Role of $O^6$-alkylguanine-DNA alkyltransferase in the cytotoxic activity of cloretazine

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Abstract

Cloretazine (VNP40101M; 101M; 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine) is a sulfonylhydrazine prodrug that generates both chloroethylation and carbamoylation species on activation. To explore the molecular mechanisms underlying the broad anticancer activity observed in preclinical studies, cloretazine and chloroethylation-only (i.e., 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine) and carbamoylation-only (i.e., 1,2-bis(methylsulfonyl)-1-[(methylamino)carbonyl]hydrazine) analogues were evaluated in five murine hematopoietic cell lines. These cell lines were separable into two groups by virtue of their sensitivity to 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-hydrazine; the sensitive group included L1210, P388, and F-MEL leukemias (IC$_{50}$s, 6–8 μmol/L) and the resistant group consisted of Ba/F3 bone marrow and WEHI-3B leukemia cells (IC$_{50}$s, 50–70 μmol/L). Resistant cells expressed $O^6$-alkylguanine-DNA alkyltransferase (AGT), whereas sensitive cells did not. A correlation existed between AGT expression and the functional status of p53; AGT$^+$ cells possessed proficient p53, whereas AGT$^+$ cells contained wild-type p53. Based on recent findings on regulation of AGT gene expression by others, we suspected that silencing of the AGT gene by promoter hypermethylation frequently occurs during tumor progression involving p53 inactivation. $O^6$-Chloroethyguanine is the initial DNA lesion that progresses to lethal interstrand DNA cross-links. Cloretazine exhibited a much higher preference toward the $O^6$-chloroethylation of guanine, as measured by the difference in IC$_{50}$s to wild-type and AGT-transfected L1210 cells, than 1,3-bis(2-chloroethyl)-1-(methylamino)carbonyl)hydrazine, which targets the same site in DNA. Preferential toxicity of cloretazine against AGT$^+$ tumor cells coupled with decreased toxicity to AGT$^+$ cells in host tissues constitute the therapeutic basis for cloretazine.

Introduction

Among the 1,2-bis(sulfonyl)-1-alkylhydrazine class of antitumor prodrugs synthesized by our laboratory, cloretazine (VNP40101M; 101M; 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine) exhibited the most efficacious antineoplastic activity against a broad spectrum of experimental tumor systems (1, 2). Cloretazine has a half-life of ~1 hour, which presumably permits the optimum distribution of this agent in vivo, a property thought to be responsible for its superior antineoplastic activity compared with other sulfonylhydrazine produgs (1, 3). The decomposition (activation) of cloretazine generates, in addition to chloroethylation species, another cytotoxic entity (i.e., methyl isocyanate) that causes carbamoylation of proteins, preferentially those possessing sulfhydryl groups; the precise role of methyl isocyanate in the antineoplastic activity of cloretazine is currently under investigation (4). Cloretazine bears a functional resemblance to chloroethylnitrosoureas, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), in that both agents generate chloroethylation and carbamoylation species; however, cloretazine was found to be more potent, more efficacious, and less toxic than BCNU in preclinical studies (2, 5). Furthermore, cloretazine exhibited significant antileukemic activity alone and in combination with cytarabine (1-β-D-arabinofuranosylcytosine) in acute myelogenous leukemia patients with relapsed/refractory disease in phase I and II clinical trials (6, 7) and is currently in a pivotal phase III trial in acute myelogenous leukemia.

Alkylation agents are electrophilic agents with affinities for nucleophilic centers in organic macromolecules. In general, soft electrophiles (i.e., agents with high Swain-Scott constants) tend to react with soft nucleophilic centers, such as the N$^7$ and N$^3$ positions of guanine in DNA, whereas hard electrophilic species energetically react with hard nucleophilic centers, such as the oxygen of phosphodiester linkages and the O$^6$ position of guanine in DNA (8). A single alkylating agent can generate a range of soft and hard electrophiles. For example, N-methyl-N-nitrosourea yields 68% and 7.5% of total alkylations at the N$^7$ and O$^6$ positions of DNA guanine, respectively (8). Likewise, chloroethylnitrosoureas form major and minor DNA adducts at the N$^7$ and O$^6$ positions of guanine, respectively (9).

