Molecular and Cellular Pharmacology of the Novel Noncamptothecin Topoisomerase I Inhibitor Genz-644282

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Abstract

Camptothecin derivatives are powerful anticancer drugs because of their ability to trap topoisomerase I (Top1)–DNA cleavage complexes. However, they exhibit clinical limitations due to the instability of their α-hydroxylactone six-membered E-ring structure. In addition, they exhibit bone marrow and intestinal toxicity, especially in adults, and are drug efflux substrates. Here, we report a novel Top1 inhibitor, Genz-644282. We show that Genz-644282 and its metabolites induce Top1 cleavage at similar, as well as unique genomic positions, compared with camptothecin. The compound also induces protein-linked DNA breaks and Top1–DNA cleavage complexes that persist longer after compound removal than camptothecin. Concentration-dependent and persistent γH2AX formation was readily observed in cells treated with Genz-644282, and was present in greater than 50% of the cell population following 24 hours compound exposure. The compound shows partial cross-resistance in cell lines resistant to camptothecin. These cell lines include the human prostate DU145RC0.1 and the leukemic CEM/C2 cells. Limited cross-resistance to Genz-644282 was also found in the Top1 knockdown colon cancer (HCT116) and breast cancer (MCF7) cell lines and in human adenocarcinoma cells (KB31/KBV1) that overexpress (P-glycoprotein, ABCB1), a member of the ATP-binding cassette family of cell surface transport proteins known to confer MDR. Together, our results provide the first molecular and cellular characterization of Genz-644282 and its clinically relevant metabolites.

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marrow suppression and diarrheas), especially in adults, which hinders the use of full therapeutic regimens. Finally, camptothecins are pumped out of the cells by ABCG2, an ABC drug efflux transporter that confers a high degree of resistance to various anticancer drugs (13).

Two chemical approaches have thus far been taken to overcome the instability and inactivation of camptothecins. The first was to enlarge the E-ring by 1 carbon atom. This limits the opening of the E-ring; however, it also prohibits its reclosure (6), which is potentially problematic, as irreversible E-ring opening inactivates the drug. Such synthetic analogues have been named the homocampothecins with diplometocan being the clinical derivative. The second approach has been to remove 1 carbon atom, which eliminates the α-hydroxylactone function and converts the E-ring from a 6- to a 5-membered highly stable ring while preserving potent inhibitory activity against Top1 (14). Those derivatives are referred to as the α-keto derivatives with S39625 selected for clinical trials (6).

Novel Top1 inhibitors have also been the focus of academic and industrial laboratories for several years (1, 4). Three classes of noncamptothecin Top1 inhibitors have reached clinical development: the indolocarbazoles, the indenoisoquinolines, and the dibenzo[a,d]naphthyridones class of noncamptothecin Top1 inhibitors (4, 15, 16). The indolocarbazoles were the first in clinical development. However, they affect other cellular targets besides Top1 and their current clinical development as anticancer drugs appears to have been put on hold. Two indenoisoquinolines have just begun clinical trials at the U.S. National Cancer Institute (NCI) with γH2AX as biomarker of genomic damage before and after treatment (17). Here, we present molecular and cellular studies of Genz-644282 and its metabolites from the dibenzo[a,d]naphthyridones class of noncamptothecin Top1 inhibitors (18, 19), which have also recently begun phase 1 clinical trial. The basis for choosing compound Genz-644282 for clinical development is that the metabolite Genz-649975 is not an active antitumor agent, likely due to rapid metabolism. Genz-649975 is an active antitumor agent but is more toxic by body weight loss than Genz-644282.

Materials and Methods

Drugs, enzymes, chemicals

Camptothecin and topotecan were obtained from the Drug Synthesis and Chemistry Branch, NCI. MJ-III-65 (NSC 706744) was synthesized as described (20). Genz-644282 citrate salt, Genz-649974, Genz-649975, and Genz-649978 were provided from Genzyme Corporation. Doxorubicin and amsacrine (m-AMSA) were obtained from Sigma. Drug stock solutions were made in 10 mmol/L aliquots in DMSO for camptothecin and topotecan, 5 mmol/L aliquots for MJ-III-65 and Genz-644282, and 1 mmol/L aliquots for Genz-649974, Genz-649975, and Genz-649978. Aliquots were stored at −20°C, and additional dilutions were made in dimethyl sulfoxide (DMSO) immediately before use. For cytotoxicity assays, additional dilutions were done in cell culture medium. The final concentration of DMSO in the reaction mixtures did not exceed 10% (v/v).

