Development of novel inhibitor probes of DNA polymerase III based on dGTP analogs of the HPUra type: base, nucleoside and nucleotide derivatives of N^2-(3,4-dichlorobenzyl)guanine

Michelle M.Butler, Lech W.Dudycz, Naseema N.Khan, George E.Wright and Neal C.Brown*
Department of Pharmacology, University of Massachusetts Medical School, Worcester, MA 01655, USA

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ABSTRACT

6-(*p*-Hydroxyphenylhydrazino)uracil (H_2-HPUra) is a selective and potent inhibitor of the replication-specific class III DNA polymerase (pol III) of Gr$^+$ bacteria. Although formally a pyrimidine, H_2.HPUra derives its inhibitory activity from its specific capacity to mimic the purine nucleotide, dGTP. We describe the successful conversion of the H_2-HPUra inhibitor prototype to a *bona fide* purine, using N^2-(benzyl)guanine (BG) as the basis. Structure-activity relationships of BGs carrying a variety of substituents on the aryl ring identified N^2-(3,4-dichlorobenzyl)guanine (DCBG) as a nucleus equivalent to H_2-HPUra with respect to potency and inhibitor mechanism. DCBdGTP, the 2′-deoxyribonucleoside 5′-triphosphate form of DCBG, was synthesized and characterized with respect to its action on wild-type and mutant forms of *B. subtilis* DNA pol III. DCBdGTP acted on pol III by the characteristic inhibitor mechanism and formally occupied the dNTP binding site with a fit which permitted its polymerization.

INTRODUCTION

6-(*p*-Hydroxyphenylhydrazino)uracil (H_2-HPUra; ref 1), 6-(phenylhydrazino)uracil (PUra; ref 2), 6-(benzylamino)uracil and 6-(3,4-trimethyleneanilino)uracil (BAU, TMAU; ref 3) are members of a family of 6-substituted pyrimidines (cf., Fig. 1A) which share a unique biologic property; they are novel dGTP analogs which selectively inhibit the replication-specific DNA polymerase III (pol III) of *B. subtilis* and other Gr$^+$ bacteria (4–6). The mechanism of inhibitory action common to this family of bases (5,6) is summarized schematically in panels B-D of Fig. 1. As shown in panel B, the inhibitor molecule consists of two essential domains; a base pairing domain equivalent to that of guanine, and an aryl domain from which it derives reactivity and selectivity for its enzyme target. Panel C displays the structure of the H-bonded, H_2-HPUra:cytosine base pair, and panel D summarizes how the base pairing and aryl domains of the molecule cooperate to effect inhibition. The inhibitor acts as a bridge between the template:primer and the polymerase, sequestering the enzyme into a reversible ternary complex. The base pairing domain forms one bridgehead with an unapposed template cytosine immediately distal to the 3′-OH terminus, while the aryl substituent forms the other through its reaction with the polymerase in the vicinity of its dNTP binding site.

We seek to characterize in *B. subtilis* DNA pol III the location and molecular structure of its inhibitor binding site and the precise relationship of the latter to the site of dNTP binding and polymerization. Our strategy has required the reconfiguration of the inhibitor prototype into a *bona fide* guanine (G) format to provide a structure useful for mutant enzyme selection and suitable for development of 2′-deoxyribonucleotidyl forms. Our first attempt to adopt the G format involved the anilino prototype and resulted in the conversion of TMAU to the N^2-(aryl)G derivative, TMPG (cf., Fig. 1,E). TMPG displayed the appropriate mechanism and requisite enzyme selectivity. However, TMPG was considerably less potent than TMAU and penetrated cells very poorly, and therefore, it was not suitable for further development. As an alternative to the N^2-(aryl)-guanine structure, we exploited the N^2-(arylalkyl)guanine format to produce a series of N^2-(benzyl)guanine (BG) derivatives. As indicated in the results presented below, the N^2-benzyl format proved to be a fruitful basis for inhibitor development, yielding the novel base, N^2-(3,4-dichlorobenzyl)guanine (DCBG), and a derivative, 2′-deoxyribonucleoside 5′-triphosphate form, DCBdGTP, which can be polymerized by its pol III target.

MATERIALS AND METHODS

Chemicals, reagents and bacterial strains

Radioactive materials were purchased from New England Nuclear. Nucleotides and synthetic polynucleotides were obtained from P-L Laboratories. Calf thymus DNA was from Worthington. All chemicals, solvents and reagents used in

* To whom correspondence should be addressed

syntheses were of analytical grade or better. *B. subtilis* strain NB841 (7), which was used as the source of pol III, was grown on minimal salts agar supplemented as described (8).

Chemical analyses

Melting points were determined on a Mel-temp apparatus. Elemental analyses were performed by the Microanalysis Laboratory, University of Massachusetts, Amherst. Phosphorus analyses were performed as described by Peterson (9). UV spectra were obtained with a Gilford Response UV-Vis Spectrophotometer. NMR spectra were obtained at 200 MHz with a Bruker AC-P200 instrument; chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (^{1}H) or external phosphoric acid (^{31}P). Only characteristic chemical shifts (ppm) and coupling constants (Hz) are given. The identity of the nucleosides as 9-β-(2-deoxyribofuranosyl) derivatives was based on the similarity of ^{1}H NMR spectra of sugar ring protons to those of deoxyguanosine and others (10).