DNA cross-linking agents occupy a key position in the currently available cancer chemotherapeutic arsenal. Although the precise mechanisms by which these agents...
preferentially kill tumor cells remain unclear, it is generally accepted that rapidly dividing cells are more susceptible to such assault, whereas normal cells possess the capacity to recover from DNA damage by genome surveillance mechanisms. Compounds that chloroethylate the O6 position of guanine are unique for the following reasons. First, studies conducted with chloroethylnitrosoureas and 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE) have provided evidence that O6-chloroethylguanine is the critical DNA lesion that progresses to lethal interstrand DNA cross-links (9–11). Secondly, the O6-alkylguanine lesion is subject to repair by O6-alkylguanine-DNA alkyltransferase (AGT; ref. 12). Finally, variability in the AGT content exists between tumor and host cells (13). Thus, a difference in AGT content in tumor and host cells constitutes an exploitable target resulting in a therapeutic basis for the antineoplastic efficacy of cloretazine.

In the present report, we have analyzed the role of the chloroethylating and carbamoylating portions of cloretazine in generating the cytotoxicity of this agent. Because AGT is expressed in various mouse organs (14), the relative tumor selectivity of cloretazine to L1210 cells derives at least in part from the absence of AGT expression in these leukemia cells and the presence of AGT in host tissues. We also show a greater capacity of cloretazine to generate an O6-chloroethylguanine lesion than the nitrosourea BCNU, which also generates this lesion, in L1210 cells, an explanation for the greater potency of the sulfonfonylhydrazine prodrug against the L1210 leukemia (2). A relationship seems to exist between the expression of AGT and the functional status of p53 in the murine cell lines employed. Recent studies by others have provided convincing evidence that the AGT gene is silenced in AGT-cell lines through promoter hypermethylation (15) and that silencing of the AGT gene by this mechanism is a common event in human primary neoplasms (16). The clinical relevance of these events in the utilization of cloretazine as a cancer chemotherapeutic agent is discussed.

Materials and Methods

Cell Lines and Growth Inhibition Assay

Five murine cell lines in suspension used in these experiments were the L1210 lymphocytic leukemia (17), the P388 lymphocytic leukemia (18), the F-MEL Friend murine erythroleukemia (19), the WEHI-3B myelomonocytic leukemia (20), and the Ba/F3 interleukin-3-dependent bone marrow-derived cell line (21). Cell lines were maintained in medium (RPMI 1640 for L1210 and P388, DMEM for F-MEL, and McCoy’s 5A for WEHI-3B) supplemented with 10% fetal bovine serum (FBS) in a humidified CO2 incubator. Ba/F3 cells were maintained in RPMI 1640 containing 10% FBS and 10% conditioned medium from WEHI-3B cells (WEHI-CM) as a source of interleukin-3. All cell lines grew with a doubling time of ~11 hours and reached saturation densities of 2.0 × 106 to 3.5 × 106 cells/mL. Stock solutions of sulfonfonylhydrazine prodrugs were made in anhydrous DMSO at a concentration of 200 mmol/L. Growth inhibition assays were carried out in 24-well plates in a volume of 1 mL/well. Cells were seeded at an initial density of 7 × 104 cells/mL and drug solution (1 μL) made in DMSO to give various concentrations was added to each well. The plate was shaken by hand at each addition to disperse the drug solution into the cell suspension. This practice was important to achieve consistent results because of the short half-lives of some of the compounds. After incubation for 3 days, cell numbers were determined using a Coulter (Hialeah, FL) counter with a Multisizer II analyzer. The percent inhibition of cell growth was calculated by the following formula: \[ \log(\text{final density of the control culture}) - \log(\text{final density of the treated culture}) \] / \[ \log(\text{final density of the control culture}) - \log(\text{initial density of the control culture}) \] × 100. DMSO at a final concentration of 0.1% had no effect on cellular growth.