Human recombinant Top1 was purified from baculovirus (17) and human recombinant Top2α was a generous gift from Dr. Neil Osheroff (Vanderbilt University, Nashville, TN).

Cell lines

Human colon HCT116 and breast MCF7 cancer cells were obtained from the NCI Developmental Therapeutics Program. The cells lines were characterized in our laboratory by DNA fingerprinting (21). The stably transfected HCT116 Top1 short interfering RNA (siRNA; HCT116siTop1) and MCF-7 Top1 siRNA (MCF7-siTop1) cells were derived in our laboratory as described (22). HCT116 cells were maintained in RPMI 1640 (Invitrogen) containing 10% FBS (Gemini Bio-Products) and MCF7 cells were maintained in Dulbecco’s Modified Eagle’s Media (DMEM) supplemented with 10% FBS. Si-Top1 cells for both HCT116 and MCF7 were maintained with the addition of 100 μg/mL hygromycin. H460 human lung cancer stable transfectants expressing wild-type ABCG2 and KB human cervical carcinoma expressing MDR-1/P-glycoprotein were a kind gift from Dr. Matthew D. Hall and Dr. Michael M. Gottesman (Laboratory of Cell Biology, Center for Cancer Research, NCI, NIH) and were maintained in RPMI supplemented with 10% FBS and either 20 ng/mL mitoxantrone or 1 μg/mL vinblastine, respectively. The DU145 cell line was obtained from the American Type Culture Collection. The RC0.1 cell subline was derived from DU145 cells as previously described (23, 24) and maintained in RPMI and 10% FBS. The CCRF-CEM cell line was obtained from the American Type Culture Collection and the CCRF-CEM C2 cell line was established as previously described (25, 26) and maintained in RPMI containing 10% FBS.

Top1/Top2-mediated DNA cleavage assays

For Top1-mediated DNA cleavage reactions, a 117-bp DNA oligonucleotide (Integrated DNA Technologies) containing a single 5′-cytosine overhang was 3′-end labeled by fill-in reaction with [α-32P]-dATP and DNA polymerase I. In addition, a shorter 23-bp 3′-end scissile strand–labeled duplex oligonucleotide (5′-AAAAAGACTT^GGAAAAATTTTTA-3′) with a single Top1 cleavage site ^ (caret) in Fig. 2A was also generated using [α-32P]-dATP and terminal deoxyribonucleotidyl transferase. For Top2 reactions, the recessed strand from the 117-bp DNA oligonucleotide was 5′ labeled using T4 polynucleotide kinase and [γ-32P] ATP. The 5′-labeled single-stranded oligonucleotide was then annealed with its complementary strand by heating for 5 minutes at 95°C and slowly cooling to room temperature. DNA cleavage reactions were prepared as previously reported (27). Briefly, approximately 2 mmol/L of radiolabeled DNA substrate was incubated with or without recombinant Top1 or Top2 in 20 μL of reaction buffer (10 mmol/L Tris-HCl, pH 7.5, 50 mmol/L KCl, 5 mmol/L MgCl2, 100 mmol/L DTT, and 0.5% nonidet-P-40).
0.1 mmol/L EDTA, and 15 μg/mL BSA) at 25°C for 20 minutes in the presence of various concentrations of drugs. The reactions were terminated by adding SDS (0.5% final concentration) followed by the addition of 2 volumes of loading dye (80% formamide, 10 mmol/L sodium hydroxide, 1 mmol/L sodium EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). The cleavage products were separated on a 16% sequencing gel. Imaging and quantification were conducted using the Typhoon 8600 and ImageQuant Software (Molecular Dynamics), respectively.

