The selective poly(ADP-ribose) polymerase-1(2) inhibitor, CEP-8983, increases the sensitivity of chemoresistant tumor cells to temozolomide and irinotecan but does not potentiate myelotoxicity

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Abstract
The effect of the potent and selective poly(ADP-ribose) (PAR) polymerase-1 [and PAR polymerase-2] inhibitor CEP-8983 on the ability to sensitize chemoresistant glioblastoma (RG2), rhabdomyosarcoma (RH18), neuroblastoma (NB1691), and colon carcinoma (HT29) tumor cells to temozolomide- and camptothecin-induced cytotoxicity, DNA damage, and G2-M arrest and on the potentiation of chemotherapy-induced myelotoxicity was evaluated using in vitro assays. In addition, the effect of the prodrug CEP-9722 in combination with temozolomide and/or irinotecan on PAR accumulation and tumor growth was also determined using glioblastoma and/or colon carcinoma xenografts relative to chemotherapy alone. CEP-8983 sensitized carcinoma cells to the growth-inhibitory effects of temozolomide and/or SN38 increased the fraction of and/or lengthened duration of time tumor cells accumulated in chemotherapy-induced G2-M arrest and sensitized tumor cells to chemotherapy-induced DNA damage and apoptosis. A granulocyte-macrophage colony-forming unit colony formation assay showed that coinubcation of CEP-8983 with temozolomide or topotecan did not potentiate chemotherapy-associated myelotoxicity. CEP-9722 (136 mg/kg) administered with temozolomide (68 mg/kg for 5 days) or irinotecan (10 mg/kg for 5 days) inhibited significantly the growth of RG2 tumors (60%) or HT29 tumors (80%) compared with temozolomide or irinotecan monotherapy, respectively. In addition, CEP-9722 showed “stand alone” antitumor efficacy in these preclinical xenografts. In vivo biochemical efficacy studies showed that CEP-9722 attenuated PAR accumulation in glioma xenografts in a dose- and time-related manner. These data indicate that CEP-8983 and its prodrugeffective chemosensitizing agents when administered in combination with select chemotherapeutic agents against chemoresistant tumors. [Mol Cancer Ther 2007;6(8):2290–302]

Introduction
The most significant cause for treatment failure and cancer mortality is radioresistance/chemoresistance. Resistance to these treatments is often a multifactorial process resulting from the following: decreased drug accumulation, defects in the mismatch repair system, low cellular energy levels, or failure of the cells to undergo apoptosis (1, 2). Agents that sensitize tumor cells to the cytotoxic effects of radiation and/or chemotherapeutics while not affecting the toxicities associated with these treatments could improve both the therapeutic index and the success rate of established cancer therapies. Poly(ADP-ribose) (PAR) polymerase (PARP)-1 belongs to the PARP enzyme family that currently includes 18 members; of these members, only PARP-1 and PARP-2 play a role in DNA damage and repair (3, 4). PARP-1 is activated by, and implicated in, base excision repair of DNA strand breaks caused by ionizing radiation, or following enzymatic repair of DNA lesions induced by methylating agents, topoisomerase I inhibitors, and other chemotherapeutic agents, such as cisplatin and bleomycin (5, 6). The role of PARP activation in cell survival and repair following sublethal DNA damage coupled with data showing an inverse correlation of PARP-1 activity with the degree of tumor cell differentiation makes PARP-1 a viable target for modulating the responsiveness of tumor cells to cytotoxic therapies (7, 8). In support of this hypothesis, studies have shown the ability of PARP-1 inhibitors to increase the in vitro and in vivo antitumor effects of radiation or chemotherapy and/or restore sensitivity to temozolomide in temozolomide-resistant tumor cells (1, 9, 10).

Previously, we reported that the PARP-1 inhibitor CEP-6800 modulated the magnitude and/or duration of DNA damage, increased the fraction and/or duration of time tumor cells arrested in the G2-M phase of the cell cycle, and potentiated the antitumor efficacy of temozolomide, irinotecan, and cisplatin against subcutaneous human tumor xenografts (7). A therapeutic index–based strategy
was used for discovering and prioritizing new PARP inhibitors with clinical potential to selectively modulate the cytotoxicity of topotecan and temozolomide and increase their therapeutic index in chemoresistant tumors. These efforts resulted in the identification of CEP-8983 (11), a novel 4-methoxy-carbazole inhibitor of PARP-1 and PARP-2 (enzyme IC₅₀ values of 20 and 6 nmol/L, respectively) with potent activity in cellular assays for inhibition of NAD⁺ depletion (IC₅₀, 300 nmol/L). This report details the in vitro and in vivo chemosensitization profile of CEP-8983 and its prodrug (CEP-9722) against chemotherapy-resistant tumor cell lines and xenografts, and the selectivity of the PARP inhibitor for malignant versus normal cells using an in vitro granulocyte-macrophage colony-forming unit (CFU-GM) myelotoxicologic assay. The toxicity to normal bone marrow progenitor cells in this validated assay is relevant to the dose-limiting myelosuppression of temozolomide and topotecan and has been proven to be highly correlated to clinical maximum tolerated dose in man (12–15).