Stable Transfection of the Murine AGT Gene into L1210 Cells

Murine AGT cDNA cloned into pCMV-SPORT6 (BC031888) was obtained from Open Biosystems (Huntsville, AL). Because the level of expression of transfected genes under the control of the cytomegalovirus promoter in murine hematopoietic cells is exceedingly low, AGT cDNA was subcloned into the expression plasmid p75/p15, which contained the human metallothionein IIA promoter (22). The AGT coding region was amplified with BamHI (5′) and XbaI (3′) sites by PCR and granulocyte colony-stimulating factor receptor cDNA located at BamHI/XbaI sites in p75/p15 (22) was replaced by the AGT coding region. The DNA fragment generated by PCR was verified by sequencing.

For transfection, 2 × 106 L1210 cells, washed with serum-free medium and suspended in 0.25 mL Opti-MEM (Life Technologies, Inc., Rockville, MD), were mixed with 1.25 mL Opti-MEM containing 5 μg p75/p15-AGT plasmid DNA and 30 μL TransFast (Promega, Madison, WI). After incubation at 37°C for 1 hour, medium (3 mL) containing 15% FBS was added and cells were incubated for 1 day for expression. Because p75/p15 contained the neo' selection marker, colonies were selected in semisolid medium containing 0.8% methyl cellulose, 15% FBS, and 0.8 mg/mL G418. Mixed populations of neo' cells were also obtained in liquid medium containing 10% FBS and 0.8 mg/mL G418. Once continuous cell growth was established in the presence of G418, the G418 was removed from the medium.

Northern and Southern Hybridization and Reverse Transcription-PCR

Total cellular RNA was extracted from 8 × 106 to 10 × 106 cultured cells using the Trizol reagent (Invitrogen, Carlsbad, CA). RNA (10 μg/lane) was separated by 1.2% agarose/6.1% formaldehyde gel electrophoresis, stained with acridine orange, and transferred to a nylon membrane according to standard procedures. Northern hybridization was done by a nonradioactive method using digoxigenin-labeled antisense RNA probes according to the manufacturer’s instructions (Roche Diagnostics Corp., Indianapolis, IN). Transcription vectors used to make antisense RNA
probes were pCRII-TOPO containing a 647-bp fragment of the murine AGT coding region, pCR2.1-TOPO containing a 1,370-bp fragment of the murine p53 coding region, and pTRI-amp18 containing a part (249 bp) of murine actin cDNA (Ambion, Austin, TX). These vectors were digested at the 5’ terminus of the coding sequence and labeled with digoxigenin-UTP by appropriate phage polymerases in the antisense orientation.

Genomic DNA was extracted from 2 × 10⁷ cells using the DNA ZOL reagent (Invitrogen). DNA was digested with HindIII/EcoRI, separated by 0.8% agarose gel electrophoresis, denatured, transferred to a nylon membrane, and hybridized with the digoxigenin-labeled antisense AGT RNA probe described above.

Reverse transcription-PCR for the coding region of p53 (1,370 bp) from Ba/F3 and F-MEL cells and the coding region of AGT (642 bp) from CLO-R3 (cloretazine-resistant) cells was carried out using total cellular RNA and the Titan One-Tube reverse transcription-PCR system (Roche Diagnostics) according to the manufacturer’s instructions.

**Western Blot Analyses**

Cells (8 × 10⁶), washed once with HBSS, were solubilized in 80 μL high-salt extraction buffer (23), except that the concentration of NP40 was 0.2% instead of 1%. Cell lysates, cleared by centrifugation at 16,000 × g for 2 minutes, were separated by 7.5% PAGE, transferred to a nitrocellulose membrane, and probed with anti-p53 antibody (sc-6243, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membrane was stripped and reprobed with anti-HSC 70 antibody (sc-1059, Santa Cruz Biotechnology) as a loading control.

**Development of Ba/F3 Cells Resistant to Cloretazine**

To develop resistance to cloretazine in Ba/F3 cells, cells were exposed to cloretazine at gradually increasing concentrations starting from 25 μmol/L (IC₅₀). After a period of 4 months, mixed cell populations growing in the presence of 44 μmol/L drug (CLO-RM) were obtained. From these populations, clones (CLO-R1–CLO-R3) were collected from semisolid medium containing 0.8% methyl cellulose, 10% FBS, 10% WEHI-CM, and 50 μmol/L cloretazine.