Detection of cellular Top1–DNA complexes

Top1–DNA adducts were isolated using cesium chloride gradient centrifugation (17, 28, 29). Briefly, 10⁶ HCT116 cells were treated with 1 μmol/L of each compound for 1 hour or left untreated. The medium was removed and cells were scraped and collected in buffer containing 1% sarkosyl, 8 mol/L guanidine hydrochloride, 30 mmol/L Tris, pH 7.5, and 10 mmol/L EDTA. Samples were homogenized with a Dounce homogenizer, cell lysates were gently layered on cesium chloride step gradients, and centrifuged at 30,700 rpm for 20 hours (ϕ = 167,400) at 20°C. DNA fractions of 0.5 mL were pooled and diluted with an equal volume of 25 mmol/L sodium phosphate buffer (8.8 and 16.2 mmol/L NaH2PO4, pH 6.5). Serial dilutions of each DNA fraction were made and blotted on Immobilon-P membranes (Millipore) using a slot-blot vacuum. Top1–DNA complexes were detected using the C21 Top1 monoclonal antibody (a kind gift from Dr. Yung-Chi Cheng, Yale University, New Haven, CT) and standard Western blotting procedures.

Alkaline elution assay for the detection of Top1–DNA cross-links

The alkaline elution assay was done to detect DNA–protein cross-links (DPC) as previously described (17, 30, 31). Human colon cancer HCT116 cells were pre-labeled with 0.2 μCi/mL of [3H]-thymidine for 1 to 2 doubling times at 37°C and chased in nonradioactive medium overnight. Cells were treated for 1 hour with 1 μmol/L of Genz-644282 or camptothecin. After compound exposure, cells were scraped in HBSS. For the reversal experiments, the cells were cultured in compound-free medium for the indicated times before scraping. DPCs were analyzed under nondeproteinizing, DNA-denaturing conditions using protein-adsorbing filters and the DPC frequencies were calculated as previously described (17, 30, 31).

Immunofluorescence microscopy detection of γH2AX

HCT116 and MCF7 cells were grown in culture medium on chamber slides. After 1 hour drug treatment, cells were fixed with 2% paraformaldehyde in PBS (Mediatech Inc.), washed in PBS, and permeabilized in ice-cold 70% ethanol. Slides were blocked with PBS containing 8% bovine serum albumin (BSA; Jackson ImmunoResearch Laboratories) for 1 hour at room temperature and incubated in 1% BSA-PBS solution with anti-γH2AX antibody (05-636; Millipore). Slides were then washed 3 times in PBS for 10 minutes, incubated for 90 minutes at room temperature with Alexa Fluor 488–conjugated goat anti-mouse IgG secondary antibody (Molecular Probes) in 1% BSA-PBS solution at a 500× dilution and washed in PBS. Slides were then washed and sealed with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vectorshield with DAPI; Vector Laboratories, Inc.) and were viewed using a BD Pathway 800 Series microscope with a 60× objective.

Two-dimensional flow cytometric analysis for DNA and γH2AX

HCT116 cells were treated with 1 μmol/L camptothecin or Genz-644282 for 1, 3, 6, or 24 hours. After treatment or the appropriate reversal time in compound-free medium, cells were harvested, washed twice with ice-cold PBS, and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Cells were then washed with 1 mL of ice-cold PBS and permeabilized with 1 mL prechilled (−20°C) 70% ethanol for 20 minutes at room temperature. Cells were again washed in PBS and further permeabilized with ice-cold 0.25% Triton X-100 in PBS for 5 minutes on ice, washed in PBS, and incubated with anti-γH2AX antibody at a 250× dilution in PBS/1% BSA for 1 hour at room temperature. Cells were washed with PBS and incubated with goat anti-mouse Alexa Fluor 488 antibody (Molecular Probes) at 250× dilution in PBS/1% BSA for 30 minutes at room temperature. Cells were washed with PBS and resuspended in 500 μL of PBS solution containing 50 μg/mL propidium iodide (PI) and 0.5 mg/mL RNase A. Analyses of FL2-A (PI) versus FL1-H (γH2AX) were done using a FACScan flow cytometer (Becton Dickinson). Cell-cycle distributions were calculated using ModFit LT software (Verity Software House, Inc.).