These studies showed that CEP-8983 caused significant sensitization of chemotherapy-resistant tumor cell lines to the effects of temozolomide and camptothecin (or SN38) in vitro by modulating the magnitude and/or duration of DNA damage, increasing the fraction and/or duration of time tumor cells arrest in the G₂-M phase of the cell cycle, and increasing the fraction of cells undergoing apoptosis. Further, administration of CEP-9722 attenuated in vivo PARP activity and resulted in significant chemosensitization of temozolomide and irinotecan in chemotherapy-resistant tumor xenografts in the absence of systemic toxicity and myelosuppressive effects. These data indicate that the scheduled administration of selective PARP-1 inhibitors with chemotherapy may be beneficial in the management of chemoresistant tumors.

Materials and Methods

Cell Lines

The HT29 human colon carcinoma cell line (American Type Culture Collection) was cultured in McCoy’s 5A (supplemented with 1.5 g/L sodium bicarbonate, Cellgro/Mediatech) containing 10% fetal bovine serum (Atlanta Biologicals); the U251MG human glioblastoma cell line (Brain Tumor Research Center Tissue Bank, University of California, San Francisco, CA) was cultured in MEM containing 10% fetal bovine serum; and the RG2 rat glioma cell lines (American Type Culture Collection), the NB1691 human neuroblastoma, and the RH18 human rhabdomyosarcoma cell line (provided by Peter Houghton, St. Jude Children’s Research Hospital, Memphis, TN) were cultured in RPMI 1640 containing 10% fetal bovine serum. All cell lines were cultured at 37°C in a humidified incubator, with 95% air/5% CO₂. Granulocyte colony-stimulating factor–mobilized peripheral blood mononuclear cells obtained from normal healthy donors who are human immunodeficiency virus and hepatitis B virus negative were purchased from Poietics, Inc. (Cambrex Bioscience).

In the studies described here, the cell lines used for in vitro experiments, NB1691 human neuroblastoma and RH18 human rhabdomyosarcoma, are reportedly defective in mismatch repair and resistant to temozolomide (16).
Attempts were made to correlate the mismatch repair status (i.e., MGMT or MSH-2 expression) of the RG2 rat glioma cell line used for in vivo studies to its temozolomide resistance. However, defects in the mismatch repair status of these cells could not be established unequivocally (data not shown). The HT29 human colon carcinoma cell line has been shown to be mismatch repair proficient and camptothecin resistant (17).

Reagents
Chemotherapeutic agents were obtained from the following sources: temozolomide (Schering-Plough), camptothecin (Sigma), SN38 (kindly provided by Dr. J.P. McGovern, Pfizer, Kalamazoo, MI), and topotecan (supplied as Hycamtin for injection; GlaxoSmithKline). Irinotecan is metabolized by carboxylesterases and the levels of cellular carboxylesterases are insufficient for inducing maximal irinotecan activation; consequently, the in vitro experiments were carried out using camptothecin, SN38, or topotecan (18). Temozolomide used for myelotoxicologic testing was resuspended in ethyl acetate and the active drug was extracted into a stabilizing formulation composed of acidified β-hydroxypropyl-cyclodextrin and propylene glycol. CEP-8983 (Cephalon, Inc.; Fig. 1A; ref. 11) was resuspended in 100% DMSO for in vitro studies and added at the initiation of the experiment in combination with or immediately after incubation with the chemotherapeutic agent. For in vivo studies, temozolomide and irinotecan were administered in 0.9% sterile saline. The prodrug CEP-9722 (patent pending) was prepared to improve solubility and deliverability of CEP-8983 in vivo and formulated in 45% (w/v) gluconic acid at an acid to base ratio of 2:1 immediately before use. This prodrug rapidly (<5 min) and nonenzymatically converts to CEP-8983.

