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Mode of action of the chloroethylating and carbamoylating moieties of the prodrug cloretazine

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Abstract

Cloretazine is an antitumor sulfonylhydrazine prodrug that generates both chloroethylating and carbamoylating species. The cytotoxic potency of these species was analyzed in L1210 leukemia cells using analogues with chloroethylating or carbamoylating function only. Clonogenic assays showed that the chloroethylating-only agent 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE) produced marked differential cytotoxicity against wild-type and *O*⁶-alkylguanine-DNA alkyltransferase–transfected L1210 cells (LC₁₀, 1.4 versus 31 μmol/L), indicating that a large portion of the cytotoxicity was due to alkylation of DNA at the O-6 position of guanine. Consistent with the concept that O-6 chloroethylation of DNA guanine progresses to interstrand cross-links, the comet assay, in which DNA cross-links were measured by a reduction in DNA migration induced by strand breaks, showed that cloretazine and 90CE, but not the carbamoylating-only agent 1,2-bis(methylsulfonyl)-1-[(methylamino)carbonyl]-hydrazine (101MDCE), produced DNA cross-links and that cloretazine caused more DNA cross-links than 90CE at equimolar concentrations. Cell cycle analyses showed that 90CE and 101MDCE at concentrations of 5 and 80 μmol/L, respectively, produced similar degrees of G₂-M arrest. 90CE produced selective inhibition of DNA synthesis after overnight incubation, whereas 101MDCE caused rapid and nonselective inhibition of RNA, DNA, and protein syntheses. Both 90CE and 101MDCE induced phosphorylation of histone H2AX, albeit with distinct kinetics. These results indicate that (a) differential expression of *O*⁶-alkylguanine-DNA alkyltransferase in tumor and host cells seems to be responsible for tumor selectivity exerted by cloretazine; (b) 101MDCE enhances DNA cross-linking activity; and (c) 90CE induces cell death at concentrations lower than those causing alterations in the cell cycle and macromolecular syntheses.

Introduction

Cloretazine (VNP40101M; 101M; 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine) is a novel sulfonylhydrazine prodrug synthesized by our laboratory and selected for clinical development because of the broad-spectrum antitumor activity at well-tolerated doses in preclinical models (1,2). Cloretazine generates two reactive electrophiles on base-catalyzed activation, one with chloroethylating activity and the other with carbamoylating activity. DNA cross-linking assays *in vitro* (3,4) and in intact cells (5) have shown that the chloroethylating species alkylates DNA at the O-6 position of guanine ultimately forming interstrand DNA cross-links with cytosine on the opposite strand, the activity believed to be essential for the antineoplastic action of cloretazine. The carbamoylating species generated from cloretazine is methyl isocyanate, a highly thiophilic agent that carbamoylates sulfhydryl groups of proteins (6). The carbamoylating moiety of cloretazine is

believed to contribute to antineoplastic activity at least in two ways. First, the presence of the (methylamino)carbonyl substitution at the N-2 position results in the sustained release of chloroethylating species ($t_{1/2}$ of cloretazine, ~1 hour) by preventing the otherwise very rapid series of activation reactions observed with the parent molecule 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-hydrazine (90CE; refs. 3,7). Second, studies using the analogues of cloretazine with chloroethylating or carbamoylating functions only have shown that the carbamoylating species not only exerts cytotoxicity by itself but also enhances the capacity of the chloroethylating species to kill tumor cells (5,8,9).

In phase I and II clinical trials, cloretazine produced complete remissions in combination with cytarabine (1- β -D-arabinofuranosylcytosine) in acute myeloid leukemia patients with relapsed/refractory disease and by itself in poor-risk acute myeloid leukemia and myelodysplastic syndromes for which no standard therapy is available, resulting in durable remissions (10–12). Cloretazine in combination with cytarabine is currently in a pivotal phase III trial in acute myeloid leukemia.

Conventional cancer chemotherapy has limited selectivity as it aims to eradicate malignant cells with cytotoxic regimens. Recently, rationally designed, molecular targeted therapeutic agents that exploit tumor sites of vulnerability have been developed for specific malignancies. For example, imatinib mesylate, a selective inhibitor of bcr-abl tyrosine kinase, has emerged as a targeted therapy for chronic myelogenous leukemia with remarkable efficacy (13). However, selective toxicity to tumor cells can also be achieved in the reverse manner if a mechanism of protection from the deleterious effects of chemotherapeutic agents is present in normal host tissues but is absent or present at a lower level in tumor cells. We have previously shown that the cytotoxic activity of cloretazine is attenuated by the expression of the repair protein, O⁶-alkylguanine-DNA alkyltransferase (AGT; reviewed in ref. 14), which directly removes alkyl groups from the O-6 position of guanine in DNA (5,8,9). Because the AGT gene is ubiquitously expressed in normal tissues (15,16), hence denoted as a housekeeping gene (17), whereas the expression of the AGT gene is silenced through promoter hypermethylation in some human neoplasms (18,19), cloretazine can be expected to exhibit tumor selectivity against neoplasms in which AGT activity is lower than in normal host tissues.

