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Inhibition of human DNA polymerase β activity by the anticancer prodrug Cloretazine

Abbie M. Frederick[1], **Marguerite L. Davis**[1], and **Kevin P. Rice**[1,*]
[1]Department of Chemistry, Colby College, 5763 Mayflower Hill Road, Waterville, ME 04901, USA

Abstract

The antineoplastic prodrug Cloretazine exerts its cytotoxicity via a synergism between 2-chloroethylating and carbamoylating activities that are cogenerated upon activation *in situ*. Cloretazine is reported here to inhibit the nucleotidyl transferase activity of purified human DNA polymerase β (Pol β), a principal enzyme of DNA base excision repair (BER). The 2-chloroethylating activity of Cloretazine alkylates DNA at the O^6 position of guanine bases resulting in 2-chloroethoxyguanine monoadducts, which further react to form cytotoxic interstrand DNA crosslinks. Alkylated DNA is often repaired via BER *in vivo*. Inhibition of the polymerase activity of Pol β may account for some of the synergism between Cloretazine's two reactive subspecies in cytotoxicity assays. This inhibition was only observed using agents with carbamoylating activity. Furthermore, while therapeutically relevant concentrations of Cloretazine inhibited the polymerase activity of Pol β, the enzyme's lyase activity, which may also participate in BER, was not significantly inhibited.

Keywords

Cloretazine; DNA base excision repair; DNA polymerase beta; DNA alkylation; DNA cross linking agents; carbamoylation; methyl isocyanate

INTRODUCTION

Cytotoxic DNA-alkylating agents are important weapons in the clinical war against cancer. Drugs with 2-chloroethylating activity, such as BCNU (1,3-bis[2-chloroethyl]-2-nitrosourea) and the preclinical Cloretazine (1,2-bis[methylsulfonyl]-1-[2-chloroethyl] -2-[(methylamino) carbonyl]hydrazine), yield interstrand DNA crosslinks between complementary G-C base pairs in DNA [1,2]. These crosslinks are believed to be responsible for the agents' therapeutic efficacy [3,4]. Both agents also yield alkyl isocyanates, which act as carbamoylating agents in a cell [5,6]. Extremely reactive with nucleophiles such as amines and sulfhydryls, isocyanates can modify amino acid side chains in cellular proteins. While the importance of the carbamoylating activity of BCNU has been controversial [7–9], sudies indicate that the identity of the carbamoylating species contributes to an anticancer agent's pharmacological profile [6,10]. The activation of Cloretazine involves an abstraction of the mildly acidic amide proton, yielding the two distinct electrophiles, methylisocyanate and 1,2-bis[methylsulfonyl]-1-[2-chloroethyl]hydrazine (90CE, Fig. 1), the latter of which further rearranges to form the 2-chloroethylating species [2,4,11]. Cloretazine is presently being tested against hematopoetic and brain cancers in clinical trials, which have been promising [12,13]. Pharmacokinetic

*Corresponding author. Fax +1 207 859 5760, kprice@colby.edu (K. Rice).

studies also support Cloretazine's potential as an anticancer agent [14], as does its demonstrable lack of nonhematological toxic consequences in clinical cancer patients [15].

A cell's primary defense against alkylation at the O^6 position of a guanine base is a direct repair mechanism via the protein O^6-alkylguanine-DNA alkyltransferse (AGT) [16], which is capable of repairing the O^6-(2-chloroethyl)guanine monoadduct caused by BCNU and Cloretazine [17,18]. The action of AGT is implicated as the primary determining factor for a cancer patient's favorable response to Cloretazine [19]. *In vitro*, cultured mammalian cell lines expressing AGT are more resistant to Cloretazine than are cells expressing little or no AGT [17]. Also evident in cell culture models is the important role of Cloretazine's carbamoylating activity. In cells expressing AGT, Cloretazine is a more effective cytotoxin than is 90CE, the derivative of Cloretazine that lacks carbamoylating activity [2]. The carbamoylating activity contributes to Cloretazine's cytotoxicity via a synergism with the 2-chloroethylating activity. When cultured cells are exposed to the Cloretazine derivative 101MDCE (1,2-bis [methylsulfonyl]-1-[(methylamino)carbonyl]hydrazine, Fig. 1B), which lacks chloroethylating activity while retaining the carbamoylating activity, the cytotoxicity of 90CE is amplified [20,21]. Given that 101MDCE can inhibit the transalkylation activity of purified AGT [18], it stands to reason that the observed synergism is due to concomitant DNA alkylation and inactivation of the direct repair protein AGT. However, cells that do not express AGT are also killed by these agents synergistically. As such, the contribution of Cloretazine's carbamoylating activity is not completely understood.

