymerase α from calf thymus. In the work reported here we utilized a sensitive gel electrophoretic assay of primer elongation to examine the base pairing potential of $S^6$dGTP during DNA synthesis catalyzed by Kf Pol. A major advantage of this technique is the ability to obtain visual evidence regarding the

specificity of incorporation of a chemically modified dNTP, including its misincorporation potential[15-17]

Our results demonstrate that $S^6$dGTP is a good substrate for incorporation in place of dGTP during primer elongation catalyzed by Kf Pol. The incorporation of $S^6$G occurred most readily opposite template C, as expected from its structure, but the gel electrophoretic assay also demonstrated that the analog exhibits ambiguous base pairing potential, being misincorporated at a low but readily detectable frequency opposite template T. No misincorporation of $S^6$G was detected opposite template A or G, however.

Kinetic analysis of $S^6$dGTP utilization by Kf Pol yielded an apparent Km value for $S^6$dGTP that is identical to that for dGTP, suggesting that the enzyme has similar affinity for the analog and the unmodified nucleotide. This result is consistent with the similar Km values for $S^6$dGTP and dGTP reported by Yoshida et al.[27] for calf thymus DNA polymerase α. However, our measurements of incorporation using both the gel electrophoretic assay and the DE81 filter assay indicated that the Vmax of the analog is approximately 20% that of dGTP. Using a different experimental approach, Maybaum and coworkers[28] also reported that Kf pol utilized $S^6$dGTP less effeciently than it did dGTP. Several possible causes for the reduced rate of DNA synthesis when $S^6$dGTP replaces dGTP can be suggested. One possibility is that once incorporation of the analog has occurred, the $S^6$G.C base pair may be thermodynamically unstable or cause structural distortion of the primer-template such that binding of the polymerase or addition of the next nucleotide is disrupted. Another possibility is that during the incorporation of the analog a conformational change in the polymerase-dNTP-DNA complex, which is believed to be a rate limiting step for phospohodiester bond formation[29] and which may involve interactions between the polymerase and the substrates[30], is slowed significantly compared to normal incorporation. Thewalt and Bugg[31] investigated the X-ray crystal structure of $S^6$G, compared with normal purines and pyrimidines, and concluded that the replacement of $O^6$ by the bulkier S atom in guanine should alter the hydrogen bonding characteristics of the purine. For example, the NH...S hydrogen bond is longer than that of NH...O, and the hydrogen bond angles also differ for these two cases. Such alterations would be expected to cause

distortions at the 3′-terminus of the primer-template and thereby slow down the incorporation of the next nucleotide, causing a reduction in the net rate of DNA synthesis. Yet another possible explanation for reduced rate of polymerization when $S^6$dGTP substitutes for dGTP stems from the fact that the net rate of incorporation by Kf Pol is the sum of an incorporation step and a 3′-exonucleolytic proofreading step[32-34]. If the $S^6$G.C pair is recognized as a mismatch by the 3′-editing activity of Kf Pol, the proofreading activity may slow down the net rate of DNA synthesis when $S^6$dGTP replaces dGTP in polymerization. However, our finding[35] that other DNA polymerases, which lack 3′-exonuclease activity, also exhibit this same phenomenon suggests that proofreading may not be the major contributor to reduced polymerization rate by Kf Pol when $S^6$dGTP substitutes for dGTP.

In this work we have shown that $S^6$dGTP exhibits misincorporation potential, specifically forming the $S^6$G.T mispair during polymerization by Kf Pol. Although the efficiency of formation of such mismatch during DNA synthesis, assessed by kinetic analysis, was only 1.35/1,000 (compared with the efficiency of A.T base pair formation), this frequency was about 25 times greater than is typical for misincorporation of normal nucleotides[36]. The mechanism of mispairing of $S^6$G with T could involve tautomerization, ionization or wobbling, as we have previously discussed[14,29]. The misincorporation of $S^6$G for A may not play an important role in the drug effect in cells since the affinity and Vmax values are considerably less than that of dATP (Table 2), i.e., the endogenous dATP would preferentially be utilized. Experiments underway to evaluate A to G mutational events may aid in determining the potential importance of such misincorporation.



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1. Supported by Grants CA-28034, AI-28213 and GM25530 from the National Institutes of Health.

2. Abbreviations: dNTP, 2′-deoxynucleoside 5′-triphosphate; ddNTP, 2′,3′-dideoxynucleoside 5′-triphosphate; EDTA, ethylenediamine tetraacetic acid; Kf Pol, Klenow fragment of E. coli DNA polymerase I; $S^6$dGTP, 2′-deoxy-6-thioguanosine 5′-triphosphate; $S^6$G, 6-thioguanine; TE, 10 mM Tris-HCl, pH 7.5 1 mM EDTA.

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