Syntheses

N^2-(3,4-Dichlorobenzyl)guanine (DCBG). Reaction of 2-bromohypoxanthine and 3,4-dichlorobenzylamine as described (10) gave, after crystallization from methanol, DCBG, in 83% yield, m.p. 282–285°C. ^{1}H NMR (Me_2SO-δ_6) δ 12.42 (1H, s, 9-H), 10.69 (1H, s, 1-H), 7.65 (1H, s, 8-H), 7.32–7.56 (3H, m, Ph), 6.82 (1H, t, 2-NH), 4.49 (2H, d, CH_2). *Anal.* Found: C, 46.31; H, 3.09; N, 22.36. $C_{12}H_9N_5OCl_2$ requires C, 46.47; H, 2.93; N, 22.58.

Synthesis of DCBdG, the nucleoside form of DCBG, was based upon the procedure described in reference 11; the approach to the synthesis of the nucleoside mono and triphosphates was based on that described by Wright and Dudycz (10).

Step 1: 2-(3,4-Dichlorobenzylamino)-6-chloropurine. Treatment of DCBG with phosphoryl chloride and crystallization from methanol yielded 84% of product, m.p. 196–198°C. ^{1}H NMR (Me_2SO-d_6) δ 8.12 (1H, s, 8-H). *Anal.* Found: C, 43.95; H, 2.62; N, 21.09. $C_{12}H_8N_5Cl_3$ requires C, 43.86; H, 2.45; N, 21.31.

Step 2:2-(3,4-Dichlorobenzylamino)-6-chloro-9-(2-deoxy-β-D-ribofuranosyl)purine. Treatment of the sodium salt of the step 1 product with 2-deoxy-3,5-di-p-toluyl-β-D-ribofuranosyl chloride in acetonitrile gave two major products which were separated by silica gel chromatography. The major desired product was deblocked by treatment with sodium methoxide in