DNA base excision repair (BER) is an important pathway for the restoration of DNA damaged by many exogenous alkylating agents, including several anticancer drugs [22]. Though it exists in many forms, BER always involves the hydrolytic excision of the damaged base by a DNA glycosylase [23]. A complex of several proteins and enzymes, including Pol β, processes the subsequent abasic site, eventually restoring the DNA to its original state. Pol β is a DNA polymerase specific to BER that also possesses lyase activity capable of severing the phosphoester bond on the 3' carbon of the abasic ribose [24]. Cells deficient in Pol β are hypersensitive to many crosslinking agents such as mitomycin C [25]. Initial reports suggested that Pol β deficient cells showed no hypersensitivity to chloroethylating nitrosoureas. However, when these cells were co-treated with benzylguanine, known to deplete AGT activity, some sensitivity to nitrosoureas was established [26].

While there have been no reports of carbamoylating agents' inhibition of purified BER enzymes, carbamoylating activity associated with nitrosoureas has been indirectly linked to inhibition of excision repair [9,18,21]. DNA synthesis in whole cells is diminished when exposed to carbamoylating agents [27,28]. It is unclear from these studies which polymerases are susceptible to inactivation by carbamoylation. The experiments described herein demonstrate that Cloretazine inhibits Pol β nucleotidyl transferase activity. This result suggests a secondary rationale to the synergistic action of Cloretazine's DNA crosslinking and carbamoylating activities beyond the inhibition of AGT-mediated direct repair of O^6 guanine alkylations.

MATERIALS AND METHODS

Bacterial strains, enzymes, and biochemicals

The expression strain for Pol β – *E.coli* BL21(DE3) with pET-Polβ-His_6 plasmid – was generously provided by Joann Sweasy at Yale University Department of Genetics. Cloretazine, 101MDCE, and 90CE were a gift from Alan Sartorelli of the Yale University School of Medicine Department of Pharmacology. BCNU, bacterial cell protease inhibitor cocktail, and type XV activated calf thymus DNA were purchased from Sigma (St. Louis, MO). Uracil-DNA glycosylase and Klenow fragment were purchased from New England BioLabs (Ipswich, MA).

Ni-NTA agarose was purchased from Qiagen (Germantown, MD). [α-^{32}P] dATP was purchased from GE Life Sciences (Piscataway, NJ). Tricarboxyethyl phosphine (TCEP) was purchased from Pierce (Rockford, IL). Oligonucleotides were purchased from Operon Biotechnologies (Huntsville, AL). All other reagents were purchased from Fisher Scientific unless otherwise specified.

Purification of DNA Polymerase β

The purification of Pol β was based on previously described methods [29] with some modifications. Briefly, 500 mL log-phase *E.coli* BL21 (DE3) cells containing the pET-Polβ-His_6 plasmid were induced with 1.0 mM IPTG for 4 hr at 30°C. Harvested cells were resuspended in 10 mL lysis buffer (40mM Tris-HCl, 500mM NaCl, and 5mM imidazole, pH 8.0) with protease inhibitors and lysed by sonication. The cell lysate was clarified by ultracentrifugation and then added to 2.5 mL Ni-NTA resin, which had been equilibrated in lysis buffer, in a 15 mL conical tube and incubated for 1 hr at 4 °C while rotating. The resin was washed with lysis buffer three times in batch format. Pol β was eluted with 40 mM Tris-HCl, 500 mM NaCl, and 1 M imidazole (pH 8.0) then dialyzed into storage buffer (50 mM Tris-HCl, 100mM NaCl, 1mM EDTA, and 10% glycerol, pH 8.0). Quantification of a Coomassie-stained SDS polyacrylamide gel revealed Pol β to be 96% pure and the total protein concentration was calculated at 5.0 mg/mL using a Bradford protein assay kit (Bio-Rad).