**Results**

**Biological Targets of Electrophiles Generated by Cloretazine**

The structures of cloretazine, 90CE, its chloroethylating-only analogue, and 1,2-bis(methylsulfonyl)-1-[(methylamino)carbonyl]hydrazine (101MDCE), its carbamoylating-only analogue, are shown in Fig. 1A. Cloretazine undergoes base-catalyzed activation with a half-life of ~1 hour at pH 7.5 and 37°C, whereas the half-lives of 90CE and 101MDCE under these conditions are ~30 seconds and 3.5 minutes, respectively (3, 4, 11). Figure 1B depicts electrophiles generated by cloretazine and potential chloroethylating species thereof. The route leading to an interstrand N1G-N3C DNA cross-link involves formation of intermediate N1,G0-ethanoguanine via intramolecular cyclization and halide loss (11).

**Growth Inhibition of Various Murine Cell Lines by Cloretazine, 90CE, and 101MDCE and Relationship between Expression of AGT and Functional Status of p53**

Cells were continuously exposed to various concentrations of cloretazine, 90CE, and 101MDCE for 3 days, and the percent inhibition of cellular growth was determined using the log of the cell density from control and treated cultures. Five cell lines were separated into two groups according to the sensitivity to the chloroethylating agent.
90CE; the sensitive group consisted of L1210, P388, and F-MEL cells (IC_{50} between 6.5 and 7.5 \mu mol/L), whereas Ba/F3 and WEHI-3B cells belonged to a group more resistant to 90CE (IC_{50} 52 and 70 \mu mol/L; Fig. 2A, middle, and B). The O^6-alkylguanine-specific repair protein AGT (12) was considered the most likely cause of the differential sensitivity. Because commercially available antihuman AGT antibody was weakly reactive with murine AGT and inadequate for Western blot analyses, we measured AGT at the level of mRNA expression by Northern hybridization. As shown in Fig. 2C, the 90CE-resistant cell lines Ba/F3 and WEHI-3B expressed AGT mRNA, whereas AGT mRNA was undetectable in the 90CE-sensitive cell lines, L1210, P388, and F-MEL. CLO-R3 and CLO-RM were derivatives of Ba/F3 cells made resistant to cloretazine; these selected cell lines will be described later.

In contrast to the differential cytotoxicity exhibited by 90CE, the carbamoylating agent 101MDCE caused growth inhibition of all cell lines at similar concentrations, with IC_{50} from 35 to 68 \mu mol/L (Fig. 2A, bottom, and B). In AGT^+ cell lines (i.e., Ba/F3 and WEHI-3B), 101MDCE expressed AGT mRNA, whereas AGT mRNA was undetectable in the 90CE-sensitive cell lines, L1210, P388, and F-MEL. CLO-R3 and CLO-RM were derivatives of Ba/F3 cells made resistant to cloretazine; these selected cell lines will be described later.

The combined effects of 90CE and 101MDCE accounted for the cytotoxicity of cloretazine. Thus, the chloroethylating activity of cloretazine predominated in AGT^- cell lines, whereas both carbamoylating and chloroethylating activities contributed to the overall cytotoxicity of this agent in AGT^+ cell lines (Fig. 2A, top, and B).

These studies showing that the absence of AGT expression is important for cloretazine to act optimally as a chloroethylating agent prompted us to investigate potential factors that regulate AGT gene expression. Previous studies have shown that AGT is induced by exposure to ionizing radiation in mice (24) and in cultured cells (25) in a p53-dependent manner, indicating that AGT expression is at least in part under the control of p53. For this reason, we determined the functional status of p53 in our murine cell lines. Excess amounts of p53 protein under nonstressed conditions indicate a mutation in p53, often resulting from the deletion of one copy and a mutation in another (26). Western blot analyses indicated that p53 in P388 and F-MEL cells is mutated (Fig. 2C). Northern hybridization, on the other hand, revealed a total lack of p53 mRNA in L1210 cells, indicating deletion of both alleles, exemplified in HL-60 human promyelocytic leukemia cells (27). Thus, the p53 gene in all of the AGT^- cell lines was either mutated or deleted, whereas both of the AGT^+ cell lines seemed to express wild-type p53. DNA sequencing of the coding region of p53 obtained by reverse transcription-PCR from Ba/F3 and F-MEL cells revealed wild-type and a point mutation (codon 283: GAA to GGA, E283G), respectively. Furthermore, exposure of Ba/F3 cells to 2 \mu mol/L doxorubicin resulted in a progressive and pronounced accumulation of p53, indicating that p53 in Ba/F3 cells was functional (data not shown).