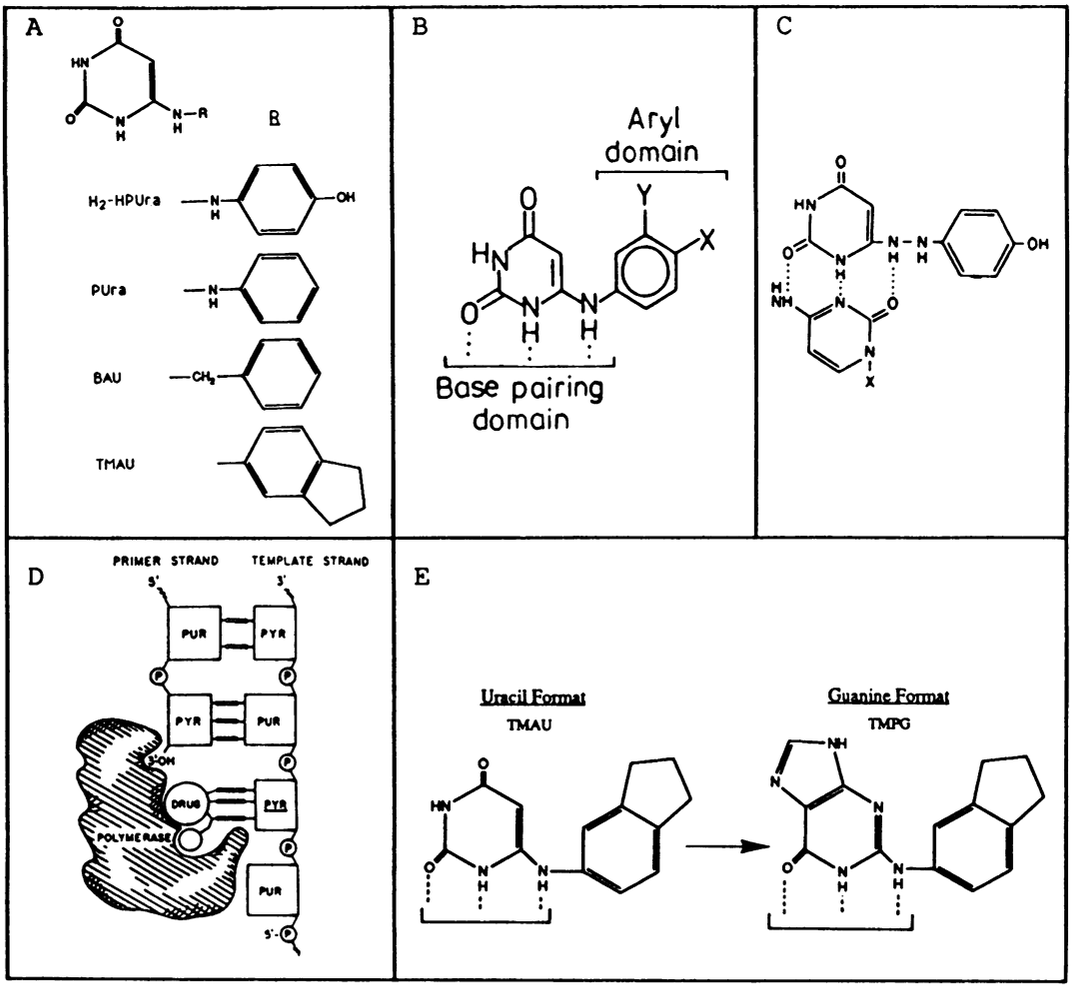

Figure 1. (A) Structures of relevant 6-substituted uracils. (B) A typical 6-anilinouracil showing relevant base-pairing and aryl domains. (C) Base-pairing of H_2-HPUra and template cytosine. (D) Inhibitor-induced sequestration of enzyme and DNA into a reversible ternary complex. (E) Conversion of the 6-anilinouracil format to the corresponding guanine format (regions involved in base-pairing are bracketed).

methanol. The product was crystallized from methanol and isolated in 56% yield, m.p. 148−150°C. ^{1}H NMR (Me_2SO-d_6) δ 8.28 (1H, s, 8-H), 6.23 (1H, t, J_{av} = 6.9 Hz, 1′-H), all other resonances as expected. *Anal.* Found: C, 45.61; H, 3.62; N, 15.47. $C_{17}H_{16}N_5O_3Cl_3$ requires C, 45.91; H, 3.63; N, 15.75.

Step 3: N^2-(3,4-Dichlorobenzyl)-9-(2-deoxy-β-D-ribofuranosyl)-guanine (DCBdG). The step 2 product was converted to DCBdG by treatment with 2-mercaptoethanol in a solution of sodium methoxide in methanol at reflux for 26h. Crystallization from ethanol and then ethyl acetate gave 73% of DCBdG with m.p. 212−214°C. ^{1}H NMR (Me_2SO-d_6) δ 11.19 (1H, s, 1-H), 7.83 (1H, s, 8-H), 7.32−7.61 (3H, m, Ph), 7.46 (1H, t, 2-NH), 6.13 (1H, t, J_{av} = 6.9 Hz, 1′-H), 5.27 (1H, d, 3′-OH), 4.87 (1H, t, 5′-OH), 4.50 (2H, d, CH_2), 4.33 (1H, m, 3′-H), 3.81 (1H, q, 4′-H), 3.57 (1H, m, 5′-H), 3.47 (1H, m, 5″-H), 2.55 (1H, m, 2′-H), 2.18 (1H, m, 2″-H). *Anal.* Found: C, 46.02; H, 3.93; N, 15.54. $C_{17}H_{17}N_5O_4Cl_2 \cdot H_2O$ requires C, 45.96; H, 4.31; N, 15.76.

N^2-(3,4-Dichlorobenzyl)-2′-deoxyguanosine 5′-phosphate (DCBdGMP). A solution of DCBdG and phosphoryl chloride in trimethyl phosphate was kept at 0−3°C for 4h during which more phosphoryl chloride was added. After chromatography on DEAE-Sephadex and elution with a linear gradient of 0.2−1.0M triethylammonium bicarbonate, pH 7.7, the product was converted to the sodium salt by dissolving in methanol and treatment with 0.5M sodium perchlorate solution. After centrifugation and extensive washing with acetone, the resulting pellet was dissolved in water and lyophilized. Recovery of DCBdGMP after conversion to the sodium salt was 55%. ^{31}P NMR (D_2O) δ 2.62 (s). *Anal.* Found: P, 5.86. $C_{17}H_{16}N_5O_7Cl_2PNa$ requires P, 5.87.

N^2-(3,4-Dichlorobenzyl)-2′-deoxyguanosine 5′-triphosphate (DCBdGTP). A solution of DCBdGMP as the triethylammonium salt in hexamethylphosphoramide, after activation by 1, 1′-carbonyldiimidazole, was treated with tributylammonium pyrophosphate. Chromatography of the diluted solution on DEAE-Sephadex and elution with a linear gradient of 0.2−1.0M triethylammonium bicarbonate, pH 7.7 gave the product, which was converted to its sodium salt by the same procedure used for DCBdGMP. The product was lyophilized to give 59% of DCBdGTP. ^{31}P NMR (D_2O) δ −6.56 (1P, d, γ-P), −10.85 (1P, d, α-P), −21.66 (1P, t, β-P). *Anal.* Found: P, 11.75. $C_{17}H_{16}N_5O_{13}Cl_2P_3Na_4$ requires P, 11.38.