3-(4-5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium Salt Assay
The ability of CEP-8983 to exacerbate the cytotoxic effects of chemotherapeutic agents on human carcinoma cells (HT29 and NB1691) was evaluated using the 3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assay (Promega). Cell number, reagent concentrations, and incubation times were optimized based on previously published data (7). Tumor cells were plated at 2,000 cells per well (HT29) or 4,000 cells per well (NB1691) and coincubated with SN38 (10–300 ng/mL) or temozolomide (50–300 μM/L), respectively, with CEP-8983 (0.1–10.0 μM/L) for 72 h (HT29) or 144 h (NB1691). Following the appropriate incubation times, effects on cell proliferation were assayed following a 2.5-h (HT29) or a 3-h (NB1691) incubation in 3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt solution (Promega). Absorbance was determined using a microtiter plate reader at 490 nm. Values presented are the mean ± SE of three independent experiments with each experiment having triplicate samples.

Figure 2. Effects of CEP-8983 in combination with camptothecin (CPT) on comet formation and stability. Exponentially growing cells were incubated with camptothecin (50 μmol/L) for 2 h, then washed, and incubated with medium containing 1 μmol/L CEP-8983 for 24, 28, 30, and 36 h. Cells were analyzed for the presence of comets using the Comet Assay (Trevigen, Inc.). Comets were counted at ×100 magnification using light microscopy; values were normalized to background (0.1% DMSO) and are expressed as percentage of total cells counted. Columns, mean of three independent experiments; bars, SE. ***, P < 0.001, camptothecin + CEP-8983 compared with camptothecin alone by Mann-Whitney rank-sum test.
**Comet Assay for Evaluation of In vitro DNA Damage**

Exponentially growing tumor cells were incubated with camptothecin (50 μmol/L; 2 h) and then incubated in medium alone with 0.1% DMSO or with CEP-8983 (1 μmol/L) for 0.5 to 30 h. Cells were harvested and counted at 24, 28, 29, and 30 h and processed using the Comet Assay kit (Trevigen, Inc.) as described previously (7). Comets (five fields per slide; ~200 cells) were counted.

**Figure 3.** Effects of CEP-8983 in combination with temozolomide and SN38 on the percentage of tumor cells arrested in the G2-M phase of the cell cycle. Exponentially growing RH18 cells (A) were incubated with temozolomide (100 μmol/L) for 3 h, then washed, and incubated with medium containing 1 μmol/L CEP-8983, or exponentially growing HT29 cells (B) were coincubated with SN38 (5 ng/mL) for the indicated time points. For both cell lines, cells were analyzed by flow cytometry to assess the percentage of cells remaining in G2 after treatment. Columns, mean of three independent experiments; bars, SE. A, ***, *P* < 0.001; ****, *P* < 0.0001, untreated compared with temozolomide; *, *P* < 0.05; **, *P* < 0.01; ****, *P* < 0.0001, temozolomide alone compared with temozolomide + CEP-8983. B, ***, *P* < 0.001, SN38 alone compared with untreated; **, *P* < 0.01; ****, *P* < 0.0001, SN38 alone compared with SN38 + CEP-8983 by Mann-Whitney rank-sum test or *t* test where appropriate.
at ×100 magnification; counts were normalized to background (0.1% DMSO). Values presented are the mean ± SE of three independent experiments.

**Flow Cytometric Evaluation of Cell Cycle In vitro**

Exponentially growing RH18 cells were incubated for 1 h with temozolomide (300 μmol/L) and then incubated with medium containing 1 μmol/L CEP-8983 for 48 h. After the appropriate incubation period, cells were analyzed for induction of apoptosis using the flow cytometry method. Plates were read using a spectrophotometer at 405 nm. Columns, mean of three independent experiments with each experiment having duplicate samples; bars, SE. A, *, *P ≤ 0.05; ***, *P ≤ 0.001, SN38 compared with 0.01% DMSO control; *, *P ≤ 0.001, SN38 compared with SN38 + CEP-8983; **, *P ≤ 0.01, CEP-8983 compared with 0.01% DMSO. B, ***, *P ≤ 0.001, temozolomide compared with 0.01% DMSO control; ***, *P ≤ 0.001, temozolomide compared with temozolomide + CEP-8983; *, *P ≤ 0.05, CEP-8983 compared with 0.01% DMSO by Mann-Whitney rank-sum test or t test where appropriate.