**Stable Transfection of AGT into L1210 Leukemia Cells**

Isogenic AGT^- L1210 cells were generated by transfection of an AGT expression plasmid into wild-type L1210 cells.
Measurement of AGT mRNA in AGT transfectants showed high levels of AGT expression (Fig. 3A). AGT transfectants exhibited greater resistance to 90CE (18-fold) than natural AGT+ cell lines (i.e., Ba/F3 and WEHI-3B) presumably due to higher levels of AGT expression (Fig. 3B, middle, and C).

The growth-inhibitory activity of 101MDCE was unaffected by AGT transfection (Fig. 3B, bottom, and C). Because the active site of AGT is a cysteine residue and AGT can be inactivated by the carbamoylating activity of cloretazine, there has been speculation that cloretazine is effective in AGT+ cells depending on the level of AGT. This possibility is particularly attractive in that it accounts for the broad-spectrum antitumor activity of cloretazine and its capacity to cure L1210/BCNU, a cell line resistant to the nitrosourea in part due to a 3-fold increase in AGT. However, the cytotoxicity of cloretazine (IC50s, 33 and 34 μmol/L) was not much greater than that of 101MDCE alone (IC50s, 36 and 37 μmol/L) in AGT transfectants. If methyl isocyanate generated by cloretazine was an effective inhibitor of AGT in AGT transfectants, one would have expected greater cytotoxicity by cloretazine due to its DNA cross-linking activity. AGT is presumed to be present to a large extent as a DNA-bound form in cells through non-sequence-specific DNA binding (28). Our previous in vitro studies have also shown that the ability of 101MDCE to inactivate AGT in the DNA-bound form is greatly diminished, although methyl isocyanate from 101MDCE effectively inhibits AGT in the absence of DNA (4).

**Extent of O6-Chloroethylation of Guanine in DNA by Cloretazine and BCNU and Modulation of Chloroethylating and Carbamoylating Activities by O6-Benzylguanine and α-Monothioglycerol, Respectively**

Because O6-chloroethylguanine is the critical DNA alkylation leading to the lethal DNA cross-links produced by cloretazine and BCNU, differences in the IC50s for these compounds in the wild-type L1210 and AGT transfectant AGT1 cells reflect the extent of O6-chloroethylation of guanine in DNA by these agents. Comparison of the extent of O6-chloroethylation of guanine by cloretazine and BCNU showed that the ratio of the IC50s (AGT1/L1210) was 7 for cloretazine and 2 for BCNU, indicating that cloretazine is a substantially more selective generator of O6-chloroethylguanine lesions than BCNU (Fig. 4A and B). Clinically useful DNA cross-linking agents, such as nitrogen mustards (melphalan and chlorambucil), mitomycin C, and cisplatin, did not show any differential cytotoxicity to AGT1 relative to L1210, indicating the uniqueness of cloretazine and BCNU among the group of DNA cross-linking agents (data not shown).

O6-Benzylguanine is a potent inhibitor of AGT, although murine AGT is reported to be less sensitive than human AGT (12, 29). Pretreatment of the AGT-expressing cell lines, Ba/F3 and AGT1, with O6-benzylguanine at a concentration of 20 μmol/L for 30 minutes resulted in marked sensitization to 90CE; thus, Ba/F3 cells became almost as sensitive as L1210 cells to 90CE, although reversal of the resistance of AGT1 cells to this agent was not complete presumably due to the relatively high content of AGT (Fig. 4C). The lack of sensitization of L1210 cells to 90CE by O6-benzylguanine is consistent with the lack of AGT mRNA expression in these cells.