Purification and assay of *B. subtilis* pol III

Purification of wild-type and mutant (azp12) pol III was performed as described previously (12); unless specified otherwise, the DNA cellulose fraction V was used in all experiments. The standard assay for DNA polymerase III was performed in a final volume of 25mL as described (7). Apparent inhibitor constants (K_i) for each inhibitor were determined in the appropriate polymerase assay conditions either conventionally or by the truncated, dGTP-deficient assay method described by Wright and Brown (13). Poly(dA):oligo(dT) and poly(dC):oligo(dG) were prepared by incubating at 50°C a mixture of 4 OD units (260nm) of polymer and 0.625 OD units of oligomer in 10mM Tris-Cl (pH 7.6) for 10min and allowing the mixture to cool to 25°C over an additional 10 min period; 0.1μL of this mixture was used in a final assay volume of 0.025ml. The labeled triphosphate (20μM, 875 cpm/pmole) was the only nucleotide used in experiments involving these homopolymeric templates.

Assay of nucleic acid and protein synthesis in intact *B. subtilis*

At time zero, [^{3}H] adenine (7×10^5 cpm/pmole, 0.1mM; for DNA and RNA) or [^{3}H] L-leucine (1.4×10^5cpm/pmole, 10^5 cpm/ml; for protein) and DCBG (75μM), HPUra (75μM) or DMSO (inhibitor diluent) were added to log phase cultures of *B. subtilis* growing at 37°C in minimal salts medium (14), and periodically, duplicate samples (1ml each) were removed to determine incorporation of radioactivity into alkali-soluble (RNA) and alkali-stable (DNA) fractions of cold TCA-insoluble material or into hot TCA-insoluble material (protein) as described elsewhere (14).

Analysis of primer extension

The method involved the autoradiographic analysis of denaturing DNA sequencing gel electropherograms of products resulting from the extension of a 5′ [^{32}P] labeled 17 residue oligonucleotide primer annealed to a 29 residue template. The structure of the primer (M13 sequencing primer #1211; Boehringer Mannheim) and template (Operon) is shown in the inset of Figure 3. The preparation of the sequencing gel (12% polyacrylamide), 5′-labeling of the primer with polynucleotide kinase, primer:template annealing, sample preparation, and the conditions for gel development and autoradiography, were performed as described in reference 15. To assay for extension 0.25 units of homogeneous pol III (Fr. VIIa, ref. 12) were incubated in 10μL of standard assay buffer (7) in the presence of [^{32}P]-primer:template in slight excess over enzyme, and in the presence or absence of selected dNTPs. After 5 min at 30°C, incubation mixtures were quenched and analyzed (15).

RESULTS

Properties of DCBG, the model benzylguanine

Following leads developed with 6-benzylaminouracil derivatives as pol III inhibitors (3), we synthesized several analogous N^2-benzylguanines substituted in the 3, 4 and 5 positions of the benzyl moiety. Of these only DCBG displayed inhibitory potency similar to those of H_2-HPUra and related compounds. The properties of DCBG are summarized in the following three subsections.

(i) Action of DCBG on the isolated polymerase is characteristic of the H_2-HPUra prototype. Our strategy for pol III inhibitor development required that in its simplest base form, the product: (a) be at least as potent as the corresponding uracils, which typically display K_is in the 0.5−2μM range; (b) be specifically competitive with dGTP like the corresponding 6-(benzylamino) and 6-(arylhydrazino) uracils; and (c) be dependent on the presence of a cytosine-containing template. The results of three sets of experiments, which are not explicitly displayed in tabulated form, indicated that DCBG fulfilled all these requirements. First, DCBG was a characteristically potent inhibitor of wild-type (wt) pol III, displaying a K_i of approximately 0.5μM. Second, its inhibition was specifically and competitively reversed by dGTP; high concentrations of dATP, dCTP, or dTTP had no effect on its inhibitory capacity. Third, when the action of DCBG on pol III was assessed, using either poly(dA):oligo(dT) or poly(dC):oligo(dG) as template:primer, it, like H_2-HPUra (16,17), inhibited only the latter reaction.

(ii) Action on intact cells: specificity for replicative DNA synthesis. DCBG, like HPUra, the oxidized azo form of H_2-HPUra which is reductively activated *in vivo*, penetrated intact *B. subtilis* and inhibited its growth on plates. In liquid medium, microscopic exam showed that 75μM DCBG arrested cell division and effected a segmented morphology typical of HPUra-treated cells which are specifically unable to synthesize DNA (14). Figure 2 summarizes the effect of DCBG on macromolecule synthesis in log-phase *B. subtilis*, exploiting analysis based on incorporation of labeled adenine into RNA and DNA, and labeled leucine into protein. The results clearly indicated that DCBG, like HPUra (1,14), inhibits only DNA synthesis and displays no significant effect on the synthesis of RNA and protein.