Nucleotidyl transferase assays

One μg of Pol β was added to 0.05M Tris, 0.02M $MgCl_2$, 0.1M NaCl, 0.2 mg/mL BSA, 0.2 mM DTT, 50 μM dNTPs, 0.2 mg/mL activated calf thymus DNA, and 0.1 Ci/L [α-^{32}P] dATP in a total volume of 10 μL. This reaction was incubated at 37°C for 10 min and stopped by the addition of 2 μL 0.5 M EDTA. Five μL of the reaction were spotted on to DE81 Whatman ion exchange membranes and allowed to air dry for 5 min. Unincorporated nucleotides were removed via three washes of at least 50 mL 0.5M Na_2HPO_4 (per membrane). The incorporation of radioactive nucleotides was then measured via Cerenkov counting of the wet membranes in a Beckman-Coulter LS6500 scintillation counter.

For experiments involving polymerase inhibition by studied agents, the enzyme was pre-incubated with the agents to allow at least three half lives of compound activation. Appropriate dilutions of Cloretazine, 101MDCE, 90CE, or BCNU were made in DMSO. One μL of agent (or DMSO) was added to 10 μg Pol β in 9 μL storage buffer (10 μL total volume) and preincubated for 3 hr at room temperature. One μL of these preincubation mixtures were then used to start nucleotidyl transferase assays. Activity was measured in triplicate and the data were normalized to the positive (no agent) and negative (no enzyme) controls. IC_{50} values were calculated by fitting the data to the following hyperbolic equation: $1/(1+(x/a)^b)$, where 'x' refers to fraction activity and 'a' is solved as the IC_{50} value. Standard deviations of the solved IC_{50} value were calculated using the de Levie 'Solver Aid' macro for Microsoft Excel [30].

Experiments with Klenow fragment were carried out similarly to those with Pol β. Two μL of DMSO, with or without agent, was added to 5 units of Klenow fragment in 18 μL 50 mM Tris-HCl, 5 % glycerol, pH 8 (20 μL total volume) and preincubated for 3 hr at room temperature. Two μL of the preincubated enzyme was added to 10 mM Tris-HCl pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 50 μM dNTPs, 0.2 mg/mL activated calf thymus DNA, and 0.1 Ci/L [α-^{32}P] dATP in a total volume of 10 μL. This reaction was incubated, stopped, measured, and analyzed as with Pol β.

Pol β AP lyase assay

The ability of Pol β to cleave a deglycoslyated oligonucleotide was tested using a 3'-^{32}P-end labeled oligonucleotide duplex. An oligonucleotide top strand containing a medial uracil in place of a thymine (5'-TACCGCGGCCGGCCGAUCAAGCTTATTGGGTAC-3') was slowly annealed in 5 mM NaCl to an equimolar quantity of its complement (5'-AATGTACCCAATAACGTTGATCGGCCGGCCGCGGTA-3') that had a 5' TAA overhang. The top strand was 3' end labeled using Klenow fragment and [α-^{32}P] dATP according to vendor protocols. The resultant labeled duplex was purified using a Qiaquick Nucleotide Removal spin column (Qiagen). Due to the susceptibility of abasic DNA to spontaneous strand cleavage, the uracil base was cleaved from the DNA substrate immediately prior to exposure to Pol β. Approximately 0.25 μM labeled oligonucleotide duplex was cleaved with 20 units/mL *E. coli* uracil-DNA glycosylase in 0.07 M Hepes-KOH (pH 7.5), 0.5 mM EDTA, 1 mM TCEP, and 5% glycerol at 37°C for 30 min. The deglycosylation reaction was distributed in 8 μL volumes for each Pol β reaction, including the negative control, which was immediately quenched with $NaBH_4$ at a final concentration of 240 mM. Pol β was pre-incubated at a concentration of 0.25 mg/mL with 1 mM or 50 μM concentrations Cloretazine, 101MDCE, 90CE, or BCNU, or with an equal volume of DMSO in 300 mM Hepes-KOH (pH 8) for 3 hr at room temperature. AP lyase reactions were initiated by adding 0.8 μL of pre-incubated Pol β to the 8 μL abasic DNA samples. The reactions were incubated at 37°C for 30 min and then stopped by the addition of $NaBH_4$. Cleaved DNA was separated from intact DNA on a 15% polyacrylamide, 7 M urea, 1x TBE gel. Radiolabeled species were visualized by phosphorimagery using a Molecular Dynamics Storm phosphorimager and quantified using ImageQuant software. Data were collected in quadruplicate and normalized to positive (no agent) and negative (no Pol β) controls and reported as a fraction of control activity.