**Figure 4.** Effects of CEP-8983 in combination with temozolomide and SN38 on the induction of tumor cell apoptosis. Exponentially growing HT29 cells (A) were incubated with SN38 (5 ng/mL) alone or in combination with 1 μmol/L CEP-8983 for 3, 4, and 5 days or exponentially growing RH18 cells (B) were incubated with temozolomide (300 μmol/L) alone or in combination with 1 μmol/L CEP-8983 for 24 to 48 h. After the appropriate incubation period, cells were analyzed for induction of apoptosis using the caspase-3/CPP-32 colorimetric assay kit. Plates were read using a spectrophotometer at 405 nm. Columns, mean of three independent experiments with each experiment having duplicate samples; bars, SE. A, *, *P ≤ 0.05; ***, *P ≤ 0.001, SN38 compared with 0.01% DMSO control; *, *P ≤ 0.001, SN38 compared with SN38 + CEP-8983; **, *P ≤ 0.01, CEP-8983 compared with 0.01% DMSO. B, ***, *P ≤ 0.001, temozolomide compared with 0.01% DMSO control; ***, *P ≤ 0.001, temozolomide compared with temozolomide + CEP-8983; *, *P ≤ 0.05, CEP-8983 compared with 0.01% DMSO by Mann-Whitney rank-sum test or t test where appropriate.
growing RH18 cells were incubated with temozolomide (300 μmol/L) for 2 h and then incubated for 24 to 48 h with 1 μmol/L CEP-8983. Cells were analyzed for induction of apoptosis using the caspase-3/CPP-32 colorimetric assay kit (Biovision). Briefly, cells were lysed in cell lysis buffer and protein lysates were quantitated (bicinchoninic acid assay, Pierce). Protein lysates (200 μg) were incubated with reaction buffer and DEVD-pNA substrate at 37°C for 2 h. Plates were read using a spectrophotometer at 405 nm. Data are the mean ± SE of three independent experiments with each experiment having duplicate samples.

**CFU-GM Colony Formation Assay to Determine Potentiation of Myelotoxicity**

**Human CFU-GM Assay**

Validation of the CFU-GM assay and IC₉₀ end points were described previously (19, 20). To eliminate intersubject variation as a source of experimental variation, cells from the same donors were used throughout the study. Cells were thawed using a European Center for the Validation of Alternative Methods procedure (19, 20), and cell number and viability were determined using trypan blue. Peripheral blood mononuclear cells (2.2 x 10⁶/300 μL) were added to MethoCult H4001 (Stem Cell Technologies, Inc.), followed by addition of CEP-8983 (1 or 3 μmol/L) and topotecan or temozolomide, and cultures were plated and incubated in a humidified, 5% CO₂/95% air environment at 37°C for 14 days.

CFU-GM colonies (focal, diffuse, and mixed) were defined as foci of clonal cell proliferation containing at least 64 myeloid cells. The colony counts from triplicate cultures at each concentration were averaged, and the percentage inhibition was determined. The endpoint of the assay for quantifying interspecies differences in susceptibility to drug toxicity is the IC₉₀ and is calculated based on log-linear regression data as detailed (European Center for the Validation of Alternative Methods, Ispra, Italy; refs. 12, 14, 15, 19).

**In vivo Pharmacodynamic and Chemosensitization Studies**

**Animals**

Female athymic nu/nu mice (8–10 weeks old, 22–25 g; Charles River) were maintained and housed as described previously (7). Animal experiments were done at Cephalon under protocol 03-023 approved by the Institutional Animal Care and Use Committee of Cephalon.

**Tumor Cell Implantation and Measurement of Tumor Growth**

Exponentially growing cells were cultured as described above, harvested, and injected (2 x 10⁶ cells per mouse) 1:1 with Matrigel (Fisher Scientific) into the right flank of nude mice. Tumor-bearing mice were randomized according to tumor size, with 10 mice per group for the chemosensitization studies or six mice per group for the PAR accumulation assay. Tumors were measured every 3 to 4 days for HT29 xenografts and 2 to 3 days for RG2 xenografts, and volumes were calculated as described previously (7). Relative tumor volumes were determined by normalizing individual tumors to their initial starting size at the initiation of dosing. Statistical analyses were calculated by the Mann-Whitney rank-sum test or t test (Sigma Stat) where appropriate with P ≤ 0.05 deemed significant.

**PAR ELISA of Tumor Xenografts**

Time course studies were carried out to determine the time point at which temozolomide-induced PAR accumulation was at its peak (7). Tumor-bearing nude mice were treated with one dose of temozolomide (68 mg/kg, p.o.) 1 h before the administration of one dose of CEP-9722 (41 or 136 mg/kg, s.c.). Mice were sacrificed 4 h after temozolomide dosing, protein lysates were made, and the PAR ELISA was carried out as described previously (7).