(iii) Utility of DCBG as an agent for selecting and identifying bacterial clones carrying mutations affecting the drug binding site of pol III. Much of the value of the H_2-HPUra inhibitor prototype as a genetic and structural probe of the replication-specific DNA pol III is derived from its 'magic bullet' capacity to clearly and specifically select, on drug-containing nutrient plates, bacterial clones which carry mutationally altered, drug-resistant forms of pol III. *B. subtilis* azp-12, a mutant displaying both an HPUra-resistant growth phenotype and an H_2-HPUra-resistant pol III (17) exemplifies this strong correspondence of *in vitro* enzyme 'phenotype' and the growth-response phenotype of its host. As shown in Table I, comparison of the action of DCBG and H_2-HPUra on the wt and azp-12-specific systems indicated that DCBG also can consistently discriminate between wt and mutant forms of pol III, both *in vivo* and *in vitro*. Table I compares the potency of H_2-HPUra and DCBG as inhibitors of the wt and azp-12-specific enzymes and their potency as inhibitors of growth of the respective wt and mutant host bacteria. The top row of data, displaying K_is, indicated that the azp-12 enzyme, relative to wt enzyme, shows approximately 55-fold *resistance* to H_2-HPUra and, interestingly, 5-fold higher *sensitivity* to DCBG. The second row of data display the same trend *in vivo*; the growth of the azp-12 mutant host, relative to that of the wt host, is 8 times more resistant to HPUra and 8 times more sensitive to DCBG.

Properties of DCBdGTP action on its pol III target

Given the promising behavior of DCBG described above, we asked whether the base would retain its H_2-HPUra-like action once converted to the dNTP form. The properties we sought in the dNTP form were: (a) a potency at least equivalent to that of the simple base form, DCBG; (b) a mechanism consistent with that of the base, and (c) a structurally 'complete' inhibitor form that will formally occupy the pol III dNTP binding site in the manner of the dNTP it mimics. The results of experiments described in subsections (i)-(iv) below summarize the properties we found.

(i) Potency and discrimination of wt and mutant pol IIIs. Table II summarizes the effects of the 2'-deoxyribonucleoside of DCBG and its 5'-mono and 5'-triphosphates on the wt and azp-12-specific enzymes. The addition of the deoxyribosyl moiety to DCBG and, in turn, the monophosphorylation of the nucleoside, decreased the potency of the inhibitors for the wt polymerase and, interestingly, had little effect on their affinity for the mutant enzyme. Addition of the $\beta-\gamma$ phosphates to produce the ultimate dNTP analog, DCBdGTP, reversed the trend to reduction of potency for the wt polymerase, yielding a K_i five times lower than that of the simple base. Similarly, DCBdGTP was four times more potent as an inhibitor of the azp-12-specific enzyme than of the wt enzyme.

(ii) The template cytosine requirement for DCBdGTP-induced inhibition is not absolute. Competition experiments, identical to those performed with DCBG, indicated that the inhibitory action of DCBdGTP, in the presence of activated natural (calf thymus) DNA, was competitively and specifically antagonized by dGTP; dATP, dCTP, and dTTP at high concentrations in identical assay conditions had no effect on its potency. Given the latter observation, we exploited the homopolymeric template:primers, poly(dA):oligo(dT) and poly(dC):oligo(dG) to determine whether the action of the dNTP form, like that of the parent base, DCBG, absolutely required template cytosine to effect inhibition. The results of the homopolymer experiments are summarized in Table III. DCBG, like H_2-HPUra (16,17), displayed no significant inhibition in the absence of template cytosine. In contrast,

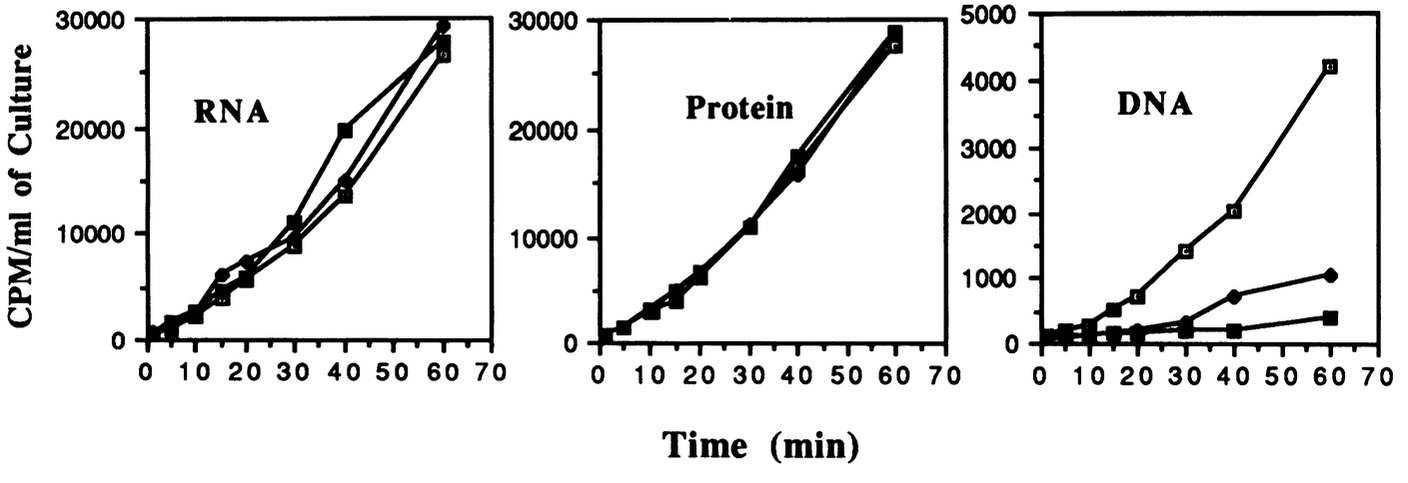

Figure 2. RNA, protein and DNA synthesis in the presence of DCBG or HPUra. Cultures of *B. subtilis* NB841 were grown and incorporation of [^{3}H] adenine into RNA and DNA and [^{3}H] leucine into protein was followed as described in the methods section. Inhibitors or control diluent were added at time zero; ⊡, control; ◆, 75μM DCBG; ■, 75μM HPUra.