DNA unwinding assays

DNA unwinding was conducted as previously described (32). Briefly, reaction mixtures (10 μL final volume) contained 0.3 μg supercoiled SV40 DNA (New England Biolabs) in reaction buffer (10 mmol/L Tris-HCl, pH 7.5, 50 mmol/L KCl, 5 mmol/L MgCl2, 0.1 mmol/L EDTA, and 15 μg/mL BSA) and 10 units Top1. Reactions were carried out at 37°C for 30 minutes and terminated by the addition of 1% SDS. A 1.1 μL of 10× concentrated compound solutions was added to relaxed DNA and incubated for 30 minutes. Proteinase K (1 mg/mL) and 1× SDS were added to samples and incubated for an additional 30 minutes at 37°C. Loading buffer (20% Ficoll 400; 0.1 mol/L Na2EDTA, pH 8.0, 1.0% SDS, and 0.25% bromophenol blue) was added and reactions mixtures were loaded onto a 1% agarose gel. After electrophoresis, DNA bands were stained with staining solution. DNA unwinding was measured by quantifying the amount of DNA unwound DNA compared to the total DNA content.
in 1 μg/mL of ethidium bromide and visualized by transillumination with ultraviolet light (300 nm).

**Cytotoxicity assays**
Briefly, cells were seeded in 96-well plates 24 hours before drug treatment. Cytotoxicity of camptothecin, topotecan, doxorubicin, and Genz-644282 in HCT116, MCF7, DU145, and their camptothecin-resistant subclones was assessed by the sulforhodamine B (Sigma Aldrich) assay. In CEM and CEM/C2 cells, H460 and H460/MX20, and KB31 and KBV1 cells, cytotoxicity was measured by the MTS (Promega) colorimetric assay (17). Compound exposures were continuous for 72 hours for both assays. Percentage of growth was calculated relative to control (vehicle-treated cells) after 3 days of culture with control taken as 100.

**Results**

**Genz-644282, Genz-649974, Genz-649975, and Genz-649978 trap Top1cc**
The primary difference between Genz-644282 and its metabolites, Genz-649975, Genz-649974, and Genz-649978, (shown in Fig. 1A) is the conversion of the methoxy to a hydroxyl group at the positions indicated by the dashed circles. To determine the effects of those compounds on Top1, the cleavage site distribution of Genz-644282 and its metabolites was tested using the 3'-end labeled 117-bp oligonucleotide in the presence of recombinant Top1 (17, 27). Figure 1B shows the induction of Top1cc by Genz-64282, Genz-649974, and Genz-649975 and to a lesser extent by Genz-649978. All 4 compounds exhibit cleavage at similar as well as unique
sites from camptothecin and the indenoisoquinoline MJ-III-65 (17). Like camptothecin, Genz-644282 and all 3 metabolites showed a strong preference for site 92. However, this cleavage occurred with differences in relative intensity (see sites 37, 44, 62, 70, 97, and 119). These results show the effectiveness of Genz-644282, Genz-649974, and Genz-649975 as Top1 inhibitors at potentially achievable concentrations. Both the similarities and differences of the DNA cleavage patterns from those of camptothecin and the indenoisoquinolines (MJ-III-65) suggest Genz-644282 and its metabolites have comparable binding modes to both camptothecin and MJ-III-65 in the Top1cc (33) but target both common and unique genomic sites.

To further investigate the relative potency of Genz-644282 for Top1cc compared to camptothecin, a 22-bp double-stranded oligonucleotide containing a high-affinity Top1–DNA cleavage site was used (27). Cleavage intensity can be measured by the generation of a 13-mer cleavage product labeled at the 30-end with [32P]cordycepin (Fig. 2A). The sequence of the 22-mer oligonucleotide is depicted with its Top1 cleavage site indicated by the caret between the T and G bases (Fig. 2A). Genz-644282 generates slightly less cleavage at the indicated site for each concentration of drug compared with camptothecin (Fig. 2B). Together the results obtained with the short oligonucleotide substrate (Fig. 2) and the long oligonucleotide substrate (Fig. 1B) show the effectiveness of Genz-644282 and its metabolites as Top1 inhibitors.