**Chemosensitization Studies**

The dose equivalents of CEP-8983 used in these studies were based on tolerability studies and on preliminary...
Results

Effect of CEP-8983 in Combination with Temozolomide and SN38 on Tumor Cell Proliferation

The ability of CEP-8983 to enhance the growth-inhibitory effects of temozolomide or SN38 was evaluated using NB1691 or HT29 cells, respectively. A 144-h coincubation of NB1691 cells with temozolomide (50–300 μmol/L) and CEP-8983 (1, 3, and 10 μmol/L) resulted in an increase in the magnitude (20–85%; P ≤ 0.05–0.0001) of tumor cell growth inhibition compared with the corresponding concentrations of temozolomide alone, whereas only coincubation of temozolomide at 250 and 300 μmol/L with 0.3 μmol/L CEP-8983 resulted in an increase (30–40%; P ≤ 0.05–0.01) of tumor cell growth inhibition compared with the corresponding concentrations of temozolomide alone (Fig. 1B). For the HT29 cell line, a 72-h coincubation of SN38 (10 and 100 ng/mL) with CEP-8983 (0.1, 0.3, 1, 3, and 10 μmol/L), SN38 (30 ng/mL) with CEP-8983 (0.1, 1, and 10 μmol/L), and SN38 (300 ng/mL) with CEP-8983 (0.1 and 1 μmol/L) resulted in (15–30%) P ≤ 0.05–0.001) an increase in the magnitude of tumor cell growth inhibition compared with the corresponding concentrations of SN38 alone (data not shown). In this assay for both cell lines, CEP-8983 alone had no effect on cell proliferation (data not shown).

Effects of PARP-1 Inhibition on DNA Damage and Repair (Comet Assay)

The effects on the kinetics of DNA damage and repair were analyzed using a single-cell gel electrophoresis assay (Comet Assay). The concentrations of the chemotherapeutic agents and the time points used in these studies were optimized based on previously published data and preliminary studies to determine a concentration resulting in maximal comet formation, in the absence of cell toxicity. In incubation with camptothecin alone showed that initial peak comet formation in 24 to 28 h, with a decrease in comet formation between 28 to 30 h followed by a peak at initial levels of 36 h. Incubation with CEP-8983 after camptothecin treatment resulted in an increase of comet formation at the 24-h (21%; P ≤ 0.001) and 28-h (66%; P ≤ 0.0001) time points compared with incubation with camptothecin alone (Fig. 2). CEP-8983 did not have significant effects on DNA damage when administered as a monotherapy. These data reproduce trends reported previously with another PARP inhibitor (CEP-6800) and indicate that CEP-8983 can potentiate the number of DNA strand breaks at the initial peak time of camptothecin-induced strand breaks but could not effectively stabilize DNA strand breaks over the time period evaluated.

Effects of PARP-1 Inhibition on the Percentage of Tumor Cells in the G2-M Phase of the Cell Cycle

The ability of CEP-8983 in combination with temozolomide or SN38 to increase the fraction of cells arrested in G2-M and/or the duration of time in the G2-M phase of the cell cycle was evaluated using temozolomide-resistant RH18 and SN38-resistant HT29 cells, respectively. Cell cycle arrest in the G2-M phase of the cell cycle peaked at 32% accumulation compared with 0.1% DMSO control (20%
accumulation) within 24 h of exposure to temozolomide (300 µmol/L). Incubation of temozolomide (300 µmol/L) with CEP-8983 (1 µmol/L) resulted in an increase in the fraction of RH18 cells accumulated at G2-M at the 24-h time point (60% accumulation; \( P \leq 0.001 \)) compared with temozolomide alone and was sustained to 72 h (53% accumulation; \( P \leq 0.001 \)). Maximal G2-M accumulation was observed at the 40-h time point (74%; \( P \leq 0.001 \)) with the magnitude of cells accumulating at G2-M decreasing at each subsequent time point (Fig. 3A).

Continuous exposure of HT29 cells with SN38 (5 ng/mL) resulted in peak accumulation between 16 to 24 h (60–68% accumulation compared with 22–45% accumulation 0.1% DMSO control; \( P \leq 0.001–0.0001 \); Fig. 3B). Following an approximate 22% decrease in the percentage of cells accumulated in G2-M at the 40-h time point, the accumulation of cells at the G2-M boundary was sustained throughout the 72-h time course (45–54% accumulation \( P \leq 0.001 \)) compared with 20–26% accumulation for 0.1% DMSO control. Coincubation with CEP-8983 resulted in the accumulation of cells in G2-M (80–90% accumulation; \( P \leq 0.001 \)) at all time points between 24 to 72 h (\( P \leq 0.001 \)) compared with SN38 alone (Fig. 3B). In both cell lines used for these studies, CEP-8983 exposure alone had no significant effects on the cell cycle.