DCBdGTP, although considerably more potent in the poly(dC)-driven reaction (IC_{50} = 7μM), displayed significant activity (IC_{50} = 200μM) in the poly(dA)-directed reaction. Given the superior structural potential of DCBdGTP-relative to that of DCBG-for template independent binding to the dNTP binding site of pol III, we hypothesize that DCBdGTP-induced inhibition of the poly(dA)-driven reaction occured simply by direct competition for the dNTP binding site with dTTP, the substrate being polymerized. The results of competition experiments employing the (dA):(dT) reaction and the four dNTPs were consistent with this hypothesis; only dTTP was competitive with DCBdGTP action in this system (results are not shown).

(iii) DCBdGTP-induced inhibition in the presence of template cytosine apparently involves a sequestration mechanism. To assess sequestration we exploited the 'scavenging' method employed to ascertain the induction of DNA:enzyme complex formation by H_2-HPUra (16,17). The specific experimental approach exploited poly(dA):oligo(dT)-driven incorporation of dTMP and simply asked whether enzyme, in the presence of inhibitor at a concentration causing minimal inhibition, can be sequestered by the addition of a small amount of a dC-containing template:primer-an amount too small *per se* to interfere with dTTP incorporation into the oligo(dT). The results of relevant experiments comparing DCBG and DCBdGTP are summarized in Table IV. Both inhibitors were used at 100μM; at this concentration DCBG and DCBdGTP, as expected, inhibited dTMP incorporation by, respectively, 0 and 40% (cf., top row Table IV). The addition of cytosine-containing activated calf thymus DNA at low concentration neither supported significant dTMP incorporation (cf., second row of data) nor drastically reduced incorporation of dTMP into poly(dA):oligo(dT) (cf., third row) in the absence of inhibitor; however, in its presence the effect was dramatic. With DCBG present, dTMP incorporation was inhibited by approximately 90%, and in the presence of DCBdGTP, inhibition of dTMP incorporation was increased from 43 to approximately 87%. To determine if this drug-induced, DNA-dependent inhibition was, as expected, sensitive to the presence of a high concentration of dGTP (0.5mM), we examined its effect and, as a control, that of dATP and dCTP. Only dGTP was effective as a competitor; it reduced inhibition induced by DCBG from 90 to 25% and that induced by DCBdGTP from 87 to 30% (cf. bottom row Table IV). In sum, these results strongly suggested that both DCBG and DCBdGTP, when provided with template cytosine in an appropriate structural context, act by a sequestration-specific mechanism typically displayed by the pyrimidine-based inhibitors.

(iv) DCBdGTP can serve as a substrate for its target enzyme. Unlike the parent base, DCBdGTP had the potential for polymerization by pol III. To investigate this potential, we exploited sequencing gel analysis of primer extension, using the template:primer depicted in the inset of Figure 3. Experimentally, we sought to determine if DCBdGTP, like dGTP, could be utilized to extend the primer by one nucleotidyl residue. The results are summarized in the gel autoradiogram in the lower part of Figure 3.

There was, as expected, no primer extension in the absence of dNTP (lane B)-only partial degradation resulting from the activity of the enzyme's resident 3'-5' exonuclease (18) which could be partially inhibited by the addition of 2 mM AMP (lane C). The 17mer was extended to its full, 29mer length when all 4 dNTPs were present (lane H), and, as expected, was extended by one residue when dGTP was present as the sole dNTP substrate (lane D). The presence of DCBdGTP as the sole dNTP, at concentrations ranging from 1 to 100 times its K_i (lanes E, F and G) resulted in the formation of a product which migrated at the position expected for that of the primer extended by one DCBdGMP residue (note: The R_f of primer extended by one DCBdGMP residue is, in the conditions of electrophoresis,

Table I. *In vitro* vs *in vivo* effects of DCBG and H_2-HPUra

	wild type system		azp-12 system	
	H_2-HPUra	DCBG	H_2-HPUra	DCBG
Isolated pol III K_i (μM)[a]	0.4±.08	0.5±.1	22±4	0.1±.02
Bacterial growth CFU_{50} (μM)[b, c]	2.5	40	20	5

[a] K_i was determined as described in Materials and Methods. [b] CFU_{50} is the concentration of inhibitor in minimal salts agar plates required to reduce the plating efficiency of *B. subtilis* to 50% of control values. [c] HPUra, the oxidized drug form, was used in the plating experiments.