In this report, we have examined the cytotoxic potency of the chloroethylating and carbamoylating portions of cloretazine separately in isogenic cell lines consisting of wild-type L1210 leukemia cells devoid of AGT and AGT-transfected L1210 cells using clonogenic assays. DNA cross-linking activity of cloretazine and its derivatives was measured using the single-cell gel electrophoresis method known as the comet assay. The effects of the chloroethylating and carbamoylating species on the cell cycle, on macromolecular syntheses, and on the induction of phosphorylated histone H2AX were ascertained to characterize the cytotoxic nature of the two electrophiles generated from cloretazine.

Materials and Methods

Cell Culture, Drug Treatment, and Clonogenic Assay

L1210 murine lymphocytic leukemia cells (20) and a clone designated AGT1 derived from L1210 cells stably transfected with a murine AGT expression plasmid (9) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified 5% CO₂ incubator. Stock solutions of various sulfonylhydrazines were prepared in anhydrous DMSO at a concentration of 200 mmol/L and stored at –20°C. These agents were stable even after repeated freezing and thawing over a 2-year period. Cells were treated with various concentrations of sulfonylhydrazines at a constant final concentration of 0.1% DMSO. At the end of drug treatments, cell numbers and median cell sizes were determined using a Beckman Coulter Counter with a Multisizer II analyzer (Hialeah, FL) as measures of cytotoxicity.

For clonogenic assays, cells in the exponential phase of growth were condensed to a density of $2 \times 10^6/\text{mL}$ in medium supplemented with 25 mmol/L of HEPES buffer (pH 7.4). One microliter of drug solution was added to 1 mL of cell suspension and incubations were carried out in a water bath at 37°C in open air. After drug exposure, 2 to 250 μL of cell suspension containing the predetermined number of cells that yielded 10 to 60 colonies per well for each treatment were mixed with 20 mL of RPMI 1640 containing 0.8% methylcellulose and 15% fetal bovine serum, divided into three wells in six-well plates, and incubated for 8 days to allow for colony formation.

The Modified Alkaline Comet Assay

L1210 cells at a density of $2.5 \times 10^5/\text{mL}$ in a volume of 2.5 mL were exposed to varying concentrations of sulfonylhydrazines for 16.5 hours. Cells were washed once with Dulbecco's PBS, suspended at a density of $5 \times 10^5/\text{mL}$, and subjected to the modified alkaline comet assay described by McKenna et al. (21). The protocol for the comet assay in general was provided by Trevigen, Inc. (Gaithersburg, MD). Briefly, cell suspensions were mixed at a ratio of 1:10 with 1% low melt agarose, immediately poured in duplicate onto the sample areas of comet slides (Trevigen), and incubated at 4°C for 10 minutes to allow the agarose to solidify. Cells were lysed by submerging slides in an alkaline lysis buffer containing 10 mmol/L of Tris-HCl (pH 10), 2.5 mol/L of NaCl, 10 mmol/L of EDTA, 1% Triton X-100, and 1% sodium *N*-laurylsarcosinate at 4°C for 1 hour and irradiated at a dose of 5 Gy to fractionate DNA. Cellular DNA was denatured in a freshly prepared alkaline solution (pH >13) composed of 300 mmol/L of NaOH and 10 mmol/L of EDTA at room temperature for 1 hour and subjected to electrophoresis at a constant voltage (32 V) at 4°C for 25 minutes in the identical alkaline solution. Cellular DNA was neutralized by successive incubations in 0.5 mol/L of Tris-HCl (pH 7.4) for 5 minutes at room temperature thrice followed by two washes in 75% and 95% ethanol each, completely dried at 37°C, and stained with SYBR Green I Nucleic Acid Gel Stain solution (Invitrogen, Carlsbad, CA) in the dark at room temperature for 1 hour. Comets derived from single cells were photographed under a Zeiss Axioskop 8 fluorescence microscope with a SPOT CCD camera (Diagnostic Instruments, Sterling Heights, MI), and head and tail moments were quantified using a CASP program (22).