RESULTS AND DISCUSSION

Carbamoylating activity from Cloretazine inhibits DNA polymerase β nucleotidyl transferase activity in vitro

The capacity of Cloretazine to affect the principal activity of Pol β, nucleotidyl transferase, was assessed using [α^{32}P]dATP and nuclease-treated calf thymus DNA as substrates. As Cloretazine is a prodrug that must be activated in aqueous solution for its electrophillic constituents to be reactive, pre-incubations of the enzyme with the agents were carried out under conditions that allowed at least three half-lives of drug activation to occur. This step also ensured that any observed difference in nucleotidyl transferase would be a function of modification of the enzyme rather than the substrates. When exposed to variable concentrations of Cloretazine, Pol β nucleotidyl transferase was inhibited in a concentration dependent manner. The IC_{50} value of this inhibition was calculated to be 74.0 ± 9.6 μM (Fig. 2). This concentration is comparable other reports of enzymatic inhibition by Cloretazine [6,18] and well within the range measured in the serum of clinical cancer patients treated with Cloretazine in pharmacokinetic studies [31].

The relative contributions of each electrophilic subspecies, the carbamoylating and chloroethylating agents, were assessed using the compounds 101MDCE and 90CE, respectively. 101MDCE, which is the analog of Cloretazine that lacks chloroethylating activity, exhibited similar inhibitory potency as Cloretazine against Pol β (IC_{50} = 92.2 ± 9.9 μM). However, 90CE, which retains the 2-chloroethylating activity rather than the carbamoylating activity, did not inhibit Pol β at all up to concentrations of 10 mM (Fig. 2). These results demonstrate that the carbamoylating activity of Cloretazine, rather than the 2-chloroethylating activity, is responsible for the enzyme inhibition. These data support previous work in which total cellular DNA synthesis, as measured by ^{3}H-labeled dNTP incorporation, is acutely depressed by carbamoylation and not 2-chloroethylation [28]. BCNU, which also possesses

both chloroethylating activity and carbamoylating activity also inhibited Pol β, albeit with a more modest IC_{50} of 271 ± 52 μM. This general pattern has been seen in other enzyme inhibition assays using Cloretazine and BCNU [6].

Although pre-clinical and clinical data largely implicate direct repair via the AGT protein as the principle repair pathway for the initial damage caused by Cloretazine, the synergistic relationship between 90CE and 101MDCE in cytoxicity assays [20,21] clearly suggests another role for methyl isocyanate in the molecular mechanism of action of Cloretazine. Given that AGT expression is widely variable among tumor types and even among patients with the same neoplasia [19], a more thorough appreciation of the cellular lesions caused by carbamoylating activity will be very important as newer cytotoxic anticancer agents, including sulfonylhydrazines, are developed. The inhibition of BER is potentially a very important piece of the mechanism of action of these agents.