Effects of PARP-1 Inhibition on Apoptosis

The ability of CEP-8983 to sensitize HT29 cells to SN38-induced apoptosis was evaluated using the caspase-3/CPP-32 assay that detects intracellular changes because of the initiation of apoptosis. Incubation of HT29 cells with SN38 (5 ng/mL) alone caused a 5- to 8-fold increase (\( P \leq 0.05–0.0001 \)) in caspase-3 activity compared with the 0.01% DMSO controls over the 5-day time course. On day 3, coincubation of SN38 with CEP-8983 resulted in an additional 2-fold (\( P \leq 0.05 \)) increase in caspase-3 activity compared with SN38 alone by day 5, the activity of the cells incubated with SN38 and CEP-8983 was essentially equivalent to cells treated with SN38 (Fig. 4A). Incubation with CEP-8983 alone resulted in a 3-fold increase (\( P \leq 0.01 \)) in cells undergoing apoptosis compared with the 0.01% DMSO control (Fig. 4A).

Similarly, the ability of CEP-8983 to potentiate temozolomide-induced apoptosis was evaluated using the RH18 cell line. Temozolomide (300 µmol/L) incubation resulted in a 10- to 20-fold increase of RH18 cells undergoing apoptosis (\( P \leq 0.001 \)) compared with the 0.01% DMSO control over the 48-h time period (Fig. 4B). Incubation of 1 µmol/L CEP-8983 following temozolomide exposure resulted in a further a 4- to 5-fold increase (\( P \leq 0.001 \)) in apoptosis compared with temozolomide alone over the 48-h time course. In this cell line, incubation of CEP-8983 alone had minimal effects on apoptosis compared with the 0.01% DMSO controls (Fig. 4B).

Effects on the Potentiation of Topotecan- or Temozolomide-Induced Myelotoxicity

The effect of CEP-8983 on the potentiation of topotecan- or temozolomide-induced myelotoxicity was evaluated in a CFU-GM colony-forming assay using human bone marrow cells. In triplicate experiments with each of two donor marrow specimens, CEP-8983 was found to possess negligible toxicity to human CFU-GM progenitors at a concentration of 3 µmol/L as shown by the fact that inhibition of colony formation did not reach the 20% effect level that would be required to exceed the experimental variation in human CFU-GM colony formation (19, 20). Coincubation of CEP-8983 (1 or 3 µmol/L) with 0.4 or 4 nmol/L topotecan did not result in any increased toxicity to human CFU-GM over that observed with topotecan alone (Fig. 5). For example, colony formation in 0.4 nmol/L topotecan plus 3 µmol/L CEP-8983 equaled that in single-agent topotecan at 0.4 nmol/L (90 ± 9% versus 97 ± 6% of vehicle-treated controls; \( P = 0.11 \)), and colony formation in 4.0 nmol/L topotecan plus 1 µmol/L PARP inhibitor equaled that in single-agent topotecan at 4.0 nmol/L (73 ± 13% versus 82 ± 7% of vehicle-treated controls; \( P = 0.10 \)). All of these topotecan-based combinations inhibited human CFU-GM colony formation by <40% (when topotecan concentration is ≤4.0 nmol/L and the CEP-8983 concentration is ≤3 µmol/L), which is the likely minimal effect level required for mild neutropenia in vivo (14, 15). The findings suggest that a combination of CEP-8983 plus topotecan will produce clinical myelosuppression no worse than that caused by single-agent topotecan, at doses that cause mild myelosuppressive neutropenia.

Coincubation of CEP-8983 with 18 µmol/L temozolomide, which was nontoxic to human CFU-GM when tested alone, did not result in any toxicity to human CFU-GM (colony formation was 93 ± 10% of vehicle-treated controls; \( P = 0.10 \); data not shown). Increasing the temozolomide concentration to 72 µmol/L in combination with 3 µmol/L CEP-8983 resulted in detectable inhibition of colony formation (81 ± 7% of vehicle controls; \( P = 0.01 \)) that remained well below the in vitro effect level associated with onset of neutropenia in vivo (40% reduction in CFU-GM). Adding 3 µmol/L CEP-8983 to the highest test concentration of temozolomide (290 µmol/L) inhibited CFU-GM by 69 ± 14%, which was an intermediate level of toxicity between that caused by temozolomide alone (50 ± 8%; \( P = 0.03 \)).