Cell Cycle Analysis

L1210 cells at a density of $2.5 \times 10^5/\text{mL}$ in a volume of 6 mL were exposed to various sulfonylhydrazines for 16 hours and subjected to cell cycle analysis according to basic protocol 2 described by Darzynkiewicz and Juan (23). Briefly, for the last 1.5 hours of drug exposure, cells were labeled with 15 $\mu\text{g}/\text{mL}$ of 5-bromodeoxyuridine, washed once with Dulbecco's PBS, suspended in 1 mL of Dulbecco's PBS, fixed by rapidly pouring the cell suspensions into 15-mL tubes containing 10 mL of ice-cold 70% ethanol, and stored at 4°C. On the day of cytometric analysis, cells were successively suspended in 0.25 mL of 2 N HCl for 20 minutes at room temperature in 1 mL of phosphate/citrate buffer (pH 7.4) twice, and in 165 μL of antibody diluting buffer to which 20 μL of FITC-conjugated mouse monoclonal anti-5-bromodeoxyuridine antibody solution (BD Biosciences PharMingen, San Diego, CA) were added. Cells were incubated for 30 minutes at room temperature, washed with antibody diluting buffer twice, and counterstained with propidium iodide followed by bivariate data analysis using a Becton Dickinson fluorescence-activated cell sorting Vantage flow cytometer.

Measurement of DNA, RNA, and Protein Syntheses

L1210 cells were treated with various sulfonylhydrazines for either 1 or 14 hours followed by a 1.5-hour labeling period. For the short-term treatment, cells in the exponential phase of growth were condensed to a density of $2 \times 10^6/\text{mL}$ in a volume of 2 mL of medium supplemented with 25 mmol/L of HEPES (pH 7.4) and exposed to the prodrugs for 1 hour.

Then the cell suspension was divided into three 0.5-mL portions and each was labeled separately with 10 $\mu\text{Ci/mL}$ of [methyl- ^3H]thymidine (MT 6038, Moravek Biochemicals, Inc., Brea, CA), 10 $\mu\text{Ci/mL}$ of [5- ^3H]uridine (MT 602E, Moravek), or 10 $\mu\text{Ci/mL}$ of [^3H]amino acid mixture (TRK440, Amersham Pharmacia Biotech UK Ltd., Piscataway, NJ) for 1.5 hours. Drug treatment and labeling were carried out in a water bath at 37°C in open air. For the long-term treatment, cells at a density of $4 \times 10^5/\text{mL}$ in a volume of 12.5 mL were treated with the prodrugs for 14 hours in a 5% CO_2 incubator, condensed to a density of $2 \times 10^6/\text{mL}$, and processed for labeling as in the short-term treatment. At the end of the labeling period, aliquots of 100 μL of cell suspension were applied to 2.4-cm Whatman 3MM filter paper circles pinned onto styrofoam in triplicate. The filters were washed thrice with ice-cold 5% trichloroacetic acid and twice with methanol, and the radioactivity thereon was determined using a Beckman LS 6500 Scintillation System. For incorporation of the [^3H]amino acid mixture into proteins, the second trichloroacetic acid wash was heated to 95°C to remove aminoacyl-tRNAs.

Induction of Phosphorylated Histone H2AX

L1210 cells at a density of $4 \times 10^5/\text{mL}$ in a volume of 6 mL were treated with various sulfonylhydrazines for 12 hours for a dose-response study and for 1 to 24 hours for a time course study. For positive controls, cells were irradiated at doses of 6 and 12 Gy and incubated for 1 hour at 37°C. At the end of the treatments, cells were washed once with Dulbecco's PBS, suspended in 1 mL of Dulbecco's PBS, and fixed by pouring the cell suspensions into 15-mL conical tubes containing 10 mL of ice-cold 70% ethanol. Staining for phosphorylated histone H2AX (γH2AX) was carried out as described by MacPhail et al. (24). Briefly, on the day of fluorescence-activated cell sorting analyses, cells were rehydrated in 1 mL of Dulbecco's PBS containing 4% fetal bovine serum and 0.1% Triton X-100 (PST) for 10 minutes on ice, suspended in 200 μL of mouse monoclonal anti-phosphohistone H2AX antibody (Upstate Biotechnology, Lake Placid, NY) diluted 1:500 in PST for 2 hours at room temperature, washed with PST twice, and resuspended in 200 μL of Alexa 488 goat anti-mouse immunoglobulin G (H+L)F(ab')₂ fragment antibody (Molecular Probes) diluted 1:200 in PST for 1 hour at room temperature. Cells were washed with Dulbecco's PBS twice and analyzed using a Becton Dickinson fluorescence-activated cell sorting Vantage flow cytometer.

