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EFFECTS OF 6-THIOPURINES ON THE TRANSFORMING ACTIVITY OF *BACILLUS SUBTILIS* DEOXYRIBONUCLEIC ACID*

BRUCE A. HARRIS, DOUGLAS A. WEIGENT and J. ARLY NELSON

Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX 77550, U.S.A.

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Abstract—6-Thioguanine (6-TG) and 6-mercaptopurine (6-MP) have been reported to produce cytotoxicity due to incorporation into DNA. Although some investigators have performed *in vitro* transcription studies with synthetic polymers containing 6-TG, the DNA isolated from a cell population exposed to thiopurines has not been evaluated for its biological activity. *Bacillus subtilis* UTH-8505 was grown in the presence of 6-TG, 6-MP, bromodeoxyuridine (BUdR), arabinosyladenine (AraA) and 2,6-diaminopurine. DNA isolated from these and control cultures was used to transform *B. subtilis* T_3, a strain lacking tryptophan synthetase, to prototrophy. 6-MP exposure decreased the transforming activity 80 per cent; 6-TG, 20 per cent; BUdR, 50 per cent; and in single determinations AraA and 2,4-diaminopurine were without effect. 6-MP was also the most effective growth inhibitor of *B. subtilis*. Base composition and melting temperatures of the DNA from 6-MP, 6-TG and BUdR cultures were not altered. Although the methods employed did not permit unequivocal demonstration of 6-TG incorporation, a maximal estimate of one 6-TG molecule substituted for every 500 guanine residues in the DNA was inferred. Thus, extremely low levels of incorporation may alter DNA function, or the reduced transforming activity is produced by another mechanism.

Purine and pyrimidine base analogs represent a major class of antitumor agents. Among these, 6-MP† and 6-TG have been used extensively in humans, and it is surprising that the precise mechanism for antitumor activity is not known. Both agents form analog nucleotides and are incorporated as 6-TG into the nucleic acids of treated cells [1]. Furthermore, 6-MP is methylated to form an active inhibitor (MMPR-5′-P) of *de novo* purine biosynthesis which potentiates 6-MP action [2, 3]. However, inhibition of *de novo* biosynthesis does not appear to account fully for the toxicity of either agent, and the effects of 6-TG on purine and pyrimidine ribonucleotide pools are temporally and quantitatively dissociable from cytotoxicity against H. Ep. #2 cells in tissue culture [4]. On the other hand, inhibition of DNA but not RNA synthesis protects the cells against 6-TG and 6-MP [4]. Therefore, in this cell system, incorporation of the base analog into DNA appears to represent a significant mechanism for the cytotoxicity. Unfortunately, little data are available to evaluate the putative altered function of nucleic acids in cells treated with thiopurines. Several reports have described effects of 6-TG on RNA metabolism and protein synthesis, which may be a result of incorporation of the drug into RNA [5–8]. Theoretical considerations suggest that incorporation of 6-TG into DNA or RNA will have deleterious effects [9–13], and synthetic polymers containing 6-TG have been shown to serve neither as templates for transcription [14] nor for translation [15] in *in vitro* systems. We describe here the reduction of transforming activity of a DNA obtained from *Bacillus subtilis* treated with 6-MP and 6-TG. A preliminary report of these observations has appeared [16].

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† Abbreviations used are: 6-MP, 6-mercaptopurine; 6-MPR, 6-mercaptopurine ribonucleoside; 6-TG, 6-thioguanine; BUdR, 5-bromodeoxyuridine; AraA, arabinosyladenine; AraC, arabinosylcytosine; dCyR, dGuR, dAdR, TdR and β-TGdR, the β-2′-deoxyribonucleosides of cytosine, guanine, adenine, thymine and 6-thioguanine, respectively; and h.p.l.c., high pressure liquid chromatography.