**Induction of persistent Top1ccs and DPCs by Genz-644282 in cancer cells**

The induction and stability of Top1cc produced by Genz-644282 were evaluated in human colon cancer carcinoma HCT116 cells using the immunocomplex of enzyme (ICE) and alkaline elution assays (17). Figure 3A shows that Genz-644282 is more potent at trapping Top1–DNA covalent cleavage complexes than either camptothecin or topotecan at the same concentration. In addition, even metabolites of Genz-644282, Genz-649974, Genz-649975, and Genz-649978 are active and effective at trapping Top1 with Genz-649978 being less potent than the others (Fig. 3B).

Reversal experiments were carried out to determine the stability of the Top1 complexes trapped by Genz-644282. Figure 3C shows the persistence of the Top1 complexes for up to 8 hours following compound removal with complete reversal of complexes at 24 hours (Fig. 3C). This cleavage complex stability is greater than that induced by camptothecin, which has been shown to reverse within 1 hour (17, 34). Alkaline elution, another method to measure topoisomerase cleavage complexes (30, 31, 35), was used to quantify Top1cc as DPCs. Genz-644282 induced slightly more DPCs than camptothecin. In addition, the DPCs induced by Genz-644282 were significantly less reversible following compound removal than those induced by camptothecin (Fig. 3D). Together these results indicate greater stability of Top1cc induced by Genz-644282 than camptothecin.

**Generation and persistence of γH2AX foci in MCF7 and HCT116 cells in response to Genz-644282**

To show the DNA damage produced by Genz-644282, γH2AX induction was assessed in cells (8, 17). Both human colon cancer HCT116 cells and breast cancer MCF7 cells were treated with 0.1, 0.5, or 1 μmol/L of Genz-644282 or camptothecin and studied by immunofluorescence after staining with γH2AX antibodies.
(Fig. 4A and B). γH2AX foci were induced by Genz-644282 at concentrations as low as 0.1 μmol/L, and the γH2AX staining increased with compound concentration. γH2AX induction was also evaluated by flow cytometry to determine the cell-cycle dependence and percentage of γH2AX-positive cells (17) following 1 hour of Genz-644282 treatment (Fig. 4C). γH2AX-positive cells were mostly in S-phase as previously showed for camptothecin.
and the indenoisoquinolines (17). Because the Top1cc induced by Genz-644282 were reversed by 24 hours (Fig. 3C), γH2AX flow cytometry was also used to study whether the DNA damage induced by these complexes was persistent. The percent of γH2AX-positive cells induced by Genz-644282 was compared between 24 hours of continuous exposure and 1 hour of exposure followed by incubation in compound-free medium for the indicated times. Left, HCT116 cells were treated as shown. Cells were fixed and incubated with γH2AX antibody and PI. Right, a quantitative comparison of γH2AX-positive cells induced by continuous compound exposure versus a 1-hour treatment followed by incubation in compound-free medium. ND, no drug; CPT, camptothecin.

Activity of Genz-644282 in camptothecin-resistant cell lines

The development of drug resistance often occurs during treatment and presents a major obstacle to curing potentially sensitive cancers (37). Table 1 shows the testing of a panel of camptothecin-resistant cells. It shows that Genz-644282 is able to partially and fully overcome resistance in 2 cell lines resistant to camptothecin that have mutations in Top1. In the prostate cancer RC0.1 cells, the Top1 is catalytically active but is highly resistant to inhibition by camptothecin and its derivatives because of an R364H mutation (24). Genz-644282 displays cross-resistance in the RC0.1 cell line (Table 1). The human leukemia cell line CCRF-CEM is 974 times more resistant to camptothecin than the parental CEM cell line. This is due to the mutation N722S (25), as the asparagine immediately flanking the catalytic tyrosine is important for the binding of camptothecin and topotecan to Top1 (10, 11, 33, 38). Genz-644282 displays almost no resistance in the resistant CCRF-CEM cell line compared with the parental (Table 1).
3-fold resistant to camptothecin and less resistant to Genz-644282 (Table 1).

The drug efflux ABC transporters, ABCG2 (mitoxantrone-resistance-associated/breast cancer resistance protein) and ABCB1 (MDR-1) also confer a high degree of resistance to various anticancer drugs (37). Topotecan and irinotecan are substrates for both. We find that Genz-644282 appears to be a substrate for the ABCG2 mitoxantrone-resistant transporter (Table 1). However, it appears to be less of a substrate for the ABCB1 pump when compared with the reference substrate doxorubicin.