The nucleotidyl transferase activity of Klenow fragment is also inhibited by carbamoylating activity

In order to examine whether the inhibitory effects of carbamoylating activity on Pol β are limited to this polymerase, similar experiments were carried out using the Klenow fragment of *E. coli* DNA polymerase I. A similar pattern of inhibition was observed. The two compounds with methyl carbamoylating activity, Cloretazine and 101MDCE, were the most inhibitory against Klenow fragment, although the IC_{50} values were approximately half an order of magnitude higher than those with Pol β (Table 1). BCNU, with its 2-chloroethyl carbamoylating activity was less active against Klenow fragment. 90CE, which lacks carbamoylating activity possessed no measurable inhibition of the polymerase. The acute diminishment of total DNA synthesis in whole cells [28] suggests a general inhibitory consequence of carbamoylating activity on DNA polymerases. The pattern of inhibition observed for Klenow fragment may support this. However, the more substantial inhibition of Pol β demonstrates, at the very least, that this pattern extends to the polymerase required for BER.

The AP lyase activity of DNA polymerase β is not significantly inhibited by Cloretazine

Pol β is a bifunctional enzyme, possessing both nucleotidyl transferase and lyase activities. Pol β therefore, can carry out two distinct steps of DNA base excision repair. The lyase activity uses two different DNA substrates. If an abasic site were hydrolyzed on the 5' side of the 5' phosphate by APE1, the resultant 5'-deoxyribose phosphate could be cleaved by Pol β (dRP lyase). Alternatively, the lyase activity could act on the abasic site itself, resulting in a strand scission event via the same chemistry (AP lyase). It is likely that the dRP lyase is the more frequent function of Pol β lyase activity in a cell and it is Pol β that is most responsible for dRP cleavage [32,33]. Given the similarities of the chemical mechanism, the AP lyase activity was assessed here given the more straightforward experimental output (strand cleavage) relative to the dRP lyase assay. The data reveal only slight inhibition of Pol β's AP lyase activity by Cloretazine and the other agents (Fig. 3). Agent concentrations of 1 mM were required to observe even the most modest inhibition. These concentrations are unlikely to be therapeutically relevant [31]. As such, the therapeutically relevant interaction with Pol β is likely limited to the polymerase activity.

In conclusion, Cloretazine's inhibition of Pol β, and consequently DNA base excision repair, is likely an important factor in the molecular mechanism of action for this preclinical anticancer agent. These data are consistent with reports that confirm the involvement of Pol β in the repair of alkylated DNA [26], the synergistic cytotoxicity between Cloretazine's reactive subspecies [21], and the cellular inhibition of DNA synthesis by carbamoylating agents [28]. Still unclear however is the mechanism of this inhibition. Most enzyme targets of carbamoylating agents

investigated to date include at least one cysteine residue in their active sites and as such, a plausible nucleophile for an isocyanate. The crystal structure and mechanism of Pol β reveals a nucleotidyl transferase active site without a catalytic cysteine [34]. Further studies are required to identify the chemical modifications specifically responsible for the inhibition of Pol β by Cloretazine.