MATERIALS AND METHODS

Materials. Purine and pyrimidine compounds were supplied as follows: 6-TG (NSC 752), Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD); [^{35}S]-6-TG (2 mCi/m-mole) prepared by radioisotope exchange [6, 17], a generous gift of Dr. G. Mandel and Dr. G. Guzzo of George Washington University; [8-^{14}C]mercaptopurine (27.8 mCi/m-mole), Schwartz-Mann (Orangeburg, NY); [^{3}H]arabinosyl adenine (18.7 Ci/m-mole) ICN (Cleveland, OH); [^{3}H]thymidine (90 mCi/m-mole), New England Nuclear (Boston, MA); 6-MPR and AraC, P.L. Biochemicals (Milwaukee, WI); BUdR, CalBiochem (LaJolla, CA); and 2,6-diaminopurine, Sigma Chemical Co. (St. Louis, MO).

The enzymes and their sources were as follows: trypsin, *Crotalus adamanteus* phosphodiesterase, 5′-nucleotidase, ribonuclease A and T_1, Sigma Chemical Co.; deoxyribonuclease I, P.L. Biochemicals; and lysozyme, Boehringer-Mannheim (Indianapolis, IN). L-Tryptophan was purchased from Fisher Chemicals (Houston, TX). Nutrient broth, casein hydrolysate and Bacto-Agar were obtained from Difco (Detroit, MI); glass-distilled acetonitrile and methanol were products from Burdick and Jackson Laboratories (Muskegon, MI).

Bacterial strains. Initial cultures of *B. subtilis* (strains UTH-8505 and T_3) were generously provided by Dr. T. S. Matney and Dr. J. T. Heard of the University of Texas Health Science Center at Houston [18]. *B. subtilis* strain UTH-8505 (trp^+) served as the source of donor DNA for transformations. *B. subtilis* strain T_3 lacking tryptophan synthetase activity and, therefore, unable to grow on indole, served as the recipient strain [19].

DNA isolation. Biologically active DNA was isolated from *B. subtilis* strain UTH-8505 according to the classical procedure of Marmur [20], or by a modification of the phenol technique of Siato and Miura [21]. Approximately 1 g of cells was obtained from a liter culture. The cells were washed with 0.15 M NaCl–0.1 M EDTA, pH 8.0, treated with lysozyme (50 μg/ml, 1 hr), and completely lysed by the addition of 1% sodium lauryl sulfate in 0.1 M Tris (10 min at 60°). Protein was precipitated with either chloroform or with Tris-SLS saturated phenol at pH 9.0. Nucleic acid was allowed to ascend through 2 vol. of 95% ethanol in a graduated cylinder. The nucleic acid was then transferred to a solution of 15 mM NaCl, 1.5 mM trisodium citrate. RNA was removed by incubation with RNase A (50 μg/ml) and RNase T_1 (16 units/ml) at 37° for 1 hr. After a second removal of protein with chloroform or phenol, the DNA was precipitated in 2 vol. of cold 95% ethanol, resuspended in 0.15 M NaCl and stored at 4°. DNA recovery was measured by the colorimetric diphenylamine assay [22].

Development of competence for transformation. B. subtilis strain T_3 was made competent for transporting exogenously supplied DNA into the cell using the procedure described by Anagnostopoulos and Spizizen [23]. Essentially, the cells were grown in medium containing 0.5% tryptophan for 5 hr at 37°, followed by growth in medium containing 0.05% tryptophan for an additional 2 hr. The competent cells were used immediately for transformation experiments or they were frozen in liquid nitrogen and stored at −70° for not more than 30 days prior to use.

Transformation procedure. Sterile DNA was added at a final concentration of 1 μg/ml to triplicate culture tubes containing competent cells in 0.9 ml minimal salt medium containing 0.5% tryptophan. This amount of DNA was found to produce near maximal transformation frequency. The cultures were shaken gently at 37° for 30 min after which the transformation was stopped by the addition of DNase I (25 μg/ml). The cultures were diluted 10-fold, plated on minimal salt medium containing 0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% sodium citrate, 0.02% $MgSO_4$, and 0.5% glucose, and incubated for 48 hr at 37°. The number of viable cells was determined by plating dilutions of each transformed culture onto nutrient agar containing 0.8% Nutrient Broth and 0.5% NaCl, followed by incubation for 24 hr at 37°.