Results

Cloretazine and Its Analogues

The structures of cloretazine and its chloroethylating-only (90CE) and carbamoylating-only (101MDCE) derivatives and the postulated activation mechanism of cloretazine resulting in the generation of chloroethylating and carbamoylating electrophiles are shown in Fig. 1. The half-lives of cloretazine, 90CE, and 101MDCE measured in Tris buffer (pH 7.5) at 37°C are ~1 hour, ~30 seconds, and ~3.5 minutes, respectively (3,4,7).

Effects of Various Sulfonylhydrazines on Cell Survival

We have previously shown that the cytotoxic activity of cloretazine is significantly attenuated by the expression of AGT using wild-type L1210 leukemia cells devoid of AGT expression and AGT-transfected L1210 cells (9). In those studies, the cytotoxic activity of cloretazine and its analogues was determined using growth inhibition assays. Because growth inhibition based on cell counts is not reflective of cellular viability, the cytotoxic activity of various sulfonylhydrazines was reexamined using clonogenic assays. L1210 cells formed colonies in semisolid medium after incubation for 8 days (Fig. 2A). Survival curves of wild-type L1210 and AGT-transfected L1210 (AGT1) cells exposed to sulfonylhydrazines for 2 hours are shown in Fig. 2B (*left*). Both cloretazine and 90CE produced exponential loss of survival in these cells. 90CE exhibited marked differential cytotoxicity (22-fold) against L1210 and AGT1 cells (LC_{10} , 1.4 versus 31 $\mu\text{mol/L}$), indicating that the cytotoxicity by 90CE was largely derived

from alkylation of DNA at the O-6 position of guanine. The degree of differential cytotoxicity exhibited by cloretazine against these isogenic cell lines was 10-fold (LC_{10} , 2.2 versus 22 $\mu\text{mol/L}$), lower than that exhibited by 90CE due to at least two factors. First, because the half-life of cloretazine is ~ 1 hour, it undergoes only a 75% activation during the 2-hour exposure period. Therefore, cloretazine was expected to exhibit less cytotoxicity than molar equivalents of 90CE in L1210 cells (LC_{10} , 2.2 compared with 1.4 $\mu\text{mol/L}$). Second, both 90CE and 101MDCE exerted cytotoxicity at similar concentrations in AGT1 cells (LC_{10} , 31 and 33 $\mu\text{mol/L}$, respectively) and contributed to the overall cytotoxicity of cloretazine, lowering the LC_{10} value to 22 $\mu\text{mol/L}$. The cytotoxicity of 101MDCE was independent of AGT expression and 101MDCE exhibited a shoulder in the survival curve. Time course studies showed that cloretazine, 90CE, and 101MDCE caused cell kill, with kinetics predicted from their half-lives of ~ 1 hour, ~ 30 seconds, and ~ 3.5 minutes, respectively. Thus, cloretazine and 90CE at 2.5 $\mu\text{mol/L}$ produced equivalent log cell kill at 4 hours due to nearly complete activation of cloretazine and a negligible contribution to cytotoxicity by the generated methyl isocyanate at this concentration (Fig. 2, *right*). Figure 2C compares the LC_{10} values from the clonogenic assays with the IC_{50} values from the growth inhibition assays (9). Cells were more sensitive when measured by clonogenic assays than by growth inhibition assays for both 90CE and cloretazine; however, similar AGT1/L1210 ratios were obtained from the two assays.

DNA Cross-Linking by Sulfonylhydrazines

The alkaline comet assay (single-cell gel electrophoresis) is a method developed to analyze DNA strand breaks in individual cells (25). The modified alkaline comet assay in which DNA cross-links are indirectly measured by analyzing the relative reduction in DNA migration induced by DNA strand breaks (21) was employed to assess the ability of the sulfonylhydrazine prodrugs to induce DNA cross-links. Because the formation of DNA cross-links by 90CE *in vitro* at 37°C is a relatively slow process requiring at least several hours, L1210 cells were treated with sulfonylhydrazines overnight (16.5 hours). These treatments caused growth inhibition, as well as an increase in cell size. 90CE caused the most striking effect on cell size (Table 1). The comet assays depicted in Fig. 3 revealed that (a) both cloretazine and 90CE cause DNA cross-links noticeably at 20 $\mu\text{mol/L}$ (the % tail moments were 76% for both cloretazine and 90CE compared with 91% for the control) and profoundly at 80 $\mu\text{mol/L}$; (b) cloretazine caused more DNA cross-links than 90CE at 80 $\mu\text{mol/L}$ (the % tail moments were 24% and 47% for cloretazine and 90CE); and (c) 101MDCE possessed no DNA cross-linking activity (the % tail moments were 91% at both 20 and 80 $\mu\text{mol/L}$). These latter findings prompted us to examine the effects of combinations of 101MDCE with 90CE on the cell kill in clonogenic assays. Against a fixed concentration of 2.5 $\mu\text{mol/L}$ of 90CE, the effects of 101MDCE were additive at 10 and 20 $\mu\text{mol/mL}$ and synergistic at 40 $\mu\text{mol/mL}$ (Table 2).