Table II. Potency of the base and higher forms of DCBG

Compound	Form	K_i (μM)† wt pol III	K_i (μM)† azp12 pol III
DCBG	Base	0.5±.1	0.1±.02
DCBdG	2'-deoxyribonucleoside	2.0±.3	0.1±.03
DCBdGMP	dNMP	2.0±.1	0.2±.005
DCBdGTP	dNTP	0.1±.02	0.025±.005

†K_i was determined as described in Materials and Methods, using activated calf thymus DNA as template:primer.

Table III. Template dependence of inhibitor action

	IC_{50} (μM)[a,b] DCBG	DCBdGTP
[α-^{32}P] dGTP + poly(dC):oligo(dG)[c]	250	7
[^{3}H] dTTP + poly(dA):oligo(dT)[c]	>1000	200

[a] IC_{50} is the concentration of inhibitor required to reduce product formation by 50% of control values. [b] Wild type pol III. [c] Polymer:oligomer assay conditions are described in the methods section.

Table IV. Demonstration of sequestration

Template:Primer	[^{3}H]dTMP Incorporation (pmole) Control	DCBG(100μM)	DCBdGTP(100μM)
poly(dA):oligo(dT)[a]	5.7	5.6	3.4
No homopolymer Activated calf thymus DNA[b] alone	<0.2	<0.2	<0.1
poly(dA):oligo(dT) + Activated calf thymus DNA	4.6	0.5	0.6
poly(dA):oligo(dT) + Activated calf thymus DNA + dGTP (0.5mM)	3.4	2.6	2.3

[a] Poly(dA):oligo(dT) experiments were performed with wild type enzyme as described in Materials and Methods. [b]Activated calf thymus DNA was used at a concentration of 9μg/ml (1:45 dilution of concentrations used in normal assays).

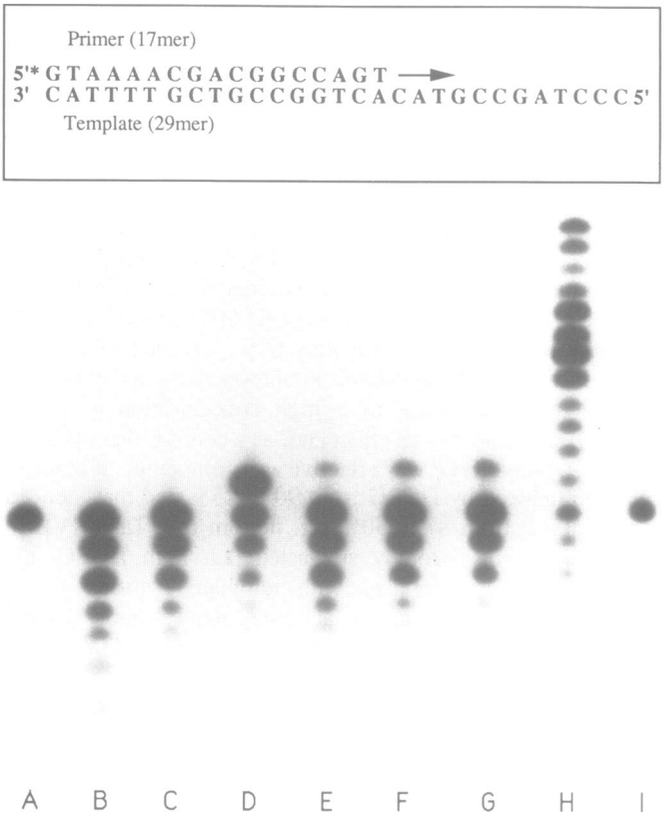

Figure 3. Analysis of the substrate potential of DCBdGTP. *Inset:* structure of template:primer. *Lower panel:* Autoradiograph of sequencing gel electropherogram of the products resulting from pol III action on the template:primer. The details of the procedures are described in the methods section. *Lanes:* A, I, Primer:template (P/T) controls; B, P/T + pol III; C, P/T + pol III + 2mM AMP (to partially inhibit 3′-5′ exonuclease). Lanes D–H contain AMP; D, P/T + pol III + 25 μM dGTP; E, F, G: P/T + pol III + DCBdGTP at 0.1, 1.0 and 10 μM, respectively; H, P/T + pol III + all 4 dNTPs at 25 μM each.

slightly, but consistently, smaller ($\sim$0.96) than that of the primer extended by one dGMP residue). Although clearly functional as a polymerizable substrate, DCBdGTP was less effective than dGTP; comparative densitometry of the N + 1 products of primer extension by 25μM dGTP (lane D) and 10μM DCBdGTP (lane G) indicated that in these conditions the rate of DCBdGMP incorporation was approximately 5–10% that of dGMP.