Control transformation frequencies obtained with non-frozen recipient cells averaged 0.2 transformants/100 viable cells. The frequency was 100-fold less with stored (i.e. frozen), competent cells. DNA isolated from controls (i.e. guanine and untreated cultures) gave transformation frequencies which were within 10 per cent of each other.

High pressure liquid chromatography. A Waters Associates model GPC/LC-204 instrument equipped with two model 6000 pumps was used. Samples (0.01 to 0.8 ml) were loaded onto a Waters Associates μ Bondapak C_{18} column (4 mm × 30 cm) via a model U_6K injector. DNA samples (10 μg) were degraded to deoxyribonucleosides by the simultaneous addition of DNase I (10 μg), phosphodiesterase (0.15 unit), 5′ nucleotidase (1.0 unit), and $MgSO_4$ (20 μmoles) in a 1-ml volume at pH 8.9. Degradation was stopped after incubation at 37° for 8 hr. Protein was removed from the samples by heat denaturation at 95° for 2 min followed by filtration through 0.22 μm membrane filters (Millipore). The deoxyribonucleosides were separated by two solvent systems. Solvent system 1 utilized isocratic elution with 10 mM KH_2PO_4, pH 5.5, and 9% acetonitrile at a flow rate of 0.7 ml/min. Solvent system 2 was a 30-min linear gradient from 10 mM KH_2PO_4, pH 5.5, to 40% methanol at a flow rate of 1.0 ml/min [24]. The gradient was produced by a model 660 Solvent Programmer (Waters Associates). The eluting deoxyribonucleoside compounds were monitored by u.v. absorbance at 254 nm using a Waters Associates model 440 absorbance detector and a Hewlett-Packard model HP 3380A electronic integrator/recorder. Absorbance at 280 and at 340 nm was measured by a Schoeffel SF 770 variable wavelength detector and a Houston Instruments Chart Recorder.

Determination of melting temperatures. Spectral measurements of T_m were performed using a Gilford model 250 u.v./vis spectrophotometer equipped with a Haake model FE circulating water bath. Denaturation of DNA was achieved at a DNA concentration of 25 μg/ml in a 40% formamide solution [25]. The increase in absorbance due to denaturation was measured at 270 nm.

RESULTS

The transforming activity of DNA obtained from 6-TG- or 6-MP-treated *B. subtilis* is reduced significantly (Table 1). The transforming activity is expressed as a per cent of control preparations evaluated simultaneously. Doses of 6-TG ranging from 1 to 30 μM reduced

Table 1. Transforming activities of *B. subtilis* DNA obtained from drug-treated cultures

Drug treatment*	n	Relative transforming activity†
Control		100
6-TG, 1 μM	2	69 ± 1
6 μM	2	80 ± 8
30 μM	3	80 ± 10
6-MP, 1 μM	2	15 ± 4
10 μM	1	45
BUdR, 1 μM	1	56
6 μM	2	15 ± 11
10 μM	1	44
AraA, 5 μM	1	120
2,6-Diaminopurine, 30 μM	1	104

* Drugs were added at the beginning of the logarithmic growth phase and DNA was isolated 5 hr later for measurement of transforming activity, as described in Materials and Methods.

† Results shown are average values ± range for separate experiments.

Table 2. Characterization of the transforming activity associated with *B. subtilis* DNA

Pretreatment*	Relative transforming activity (%)
Control	100
Deoxyribonuclease I, 50μg/ml	0
Heat, 85°	0
Ribonuclease A, 50μg/ml	63
Trypsin, 50μg/ml	100

* Isolated DNA was incubated with the enzymes shown for 1 hr at 37° or heated for 10 min. Evaluation of transforming activity was determined immediately thereafter, as described in Materials and Methods.