The activity of most alkylating agents is enhanced by glutathione depletion (30). Conversely, an increase in cellular glutathione content is one of the most common mechanisms associated with resistance to alkylating agents. We employed α-monothioglycerol (MTG) to examine whether the cytotoxicity of the chloroethylating

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**Figure 3.** Stable transfection of AGT into L1210 cells. A, representative Northern blots for AGT and actin from AGT transfectants. AGT1, AGT2, and AGT3 were clones of L1210 cells selected in semisolid medium containing G418. AGTM was a mixed population selected in G418 containing liquid medium. B and C, growth inhibition curves and IC50s were determined as in Fig. 2A and B.
Characterization of Ba/F3 Cells Resistant to Cloretazine

The carbamoylating agent 101MDCE (IC_{50}, 35 \mu mol/L) is slightly more toxic than the chloroethylating agent 90CE (IC_{50}, 52 \mu mol/L) in AGT-expressing Ba/F3 cells. The resistant Ba/F3 cells were developed by exposure to cloretazine in gradually increasing concentrations starting at a concentration of 100 \mu mol/L, protected L1210 cells from the cytotoxicity of methyl isocyanate generated by the decomposition of 101MDCE, the cytotoxicity of 90CE was barely influenced by MTG (Fig. 4D). These findings are an indication that 90CE generates hard chloroethylating electrophiles that preferentially react with hard nucleophilic centers, such as the O^6 position of guanine in DNA, rather than soft thiol nucleophiles, such as MTG.

**Discussion**

The availability of cloretazine analogues with the capacity to chloroethylate only (90CE) and carbamoylate only (101MDCE) made a dissection of the contribution of the two reactive components of cloretazine to the overall cytotoxicity of this agent possible. With respect to AGT, human cell lines historically have been called mer^+/− (N-methyl-N’-nitro-N-nitrosoguanidine damage repair; ref. 31) or mex^+/− (methyl excision; ref. 32) phenotypes. We have characterized several murine cell lines by their sensitivity to the chloroethylating agent 90CE relative to the cellular content of AGT. L1210 cells seemed to be completely deficient in AGT expression as assessed by the absence of AGT mRNA expression and the lack of sensitization to 90CE by O^6-benzylguanine, the potent inactivator of AGT. The chloroethylating agent 90CE caused growth inhibition at concentrations considerably below the growth-inhibitory concentrations of 101MDCE in AGT^+ cell lines, whereas the carbamoylating agent, 101MDCE, was slightly more cytotoxic than 90CE, and both carbamoylating and chloroethylating actions seemed to contribute to the overall cytotoxicity of cloretazine, in AGT^+ cell lines. These findings imply that the cellular content of AGT is the single most important predictor of tumor susceptibility to cloretazine. Furthermore, these results are compatible with the recent clinical observation that the highest response rates to cloretazine in acute myelogenous leukemia patients are associated with the lowest AGT levels in leukemic blasts (33). Application of temozolomide to deplete AGT before cloretazine treatment is currently in a phase I trial with hematologic malignancies (33).

Stable transfection of AGT in L1210 cells produced cell lines expressing AGT at a level significantly higher than that found in natural AGT^+ cell lines. These isogenic AGT^- and AGT^+ cells provided a system to measure the extent of cytotoxicity due to alkylations at the O^6 position of guanine in DNA. Comparison of cloretazine and BCNU in these cell lines revealed that cloretazine generated a higher yield of O^6-chloroethylguanine DNA lesions than BCNU; the ratio of the IC_{50} for AGT^−/AGT^+ cell types was 7-fold for cloretazine versus 2-fold for BCNU. These findings are compatible with several previous observations. Thus, BCNU alkylates the N^7 position of DNA guanine to a