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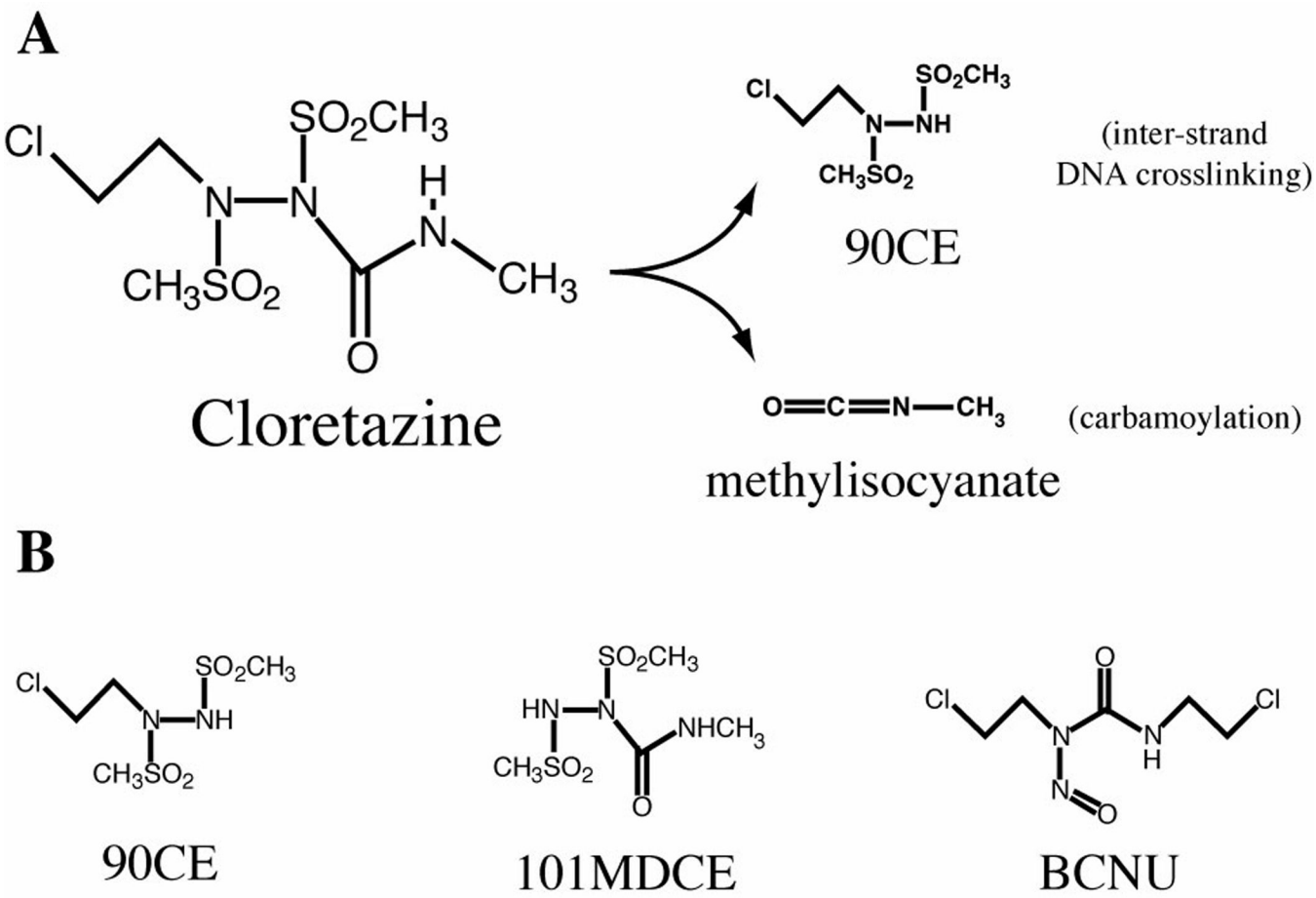

Fig. 1.

(A) The activation of Cloretazine into its two reactive subspecies, 90CE and methylisocyanate. (B) Other molecules of interest including the Cloretazine analogs 90CE and 101MDCE and the nitrosourea BCNU.