Because the structure of CEP-8983 does not confer any intrinsic chemical toxicity to normal human progenitor cells, the small increase in temozolomide suppression of CFU-GM colony formation detected by the in vitro assay likely represents the consequences of actual modulation of PARP function in response to temozolomide myelotoxicity. Although the difference in suppression of colony formation by temozolomide in the presence and absence of CEP-8983 may seem small (69% versus 50% inhibition), it is noted that the in vitro adverse effect level of the combination is associated with moderate neutropenia in vivo, whereas the in vitro effect level in the absence of the PARP inhibitor is associated with mild neutropenia in vivo (19, 20).

PAR Accumulation Assay

The ability of CEP-9722 to attenuate PAR accumulation (PARP activation) was evaluated using U251MG glioma xenografts. The dose and time points used in this study
were based on previous data using temozolomide in other glioma xenograft models (7). Temozolomide (68 mg/kg/dose, p.o.) administration resulted in a 41% increase in PAR accumulation compared with vehicle-treated mice (Fig. 6). Administration of temozolomide in combination with CEP-9722 at 41 or 136 mg/kg/dose, s.c. attenuated PAR accumulation by 58%, CEP-9722 (41 and 136 mg/kg) administered as a monotherapy attenuated PAR accumulations significantly (86% and 70%, respectively; \( P < 0.01 \)) compared with vehicle treatment. Data have been presented showing that tumor tissues have increased levels of PARP activity (8); the basal levels of PAR activity obtained in our studies are supportive of these claims. The fact that CEP-9722 administration inhibited basal levels of PAR activation is suggestive that this inhibitor could have ‘‘stand-alone’’ effects. These combined data indicate that CEP-8983 prodrug can attenuate PAR accumulation in U251MG tumor xenografts.

Chemosensitization of Temozolomide Using RG2 Rat Glioma Xenografts

The effect of alternate dosing schedules of temozolomide (68 mg/kg/dose q.d, p.o., 5 days) and CEP-9722 (136 mg/kg/dose; administering CEP-9722 1 h before administration of temozolomide compared with administration 1 h after temozolomide treatment) was evaluated using chemotherapeutic refractory RG2 rat glioblastoma xenografts in nude mice (21). Temozolomide monotherapy resulted in significant but moderate tumor growth inhibition compared with the vehicle control-treated tumors beginning on day 5 (21%; \( P < 0.05 \)) and continuing through day 12 (32%; \( P < 0.01 \); Fig. 7A). Significant inhibition of tumor growth was lost on day 15 but returned on day 17 (30%; \( P < 0.05 \)), the termination of the study (Fig. 7A). Administration of CEP-9722 1 h after temozolomide administration on the 5-day dosing regimen resulted in tumor growth inhibition (32–60%; \( P < 0.001 \–0.0001 \); Fig. 7A) compared with temozolomide monotherapy beginning on day 3 and continuing until the end of the experiment (day 17). Maximal tumor growth inhibition of this group compared with temozolomide alone was observed on days 12 and 15 (60%; \( P < 0.0001 \)). Similarly, administration of CEP-9722 1 h before temozolomide administration on the 5-day dosing regimen resulted in tumor growth inhibition (32–60%; \( P < 0.001 - 0.0001 \); Fig. 7A) compared with temozolomide monotherapy beginning on day 3 and continuing until the end of the experiment (day 17). The magnitude of the effect from the latter temozolomide dosing schedule was substantially diminished compared with that observed with giving temozolomide before CEP-9722 (26–36% CEP-8983 + temozolomide compared with 32–60% CEP-8983 + temozolomide compared with vehicle treatment).
Figure 7 Continued. B, on HT29 xenografts reaching mean volume between 250 to 260 mm³, mice were treated on day 1 to day 5 with vehicle (sterile saline, 100 μL/dose, p.o., qd + gluconic acid, 100 μL/dose, s.c., qd), irinotecan (10 mg/kg, i.p., qd) as a monotherapy, irinotecan (10 mg/kg, i.p., qd) + CEP-9722 (136 mg/kg/dose, s.c., qd), CEP-9722 (136 mg/kg/dose, s.c., qd) + irinotecan (10 mg/kg, i.p., qd), or CEP-9722 (136 mg/kg/dose, s.c., qd) as a monotherapy. For the combination groups, the first agent was given 1 h before the second agent. Tumor volumes were determined every 3 to 5 d. Relative tumor volumes were determined by normalizing individual tumors to their initial starting size at the initiation of dosing. Statistical analyses were done by t test or Mann-Whitney rank-sum test where appropriate.