Table 3. Growth inhibition of *B. subtilis* by purine and pyrimidine base analogs

Analog	Growth inhibition* (%) 10μM	100μM
6-MP	64	68
6-TG	20	21
AraA	23	16
BUdR	3	0
AraC	16	12
6-MPR	67	70

* Growth was measured as the increase in turbidity at 615 nm in a 4-hr incubation during logarithmic growth.

the transforming activity by only 20 per cent, whereas 6-MP reduced the activity by 80 per cent. Increasing the DNA concentration above 1μg/ml up to 3μg/ml provided a gradual increase in transformation frequency for control preparations. Conversely, 6-TG DNA preparations plateaued at 1μg/ml, and transformation frequency decreased somewhat at the higher DNA levels. BUdR-treated cells also yielded a DNA with less transforming activity. The amount of BUdR incorporated into the DNA was probably small, since liquid chromatographic and sucrose gradient profiles failed to reveal any marked physical alteration and no artificial thymidine starvation was created (data not shown). Single experiments with DNA isolated from cells treated with AraA and 2,6-diaminopurine showed no decrease in the transforming activity of the DNA. In the experiment shown, the amount of [^{3}H]arabinosyl adenine associated with the DNA was only 0.015 pmole/μg of DNA. In each of these experiments, there was no toxic effect associated with the DNA obtained from drug-treated cultures, i.e. determined by cloning DNA-treated *B. subtilis* T_3 in complete medium. Evidence that the transforming principle in these experiments was indeed DNA consists of the observations that deoxyribonuclease and heat destroyed the activity (Table 2). Trypsin and ribonuclease were not effective in destroying the activity. The slight effect of ribonuclease might be the result of contaminating deoxyribonuclease activity.

Several base analogs which are markedly cytotoxic to mammalian cells in culture inhibited the growth of *B. subtilis* (Table 3), but failed to reduce viability, as determined by cloning in drug-free medium (data not shown). Interestingly, 6-MP and 6-MPR were the most effective agents tested as inhibitors of growth, reducing

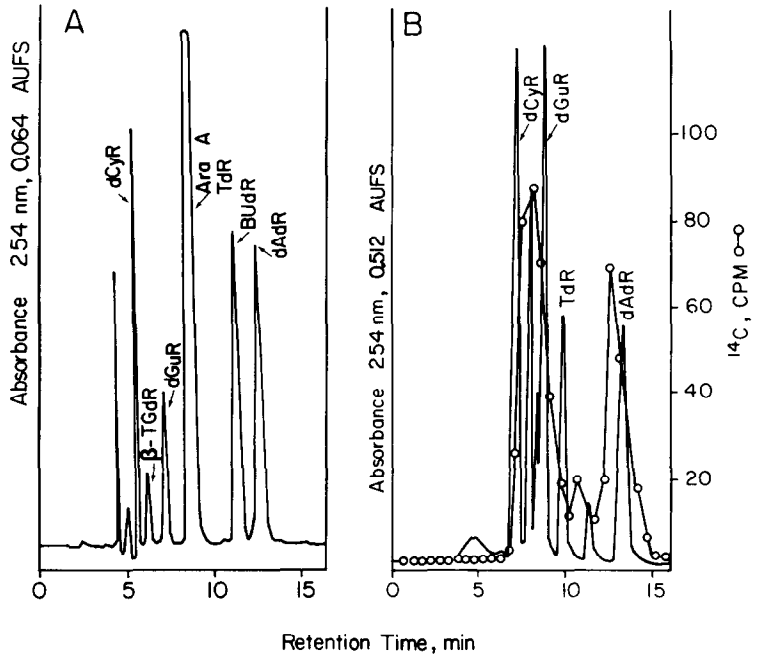

Fig. 1. High pressure liquid chromatography separation of deoxyribonucleosides. A mixture of standard deoxyribonucleosides (A) or deoxyribonucleosides obtained via hydrolysis of *B. subtilis* DNA (B) was separated using solvent system 1 (see Materials and Methods). The hydrolyzed DNA shown was obtained from a culture grown in the presence of 1.0μM [8-^{14}C]mercaptopurine. Radioactivity (○——○) was measured in the eluant collected at 0.4-min intervals.