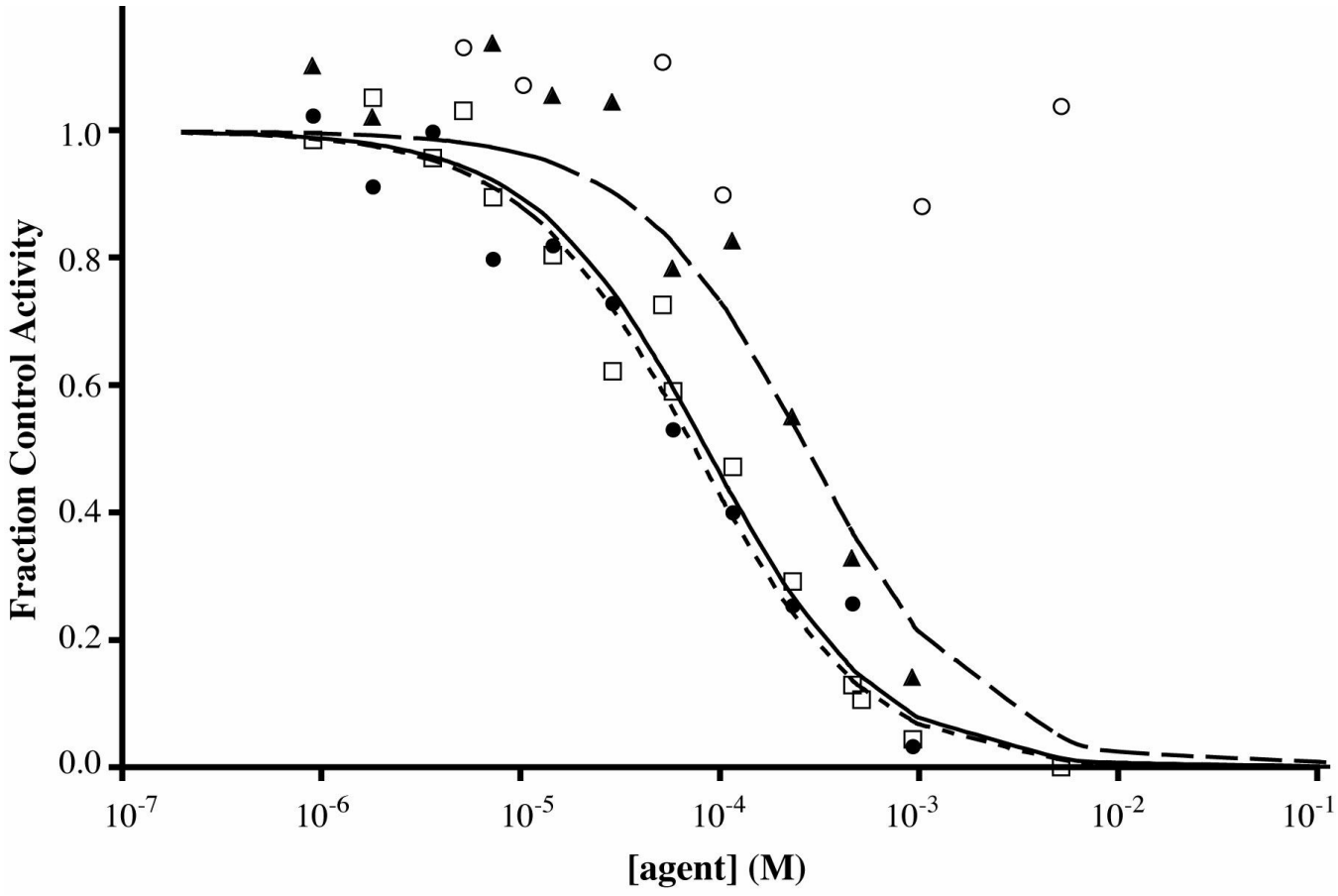

Fig. 2.

Inhibition of purified human DNA Pol β nucleotidyl-transferase activity by Cloretazine (- - ● - -), 101MDCE (—□—), BCNU (— – ▲ – —), and 90CE (○). Agents were pre-incubated with purified Pol β for 3 hrs at 25°C, pH 8 before the addition of substrates. Polymerase activity was measured via ^{32}P-labeled nucleotide incorporation into gapped calf thymus DNA and expressed as fraction of positive control (no agent) activity.