DISCUSSION

The benzylguanine format provides structural versatility without disruption of the characteristic inhibitor mechanism

The goal of this work was to expand the utility of the H_2-HPUra inhibitor class as pol III-specific genetic and structural probes. Specifically, we sought to develop a rationally structured series of inhibitor forms of increasing complexity, ranging from the simple base, which can specifically access and sense wild-type and mutant forms of pol III in the intact host, to the 2′-deoxyribonucleosidyl and nucleotidyl forms, whose structure can be manipulated appropriately to probe the structure and function of the dNTP and inhibitor binding site of isolated enzyme.

The N^2-benzylguanine format, represented specifically by DCBG, provides an excellent basis for the inhibitor series we seek. The properties of the simplest, base form are essentially identical to those of H_2-HPUra. DCBG penetrates cells effectively (cf., Table I), and chemically it is clearly adaptable to 9-β-D-nucleosidation and nucleotidation without loss of its essential H_2-HPUra 'character', including the ability to distinguish wild-type and mutant forms of pol III (cf., Tables I and II).

Significance of the polymerization of DCBdGTP

Although DCBdGTP is less effective as a pol III substrate than dGTP, it, nevertheless, participates successfully in the catalysis of polymerization (cf., Fig. 3). The polymerization of DCBdGTP indicates that it formally occupies the enzyme's dNTP binding site with a 'fit' approximating that of the dNTP it mimics. This fit, when considered in the context of the characteristic, base (HPUra/DCBG)-like behavior of DCBdGTP, permits several important conclusions regarding the structure of the enzyme:inhibitor:DNA complex and specific features of the enzyme's dNTP binding domain on which complex formation depends.

First, one may firmly conclude that the geometry of the base pair formed between the template cytosine and the N^2-benzylguanine substituent closely approximates that of the G:C pair formed in the process of dGTP polymerization. Second, one may conclude that the N^2-benzyl substituent does not formally occupy sites or space critical to the catalysis of dNTP polymerization; these include: (a) the substituents involved in the binding of the deoxyribosyl ring and its 5′ phosphates; (b) the space about the 3′OH of the primer terminus, and (c) the path between this OH group and the a phosphate of the incoming dNTP. Finally, as a corollary to the above conclusions, one also may envision more precisely the location of the major site of inhibitor:enzyme interaction-the so-called aryl binding site (6). Specifically, one may conclude that this site lies on the 'edge' of the dNTP binding domain in space immediately surrounding the unpaired H constituent of the exocyclic 2-amino group of an appropriately base-paired dGTP molecule.

Potential of DCBG derivatives as probes for *in vitro* analysis of the structure of pol III and its dNTP binding site

The four major forms, DCBG, DCBdG, DCBdGMP, and DCBdGTP, provide a rich variety of options for fitting the inhibitor with useful 'reporter' groups, in particular groups capable of forming covalent bonds with the inhibitor/dNTP binding site. Among the positions most amenable to substitution are the carbons of the N^2-aryl ring, the 7, 8, and 9 positions of the base and, in the nucleoside and nucleotide forms, the 2′, 3′, and 5′ carbons and the 5′-phosphoryl groups. Application of such derivatives in combination with the manipulation and *in vitro* expression of the pol III structural gene (19–21) should provide a facile means to map and directly identify the side chains of amino acids comprising the dNTP/inhibitor binding sites. Even in the absence of covalently reactive substituents, DCBG derivatives can be useful as probes of the dNTP binding site of pol III. For example, DCBdGMP or non-polymerizable dNTP forms thereof (i.e. α thio or β,γ-methylene) have considerable potential as reagents that can be exploited to promote the crystallization, and in turn, the 3-D structural analysis of a pre-polymerization DNA:enzyme:deoxyribonucleotide complex.

The availability of DCBG and structural variants increases the utility of the HPUra inhibitor class as genetic *in vivo* probes of pol III structure

A rationally designed series of structural variants of a mechanistically 'pure' inhibitor class can serve as a powerful set of tools with which to probe the structure of inhibitor-specific

binding sites within a relevant target protein. The utility of multiple inhibitor forms becomes even greater when their use can be combined with manipulation of the cloned gene encoding the target protein and the selection of gene mutants displaying appropriate resistance/sensitivity phenotypes for the relevant inhibitor variants. *B. subtilis* wt and *B. subtilis* azp-12 and their respective responses to HPUra and DCBG exemplify the potential of such a combined approach. *B. subtilis* azp-12 (serine to alanine in codon 1175 of *pol C*; cf., refs. 19–21) was obtained by selection on HPUra; azp-12 confers upon pol III approximately 50-fold resistance to HPUra and, as shown in Table I, ≥ 8-fold hypersensitivity to DCBG. The HPUra^r/DCBG^s phenotype of bacteria carrying azp-12 permits counterselection of an appropriately mutagenized population of *B. subtilis* azp-12 on DCBG (Butler and Brown, unpublished results), and, thus, provides a very convenient means to isolate and map both intracodon- and extracodon-specific *pol C* mutations accompanying the development of DCBG resistance. Replication of similar approaches with appropriate combinations of *pol C* mutants and the several score of inhibitor variants now available should provide a wealth of detail about the location and molecular structure of both the inhibitor and dNTP binding domains of the enzyme's active site.

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