Table 4. Purine and pyrimidine composition of *B. subtilis* DNA

Treatment*	dCyR	dGuR	TdR	dAdR	β-TGdR
	(% of total)†				
Control	36 ± 1	28 ± 1	18 ± 2	22 ± 7	
6-MP, 1 μM	31	28	18	21	0.1
10 μM	28 ± 3	25 ± 1	16 ± 1	22 ± 4	0.05
6-TG, 1 μM	37	29	19	15	0.1
BUdR, 1 μM	40	27	18	15	

* DNA was isolated from cells grown in the presence of the drug shown, as described in Materials and Methods.

† Deoxyribonucleosides obtained by enzymatic hydrolysis were measured by h.p.l.c. (see Materials and Methods). Values shown are means ± range for two separate determinations or single experiments.

the growth rate by 60–70 per cent in cells treated during logarithmic growth.

High pressure liquid chromatography was utilized in an effort to establish the extent of base analog incorporation (Fig. 1). Facile separation of the deoxyribonucleosides was obtained using a water–acetonitrile or a water–methanol system and a reversed-phase packing material. Part A of Fig. 1 illustrates the 15-min separation of deoxyribonucleoside standards. Part B of Fig. 1 is a chromatogram of the deoxyribonucleosides obtained after enzymatic hydrolysis of 20 μg of DNA from *B. subtilis* treated with [8-^{14}C]mercaptopurine. Collection of eluting solvent and the subsequent determination of radioactivity indicated desulfuration of the [8-^{14}C]mercaptopurine. Using this methodology, the limit of detection (340 nm) for β-TGdR is approximately 2.5 pmoles/μg of DNA. Since β-TGdR was not detected in any sample analyzed, we assume this level to represent the maximal amount possible, i.e. 0.05 per cent in Table 4. This corresponds to approximately 1 molecule of β-TGdR in every 500 guanine residues. Since β-TGdR was not detected, the radioactivity associated with DNA from [8-^{14}C]mercaptopurine-treated cultures is almost totally due to substances other than 6-MP or 6-TG. The amount of radioactive material associated with the DNA isolated from [^{35}S]thioguanine-treated cells was 1.5 and 15 pmoles/μg of DNA in two separate experiments. Again, β-TGdR was not detected by u.v. in hydrolysates of the DNA in experiments similar to that shown for 6-MP. That β-TGdR is stable through the enzymatic treatment of the DNA was determined by supplementing a DNA preparation with a known amount of β-TGdR. This procedure also confirmed the 340 nm sensitivity for the compound.

Table 5. Melting temperatures of DNA obtained from *B. subtilis* grown in the presence of base analogs

Source of DNA	T_m*
Control	58 ± 1
6-TG, 1 μM	59 ± 1
6-MP, 10 μM	58 ± 2
BUdR, 10 μM	60 ± 1
AraA, 5 μM	59 ± 1
Calf thymus	50 ± 2

* Data represent the average of two experiments for each DNA preparation.

The melting temperature of the DNA from drug-treated cultures was not altered, i.e. each DNA melted within ±1° of that observed with control DNA (Table 5). This suggests that the hydrogen bonding, hyrophobic characteristics and the base composition of the DNA have not been greatly altered from exposure to these base analogs.

DISCUSSION

Numerous experiments have been performed which suggest that incorporation of 6-TG into DNA is a mechanism of cytotoxicity in mammalian cells (see review, Ref. 1). If true, 6-MP may be effective by the same mechanism, since it is incorporated into DNA after conversion to 6-TG nucleotides [26]. A problem in studying the function of a DNA containing 6-TG is that the agent is highly cytotoxic, an effect which is often difficult to dissociate from other drug effects. For this reason, the *B. subtilis* transformation system was selected in an attempt to discern the effect of base analog substitution on a biological effect of DNA. The reduced transforming activity (Table 1) of DNA from 6-TG- and 6-MP-treated *B. subtilis* is the first direct evidence which demonstrates that a function of DNA is altered by these agents. Several studies have been reported which indicate changes in the function of synthetic polymers [13, 14] or which give theoretical reasons why 6-TG would alter DNA function [11, 12].