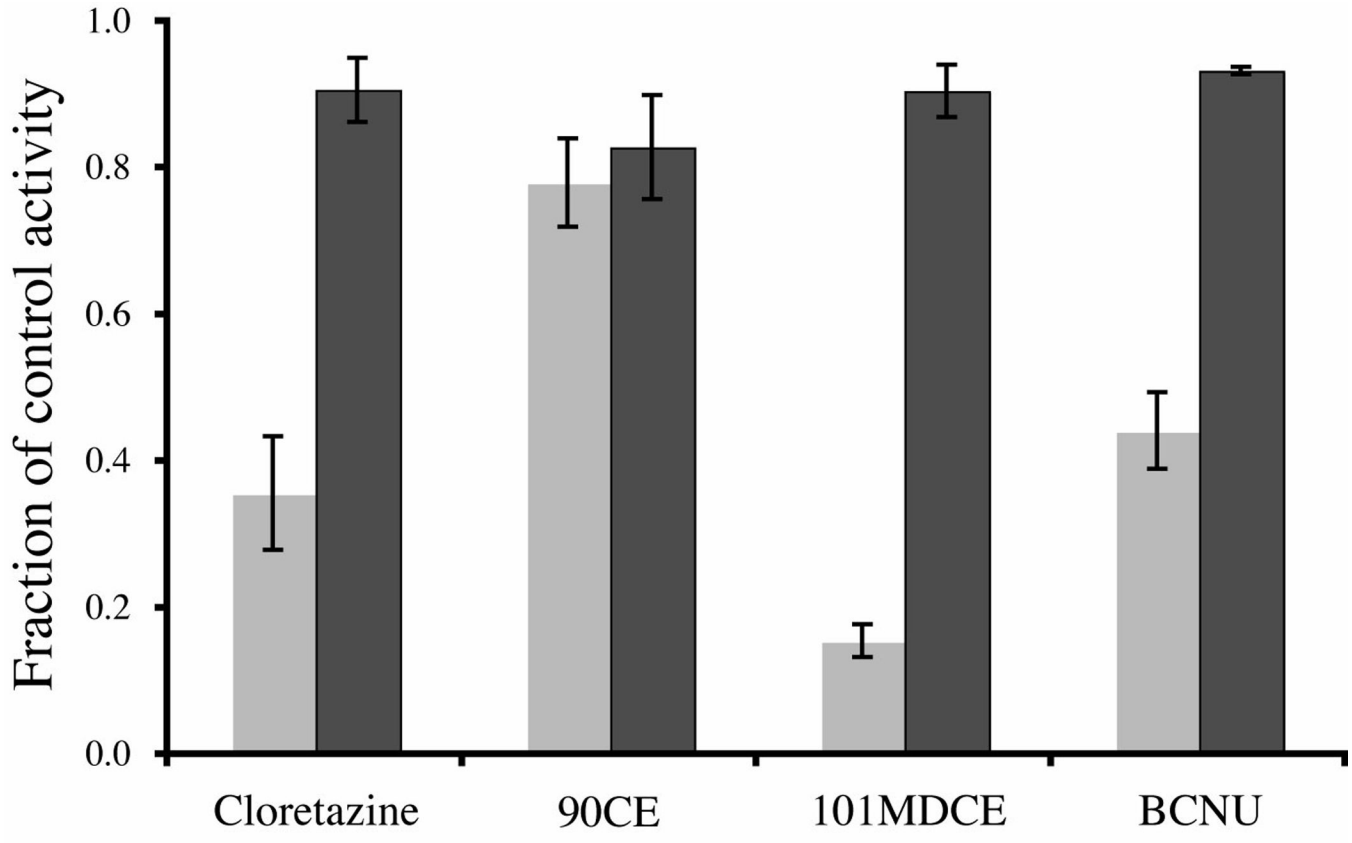

Fig. 3.

Inhibition of purified human DNA Pol β AP lyase activity by 50 μM (dark gray bars) and 1 mM (light gray bars) Cloretazine, 90CE, 101MDCE, and BCNU. Agents were pre-incubated with purified Pol β for 3 hrs at 25°C, pH 8 before the addition of ^{32}P-labeled abasic DNA substrates. AP lyase activity was observed via denaturing electrophoretic separation and phosphorimagery. Data are expressed as fraction of positive control (no agent) activity with standard deviations.

Table 1

Inhibition of DNA polymerases by sulfonylhydrazines and BCNU

	IC_{50} value in μM [a]			
	Cloretazine	**101MDCE**	**90CE**	**BCNU**
Pol β	74.0 ± 9.6	92.2 ± 9.9	>10,000	271 ± 52
Klenow fragment	387 ± 133	307 ± 57	>10,000	1,435 ± 204

[a] Purified polymerases were pre-incubated with agents for 3 hrs at 25°C, pH 8 before addition of substrates. Enzyme activity was measured and IC_{50} values were calculated as described in Materials and Methods.