Effects of Sulfonylhydrazines on the Cell Cycle

To determine whether the sulfonylhydrazine prodrugs cause perturbation of the cell cycle in L1210 cells, the leukemia cells were exposed to these agents for 16 hours (including the DNA labeling with 5-bromodeoxyuridine for the last 1.5 hours) and subjected to flow cytometric analysis for DNA synthesis and DNA content. The chloroethylating agent 90CE produced pronounced G_2 -M arrest; the percent of cells in the G_2 -M phase was 3.4%, 44%, 60%, and 69% at concentrations of 90CE of 0, 5, 20, and 80 $\mu\text{mol/L}$, respectively (Fig. 4). The carbamoylating agent 101MDCE did not significantly affect the cell cycle at 20 $\mu\text{mol/L}$ but produced significant G_2 -M arrest (39%) at 80 $\mu\text{mol/L}$. Cloretazine exhibited the combined effects of 90CE and 101MDCE on the cell cycle of L1210 cells.

Effects of Sulfonylhydrazines on DNA, RNA, and Protein Syntheses

The sequences in which 90CE produces DNA cross-links involve initial chloroethylation at the O-6 position of guanine, the relatively rapid formation of the intermediate N^1,O^6 -ethanoguanine via intramolecular cyclization and halide loss, and the slower reaction (completed within 12 hours) of this intermediate with a cytosine on the opposite strand resulting in an N^1G-N^3C cross-link via an ethylene bridge (see Fig. 1B in ref. 9 for postulated sequences). With these reaction sequences taken into account, the effects of the sulfonylhydrazines on macromolecular syntheses were measured at two different incubation times (Table 3). In short-term incubation experiments (i.e., exposure to the sulfonylhydrazines for 2.5 hours, including a 1.5-hour labeling period), 90CE did not affect any type of macromolecular synthesis even at the highest concentration examined (80 $\mu\text{mol/L}$), whereas 101MDCE caused nonselective moderate and profound inhibition of DNA, RNA, and protein syntheses at 20 and 80 $\mu\text{mol/L}$, the results expected from the promiscuous nature of carbamoylation chemistry (6). In contrast, 90CE caused selective inhibition of DNA synthesis in the long-term incubation experiments (i.e., 14 hours of incubation, including a 1.5-hour labeling period), compatible with DNA cross-link formation resulting in blockage of DNA synthesis at DNA replication forks. 101MDCE was less inhibitory in the long-term incubation experiments than in the short-term incubations, suggesting that cells had the capacity to recover from the damage produced by 101MDCE.

Induction of Phosphorylated Histone H2AX

H2AX is believed to be one of the guardians of the genome, phosphorylated in chromatin domain flanking sites of DNA double-strand breaks (26). To examine whether treatment with various sulfonylhydrazine pro-drugs induces γ H2AX formation, L1210 cells were exposed to these agents for 12 hours, stained with anti-phosphohistone H2AX antibody, and the extent of γ H2AX induction was analyzed by flow cytometry. L1210 cells irradiated at 6 and 12 Gy and incubated for 1 hour at 37°C were used as positive controls. Both 90CE and 101MDCE induced γ H2AX formation although a relatively high concentration of 101MDCE (80 $\mu\text{mol/L}$) was required to induce this effect (Fig. 5A). Time course (0–24 hours) studies using the fixed concentrations of 50 $\mu\text{mol/L}$ of 90CE and 80 $\mu\text{mol/L}$ of 101MDCE revealed a distinction in the kinetics of γ H2AX induction. Thus, induction by 90CE progressively increased up to 10 hours, whereas that by 101MDCE was highest at 1 hour and gradually returned toward the noninduced state within 24 hours (Fig. 5B).