**Discussion**

Here, we show that Genz-644282, which is undergoing phase 1 clinical trial, and its metabolites are potent Top1 inhibitors. Genz-644282 appears to overcome some of the limitations of topotecan and irinotecan, the only clinically approved Top1 inhibitors. One of the primary therapeutic limitations of camptothecin derivatives is the conversion of their α-hydroxylactone into an inactive carboxylate form at physiologic pH. This happens rapidly within minutes and limits the availability of active drug. Unlike camptothecin, the metabolites of Genz-644282 trap Top1cc both in vitro and in cells (Figs. 1B and 3B). The metabolites of Genz-644282 that are most active against Top1 (Genz-649975 and Genz-649974) differ from the parent compound by the demethylation of Genz-644282 (see Fig. 1A). In the least active metabolite, Genz-649978, the side chain of the compound (designated by R) is hydroxylated. The side chain in the parental compound, Genz-644282, and its 2 active metabolites, Genz-649975 and Genz-649974, is critical for achieving the desired properties of the compound as a Top1 inhibitor and increases the affinity of the compounds for DNA.

The similarities of the DNA cleavage patterns compared with those of camptothecin and the indenoisoquinolines and the chemical similarity between Genz-644282 and the indenoisoquinoline derivatives suggest that Genz-644282 and its metabolites have comparable binding modes to both camptothecin and MJ-III-65 in the Top1cc but may have potential uniqueness in the way they target genomic sites (33). The ternary complex structures of camptothecin, indenoisoquinolines, and indolocarbazoles show flat planar ring structures that intercalate between the −1 and +1 bps at the site of DNA–enzyme cleavage (6, 10, 11, 33, 38). In addition, each possesses a major groove substituent and a hydrogen bond acceptor that faces the minor
groove. Genz-644282 also has a relatively flat planar ring structure, and it is possible that the substituents on the nitrogen in the lactam ring in Genz-644282 may be directed into the major groove.

Another limitation of the camptothecins is their rapid dissociation and diffusion from the Top1cc due to their noncovalent binding. This creates a requirement for extended clinical infusions to maintain the persistent cleavage complexes necessary for killing cancer cells (1, 6). Genz-644282 appears to overcome this limitation by creating persistent Top1–DNA cleavage complexes for up to 8 hours following drug removal (Fig. 3C). The stereochemistry produced by the methoxy substituents at the 8 and 9 position tends to limit intercalation. One indication of intercalation is DNA unwinding (32), which we found at a concentration of Genz-644282 of 100 µmol/L (Supplementary Fig. S1A). This dual effect is reminiscent of intercalating Top2 inhibitors such as acridines (amsacrine) and anthracyclines (doxorubicin; refs. 39, 40). Thus, it is plausible that Genz-644282 primarily targets Top1 cleavage sites in DNA at low concentrations that may be clinically relevant. At higher concentrations, the compound may intercalate into DNA at other locations thereby preventing proteins, such as Top2, from binding (Supplementary Fig. S1B). This additional effect might account at least in part for the low cross-resistance of the camptothecin-resistant cells to Genz-644282.

Histone γH2AX has been found to form rapidly in response to Top1 inhibitors (7) and is considered to be a sensitive and selective marker for DNA double-strand breaks (8). The present data show that Genz-644282 induces significant replication-dependent γH2AX at nanomolar concentrations within 1 hour of treatment. Foci could be detected at concentrations as low as 0.1 µmol/L. In addition, the induction of γH2AX by Genz-644282 is persistent and actually increases following drug removal indicating continual DNA damage. This increase in signal could be due to irreversible DNA damage or secondary to apoptosis, which has recently been shown to induce a typical γH2AX ring pattern by confocal microscopy at the periphery of the nucleus as cells initiate their apoptotic response (41, 42). Thus, our data suggest that γH2AX may be used as a pharmacodynamic biomarker to clinically evaluate and monitor the efficacy of Genz-644282 several hours following infusion.

Disclosure of Potential Conflicts of Interest

Beverly A. Teicher was a full-time employee of Genzyme Corporation.

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