**Figure 4.** Differential cytotoxicity of cloretazine and BCNU in L1210 and AGT1 cells and modulation of the cytotoxicity of 90CE and 101MDCE by O^6-benzylguanine and MTG. A and B, growth inhibition curves of cloretazine and BCNU in L1210 and AGT1 cells and their IC_{50} were determined as in Fig. 2A and B. C, cells were continuously exposed to 90CE alone or 90CE plus O^6-benzylguanine (BG). O^6-Benzylguanine at a concentration of 20 \mu mol/L was added to cells 30 min before the addition of 90CE. D, the concentration of MTG that was not growth inhibitory by itself was predetermined (100 \mu mol/L) and added to cells before addition of 90CE or 101MDCE.
greater extent than the $O^6$ position (9). The difference in the LD$_{50}$ values for BCNU in wild-type and AGT knockout mice is <2-fold (34). In an in vitro DNA cross-linking assay, BCNU caused a relatively large number of DNA strand breaks due to depurination resulting from the alkylation of the $N^7$ position of guanine, whereas cloretazine did not (11). That the cytotoxicity of 90CE in L1210 cells was barely modulated by the presence of the thiol, MTG, is another indication that 90CE produces hard nucleophiles with a preference for hard nucleophilic centers in DNA, such as the $O^6$ position of guanine. Thus, the antitumor superiority of cloretazine over that of BCNU against the L1210 leukemia (2) can be explained in part by the greater tumor selectivity of cloretazine exerted on AGT$^+$ host cells. The cytotoxicity of the clinically useful DNA cross-linking agents, melphalan, chlorambucil, mitomycin C, and cisplatin, was not affected by the cellular content of AGT, a finding that points to the uniqueness of the DNA cross-link produced by cloretazine and BCNU among the DNA cross-linking agents.

In light of AGT being a decisive factor in determining the degree of tumor susceptibility to cloretazine, the mechanism(s) underlying the variability of AGT expression in tumor and normal cells warrants intense investigation. In five murine cell lines studied in this report, a correlation existed between AGT expression and functional status of p53, such that p53 was absent or nonfunctional in AGT$^-$ cells. Because a deficiency in AGT expression in cultured cells, as well as the loss of p53 function in tumors, occurs at high frequency (26, 35), the relationship can be fortuitous. However, such a relationship occurs in several other cell lines, such as Chinese hamster ovary, 3T3, and HCT-116, and p53 mutations are observed in AGT$^+$ human primary brain tumors, cell lines, and xenografts (36). Transformation and/or immortalization of cultured cells with the SV40 large T antigen, a process that inactivates tumor suppressor genes, such as p53 and pRb, often results in a loss of AGT expression (37). Recent studies have produced convincing evidence that the AGT gene is silenced by promoter hypermethylation in AGT$^+$ cultured cells as well as in AGT$^+$ primary tumors (15, 16, 37). Such a mechanism is compatible with the observation that AGT$^+$ cultured cells completely lack AGT expression at the levels of protein (38) and mRNA. Because it has become clear that epigenetic malfunctions occur during the development of malignancy (39), we conclude that the p53-inducible gene AGT often becomes silenced during tumor progression that involves loss of p53 function. Because inactivation of the p53 gene itself does not abrogate the expression of AGT (24), silencing of the AGT gene must be dependent on other factors involved in tumor evolution.

In this report, we show that cloretazine is a more selective generator of $O^6$-chloroethylguanine alkylations than BCNU and that these lesions account for a greater portion of the cytotoxicity of cloretazine based on the attenuation of the cytotoxicity of this agent by the expression of AGT. These conclusions are consistent with previous findings from our laboratory conducted with Chinese hamster ovary cells transfected with human AGT (40), human mer$^{-/-}$ cell lines (2, 41), and in vitro DNA cross-linking assays (4, 11). $O^6$-Chloroethylguanine is a lesion that progresses to DNA interstrand G–C cross-links. The formation of such DNA cross-links is quite a cytotoxic lesion in that it is difficult to repair and therefore persists in cells; for this reason, cytotoxicity can be delayed for days to weeks following exposure to agents that produce $O^6$-chloroethylation of DNA guanine (42). Thus, for therapeutic efficacy, it is extremely important for normal host tissue cells to possess a mechanism of protection from such a lethal event. AGT provides an effective
mechanism of protection for host cells, allowing cloretazine to selectively target AGT tumor cells or those malignant cells with relatively low concentrations of AGT. Among the DNA cross-linking agents currently available for cancer chemotherapy, cloretazine and the chloroethylnitrosoureas are the only agents whose cytotoxicity is intervened by AGT. Although combination therapy of BCNU with an AGT inhibitor to enhance efficacy has been clinically pursued, this approach does not address the issue of tumor selectivity, as host cells can also be sensitized. Because cloretazine is a more selective generator of issue of tumor selectivity, as host cells can also be sensitized.

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