Discussion

The availability of analogues of cloretazine with chloroethylating and carbamoylating functions only made a dissection of the cytotoxic potency and cytotoxic nature of the two reactive components generated by the activation of cloretazine possible. Clonogenic assays of the isogenic cell lines consisting of wild-type (AGT⁻) and AGT-transfected (AGT1) L1210 cells showed that the chloroethylating-only analogue 90CE exhibited differential toxicity against the two cell types by 22-fold (LD_{10} , 1.4 versus 31 $\mu\text{mol/L}$), indicating that a large portion of the cytotoxicity was due to O^6 -chloroethylation of guanine in DNA. Consistent with the concept that O^6 -chloroethylation of guanine progresses to interstrand DNA cross-links, the alkaline comet assays showed that cloretazine and 90CE, but not the carbamoylating agent 101MDCE, produced DNA cross-links. Compared with the LC_{10} values of cloretazine and 90CE (2.2 and 1.4 $\mu\text{mol/L}$, respectively) in L1210 cells, however, DNA cross-links induced in these cells by these agents were detectable at 20 $\mu\text{mol/L}$ by the comet assay, approximately a magnitude higher concentration. We infer that detection of DNA cross-links in cells by the comet assay method is not sufficiently sensitive to detect the few DNA cross-links per cell that we surmise are sufficient to induce clonogenic cell death.¹

¹Penketh et al., manuscript in preparation.

The LC₁₀ value of 90CE in L1210 cells (1.4 $\mu\text{mol/L}$; Fig. 2) in clonogenic assays is significantly lower than the IC₅₀ value of 6.0 $\mu\text{mol/L}$ obtained from the growth inhibition assays (9) and considerably lower than the minimum concentration required to induce G₂-M cell cycle arrest (5 $\mu\text{mol/L}$; Fig. 4) or to induce inhibition of DNA synthesis (5 $\mu\text{mol/L}$; Table 3, long-term incubation). This characteristic of 90CE contrasted with that of the carbamoylating agent 101MDCE, which affected clonogenic survival (LC₁₀, 35 $\mu\text{mol/L}$; Fig. 2), cellular growth (IC₅₀, 38 $\mu\text{mol/L}$; Fig. 2), G₂-M cell cycle arrest (80 $\mu\text{mol/L}$; Fig. 4), and macromolecular syntheses (20–80 $\mu\text{mol/L}$; Table 3) at higher and narrower concentration ranges. In addition, unlike 90CE, the cytotoxic activity of 101MDCE was independent of AGT expression (LC₁₀, 33–35 $\mu\text{mol/L}$ in L1210 and AGT1 cells). These findings imply that the cellular AGT content is the single most important determinant of tumor susceptibility to cloretazine, and that the chloroethylating moiety of cloretazine, presumably due to its ability to cause DNA cross-links, is extremely potent in inducing irreversible loss of the proliferative potential of cells.

Mammalian cells accomplish the tremendous feat of duplicating their 3 billion bases of DNA rapidly and accurately in the S phase of the cell cycle. To coordinate DNA replication to occur only once in a single S phase, the cell uses a sophisticated mechanism called replication licensing that triggers the initiation of DNA replication, as well as prevents the reinitiation of DNA replication on DNA which is already replicated (27). Agents such as cloretazine that generate interstrand DNA cross-links are expected to elicit catastrophic consequences to the replication licensing machinery by blocking the separation of the two strands at replication forks and causing an imbalance in the licensing system, resulting in the activation of a “cell-cycle checkpoint,” the mechanisms by which the cell actively halts progression of the cell cycle in an attempt to correct lesions (28). The ATM(ATR)/CHK2(CHK1)-controlled checkpoint network delays cell cycle progression in G₁, S, and G₂-M phases (28). In our experiments, prominent G₂-M arrest was induced in L1210 cells, which are deficient in p53 (9), the player in the cell cycle checkpoint network responsible for inducing G₁ arrest (28), after exposure to the chloroethylating agent 90CE (Fig. 4). Cells with irreparable DNA damage undergo permanent growth arrest with phenotypic features of cell senescence or die through the process of mitotic catastrophe (29) in an ATM/ATR-dependent manner (30). 90CE induced cell death at concentrations lower than those causing alterations in the cell cycle and macromolecular synthesis. We believe that 90CE causes cell death by forming irreparable DNA damage (interstrand DNA cross-links) thereby causing a collapse of genomic surveillance mechanisms, and that very small numbers of interstrand cross-links are sufficient to elicit a collapse of the genomic surveillance systems, generating a gap between lethal concentrations and concentrations causing inhibition of DNA synthesis and arrest in the cell cycle. The G₂-M cell cycle arrest and cell death that occur through mitotic catastrophe seem to be a common consequence in tumor cells exposed to DNA-damaging chemotherapeutic agents (28,29).