The fact that 6-MP and 6-TG inhibited the growth of *B. subtilis* but were not cytotoxic is consistent with the studies of Mandel *et al.* [27] with *Bacillus cereus*. Recovery from toxicity may occur due to desulfuration of the drug. The differences in effects on growth (Table 3) and DNA transforming activity (Table 1) between 6-TG and 6-MP are of interest. The difference does not appear to be a result of different affinities or activities of activating enzymes, since *B. subtilis* hypoxanthine and guanine phosphoribosyltransferase activities using 6-MP and 6-TG as substrates yielded identical K_m and V_{max} values (data not shown). Thus, the reason for the greater bacteriostatic and reduced DNA transforming activity after 6-MP treatment remains obscure.

The literature is not in total agreement on the activity of BUdR in the *B. subtilis* transformation system. One report by Szybalski *et al.* [28] in 1960 indicated that substituting *B. subtilis* strain SB23 with up to 50% BUdR did not alter the transformation frequencies of several mutants. Yet, in 1966, Gimlin *et al.* [29] reported that BUdR-DNA resulted in a lethal transforma-

tion. With *B. subtilis* UTH-8505, a strain which is stable to hydroxylamine treatment for 30 min [18], a decrease in biological activity (presumably in the tryptophan synthetase gene) was noted (Table 1).

The reduced transforming activity of the DNA from cells treated with 6-TG or 6-MP (Table 1) could be due to: (1) differences in the ability of the recipient strain to incorporate the DNA; (2) inability of RNA polymerases to utilize the base analog DNA; (3) degradation of the base analog DNA, thereby releasing the drug into the cell; (4) a mutagenic event such as mispairing of bases during transcription; or (5) effects other than base analog substitution. We were unable to measure the amount of DNA taken up by a population of cells quantitatively to assess any differences in transport between DNA from 6-TG-treated cells and control DNA. In a single experiment, the reduced transformation frequency observed with a 6-TG DNA was not increased when 3 μg/ml rather than 1 μg/ml of DNA was used in the transformation procedure. Should the 6-TG DNA be rapidly degraded after uptake, the amount of drug liberated would be so small that the decrease in transformation frequency could not be attributed to reduced viability. Mouse RNA polymerase B is unable to transcribe a synthetic polymer containing deoxythioGMP [14], a finding which suggests that base analog substitution may alter transcription.

Barranco and Humphrey [30] have reported that Chinese hamster ovary cells are most sensitive to β-TGdR during early and mid-S phase, and Tidd and Paterson [31, 32] have described the "delayed" cytotoxic effect of 6-TG and 6-MP in L5178Y cells. These observations are consistent with the hypothesis that 6-TG incorporation into DNA results in a lethal mutation during subsequent replications. 6-MP has been shown to be mutagenic in several *in vitro* assays (i.e. Ref. 33) and is active in dominant lethal tests in mice [34]. However, since our methodology did not permit unequivocal measurement of 6-TG in internucleotide linkage, the reduced transforming activity of the DNA from 6-MP- and 6-TG-treated cells may be a result of effects other than incorporation. 6-ThioIMP and other ribonucleoside 5′-monophosphates have been shown recently to inhibit the endonuclease associated with *Escherichia coli* DNA polymerase [35]; thus, the analog nucleotide may alter the normal repair mechanisms or by some other means increase the frequency of lethal mutations. In spite of these difficulties, the current study demonstrates that an altered biological activity of a DNA from 6-MP- and 6-TG-treated cells occurs, providing further evidence for an effect of these agents on DNA function.

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