Histone H2AX, representing 2% to 25% of the mammalian histone H2A pool depending on the cell type, is believed to be one of the guardians of the genome, phosphorylated in chromatin domain flanking sites of DNA double-strand breaks associated with γ -irradiation, meiotic recombination, and V(D)J gene rearrangement (26). Although phosphorylation of H2AX is generally accepted as a specific marker for DNA double-strand breaks, recent findings indicate that it occurs in broader contexts such as stressed DNA synthesis beside DNA double-strand breaks (31). In our studies, both 90CE and 101MDCE induced γ H2AX formation in L1210 cells. However, the kinetics of γ H2AX induction by the two agents were different in that induction by 90CE was progressive up to 10 hours, whereas that by 101MDCE was maximal at 1 hour. The kinetics of γ H2AX induction by 101MDCE resembles that of X-ray irradiation (24). Phosphorylation of H2AX may be induced from severe inhibition of DNA synthesis by 101MDCE at 80 $\mu\text{mol/L}$. Phosphorylation of H2AX was also detected in L1210 cells exposed to 90CE at 50 $\mu\text{mol/L}$ for 1 hour. Because progression of the initial alkylation of DNA by 90CE to cross-link formation is a relatively slow process requiring at least several hours (3,4) and

90CE caused no inhibition of DNA synthesis in L1210 cells even at 80 $\mu\text{mol/L}$, neither DNA cross-link formation nor inhibition of DNA synthesis accounted for the induction of γH2AX . These results imply that primary and secondary alkylations of DNA caused by 90CE are recognized by DNA damage sensors that cause γH2AX formation.

Unlike conventional cytotoxic cancer chemotherapeutic agents with limited tumor specificity, cloretazine is exceptional, fulfilling the criteria of targeted therapy because it can selectively destroy tumor cells in which the AGT expression is absent or relatively low, whereas its cytotoxicity is not markedly expressed in normal host tissues by the expression of the AGT repair protein. The preclinical tumor models in which cloretazine is curative, including the L1210 and P388 murine leukemias (9), C26 murine colon carcinoma (32), and U251 human glioma (33), are negative in AGT expression. Recent clinical data also indicate that the highest response rates to cloretazine in acute myeloid leukemia patients are associated with the lowest AGT levels in leukemic blasts (12). With respect to AGT activity in human neoplasms, a wide variability is documented (reviewed in ref. 34). For instance, histochemical studies have shown that breast carcinoma cells often contain elevated levels of AGT relative to corresponding normal ductal cells (35). In contrast, 22% to 27% of human primary brain tumors completely lack AGT activity (36,37) and these tumors are promising candidates for targeted therapy by cloretazine. The loss of AGT expression seems to be due to gene silencing through promoter hypermethylation of the *AGT* gene (18,19), resulting from epigenetic malfunction occurring during tumor evolution (38,39). Cloretazine can be compared with the chloroethylnitrosoureas such as 1,3-bis(2-chloroethyl)-1-nitrosourea because the latter agent also generates chloroethylating and carbamoylating species. However, cloretazine is more selective in producing *O*⁶-chloroethylguanine, as evidenced by the greater differential cytotoxicity against L1210 and AGT1 cells [7- and 2-fold for cloretazine and 1,3-bis(2-chloroethyl)-1-nitrosourea, respectively; ref. 9]. Cloretazine surpasses 1,3-bis(2-chloroethyl)-1-nitrosourea in antineoplastic activity in preclinical studies (1,2). Our findings point to the importance of (a) developing a rapid, relatively simple assay for the accurate quantification of AGT and (b) the measurement of tumor and normal tissues of patients for expression of AGT to select those individuals with the highest probability of responding to cloretazine.

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Figure 1.

Cloretazine and its analogues, and activation mechanism of cloretazine. Structures of cloretazine, its chloroethylating-only derivative 90CE, and its carbamoylating-only derivative 101MDCE (*left*), and activation of cloretazine generating the initial chloroethylating and carbamoylating species (*right*).

Figure 2.

Survival of L1210 and AGT1 cells exposed to various sulfonylhydrazines using clonogenic assays. **A**, colonies of L1210 cells plated at 27 and 81 cells/well for the left and right rows, respectively, and incubated for 8 d. **B**, dose-response curves of cloretazine, 90CE, and 101MDCE in L1210 and AGT1 cells incubated with each sulfonylhydrazine for 2 h (left). Time course of cell kill by cloretazine, 90CE, and 101MDCE in L1210 cells (right). The data from a representative experiment are shown; measurements were repeated at least twice with similar results. **C**, comparison of LD₁₀ values from clonogenic assays with IC₅₀ values from growth inhibition assays (the latter data from ref. 9).

Figure 3.

DNA cross-links produced by cloretazine and 90CE in L1210 cells examined by the modified alkaline comet assays. Cells were exposed to various sulfonylhydrazines for 16.5 h, lysed in alkaline lysis buffer, irradiated at a dose of 5 Gy (i.e., the predetermined dose which caused distribution of 10% and 90% of total fluorescence in the head and tail moments, respectively, in the control cells), and subjected to electrophoresis. DNA was visualized by SYBR Green nucleic acid gel stain.

Figure 4.

Effects of sulfonylhydrazine prodrugs on the cell cycle of L1210 leukemia cells. Cells were exposed to each sulfonylhydrazine for 16 h. 5-Bromodeoxyuridine was added for the last 1.5 h of incubation to probe for the capacity for DNA synthesis. Cells were fixed, stained with FITC conjugated anti-5-bromodeoxyuridine antibody, counterstained with propidium iodide, and analyzed for DNA synthesis (*y axis*) and for DNA content (*x axis*) by flow cytometry. R2 and R3, cells in G₂-M and G₁ phase, respectively. R1, cells in G₁, S, and G₂-M phases.

Figure 5.

Induction of γ H2AX by sulfonhydryl prodrugs in L1210 cells. **A**, cells were treated with sulfonhydryls for 12 h, fixed, stained with anti – phosphohistone H2AX antibody, and analyzed by flow cytometry. Cells irradiated at doses of 6 and 12 Gy and incubated for 1 h at 37°C were used as positive controls. **B**, time course (0 –24 h) of γ H2AX staining in cells exposed to 90CE and 101MDCE at concentrations of 50 and 80 μ mol/L, respectively.

Table 1

Effects of sulfonylhydrazine prodrugs on cell growth and cell size

	$\mu\text{mol/L}$	Cell number (cells/mL $\times 10^{-5}$)	Size (μm)
None		8.6	10.6
Cloretazine	5	4.0	12.8
	20	3.1	12.5
	80	2.5	10.4
90CE	5	4.0	13.1
	20	3.8	13.5
	80	3.2	13.0
101MDCE	20	4.3	11.1
	80	2.6	11.1

NOTE: L1210 cells were treated with each agent for 16.5 hours, and the cell number and size (median cell diameter) were determined.

Table 2

Effects of combinations of 90CE and 101MDCE on cell survival

90CE ($\mu\text{mol/L}$)	101MDCE ($\mu\text{mol/L}$)	Surviving fraction	Combination index
2.5		0.0443	
	10	0.618	
	20	0.379	
	40	0.0412	
2.5	10	0.0283	1.139
2.5	20	0.0129	1.067
2.5	40	0.00125	0.680

NOTE: L1210 cells were exposed to 90CE, 101MDCE, or the combination of both agents for 2 hours and subjected to clonogenic assays. The data were analyzed by CalcuSyn software (Ferguson, MO). Combination index: 1, additive; <1, synergistic.

Table 3
Effects of various sulfonylhydrazines on DNA, RNA, and protein syntheses

1 h treatment-1.5 h labeling, cpm/2 × 10 ⁵ cells (% of control)					14 h treatment-1.5 h labeling, cpm/2 × 10 ⁵ cells (% of control)		
	μmol/L	[³ H]dThd	[³ H]Urd	[³ H]AAs	[³ H]dThd	[³ H]Urd	[³ H]AAs
Control	0	17,700 (100)	10,800 (100)	6,380 (100)	23,500 (100)	13,600 (100)	4,200 (100)
Clotetazine	5	20,700 (117)	11,500 (107)	5,520 (86)	14,000 (59)	13,700 (100)	5,290 (126)
	20	17,700 (100)	9,180 (85)	5,430 (85)	6,180 (29)	12,200 (90)	4,890 (117)
	80	6,470 (37)	6,020 (56)	3,370 (53)	5,050 (22)	4,140 (30)	2,200 (52)
90CE	5	19,200 (109)	12,500 (116)	6,460 (101)	13,800 (59)	13,600 (99)	4,870 (116)
	20	18,100 (102)	11,800 (109)	6,290 (98)	7,120 (30)	15,300 (112)	5,320 (127)
	80	18,200 (103)	9,850 (92)	5,800 (91)	5,580 (24)	10,700 (79)	4,320 (103)
101MDCE	20	12,500 (71)	6,560 (61)	4,280 (67)	19,400 (83)	11,500 (84)	3,590 (86)
	80	207 (1)	1,300 (12)	954 (15)	6,990 (30)	6,100 (45)	2,710 (64)

NOTE: Values represent acid-insoluble counts and triplicate averages with SEs <10% in the representative experiment. Measurements were repeated at least twice with reproducible results.
Abbreviations: dThd, thymidine; Urd, uridine; AAs, amino acid mixture.