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<th>Day of Dosing</th>
<th>Vehicle vs. Irinotecan</th>
<th>Irinotecan vs. CEP-9722</th>
<th>Irinotecan vs. CEP-9722 + Irinotecan</th>
<th>Irinotecan + CEP-9722 vs. CEP-9722 + Irinotecan</th>
<th>Vehicle vs. CEP-9722</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.0001</td>
<td>NS</td>
<td>NS</td>
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</tr>
<tr>
<td>8</td>
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<td>P &lt; 0.0001</td>
<td>NS</td>
<td>NS</td>
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</tr>
<tr>
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<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.05</td>
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<tr>
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<td>P &lt; 0.0001</td>
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<td>NS</td>
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<tr>
<td>26</td>
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<td>P &lt; 0.0001</td>
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<td>NS</td>
<td>P &lt; 0.0001</td>
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</tbody>
</table>

Temozolomide + CEP-9722; P ≤ 0.01; Fig. 7A). In this study, the CEP-9722 monotherapy also resulted in tumor growth inhibition of 35% to 70% (P ≤ 0.05–0.001) beginning on day 5 and continuing until the end of the study. The major toxicity after 5 days of dosing with temozolomide alone and in combination with CEP-9722 was transient body weight loss. The maximal weight loss observed was ~8.0% by day 8; this magnitude of weight loss was reversible with mice beginning to regain weight by day 9 of the study (data not shown).

**Chemosensitization of Irinotecan Using HT29 Human Colon Carcinoma Xenografts**

The effect of alternate dosing schedules of irinotecan (10 mg/kg/dose) and CEP-9722 (136 mg/kg/dose; administering CEP-9722 1 h before administration of irinotecan compared with administration 1 h after irinotecan treatment) was evaluated in nude mice bearing irinotecan-resistant HT29 human colon carcinoma xenografts (22). Irinotecan monotherapy resulted in significant but moderate tumor growth inhibition compared with vehicle control-treated tumors beginning on day 5 (14%; P ≤ 0.05) and continuing until the termination of the study (day 26; 50%; P ≤ 0.001; Fig. 7B). Maximal tumor growth inhibition of 56% (P ≤ 0.0001) for this treatment group was observed on day 19. Administration of irinotecan 1 h before CEP-9722 also resulted in tumor growth inhibition (54–90%; P ≤ 0.0001; Fig. 7B) compared with irinotecan monotherapy beginning on day 5 and continuing until the end of the experiment (day 26). Maximal tumor growth inhibition of this group compared with irinotecan alone was observed on days 19 to 26 (~90%; P ≤ 0.0001). Similarly, administration of irinotecan 1 h after CEP-9722 administration resulted in tumor growth inhibition (51–83%; P ≤ 0.05–0.001) compared with irinotecan monotherapy beginning on day 5 and continuing until day 26 (Fig. 7B). There were no significant differences in tumor growth inhibition between the two combination treatment groups. Although both groups showed a nadir of 25% loss in body weight relative to initial starting weight (P ≤ 0.001–0.0001) by day 8, administration of irinotecan 1 h after CEP-9722 treatment resulted in mortality beginning on day 8 and reaching a maximal 60% mortality by day 12 compared with 20% mortality by day 12 in the combination treatment group in which irinotecan administration preceded treatment with the CEP-9722. The mice receiving irinotecan 1 h before CEP-9722 began to gain weight starting on day 12. In this study, neither irinotecan alone or CEP-9722 alone had any...
effect on body weight. As observed in the RG2 glioma xenografts, CEP-9722 monotherapy resulted in inhibition of tumor growth compared with the vehicle-treated tumors beginning on day 5 (35%; P ≤ 0.01) and continuing to day 26 (~70%; P ≤ 0.0001; Fig. 7B) indicative of a "stand alone" antitumor effect of CEP-9722 in the preclinical models used in these studies.

Discussion

The most significant cause for treatment failure and cancer mortality is radioresistance/chemoresistance, necessitating the unmet medical need to identify agents that will sensitize resistant cancer cells to traditional cytotoxic therapies and while maintaining an acceptable therapeutic index when used in combination. PARP-1 inhibition has been the focus of extensive research as a radiosensitizer/chemosensitizer based on evidence implicating a role for PARP-1 (and PARP-2) in DNA damage repair and survival of cells under genotoxic stress (3, 9). The potential application and preclinical development of PARP inhibitors as radiosensitizers/chemosensitizers, until recently, was limited by their potency, selectivity, and pharmaceutical properties. These issues lead to the development of more potent and selective PARP-1 inhibitors with acceptable toxicity profiles and the ability to sensitize tumor cells to the effects of radiation and established chemotherapies using both human and murine solid and/or hematologic tumor models (1, 7, 9, 23–25). A PARP inhibitor capable of sensitizing tumor cells to different classes of chemotherapeutic agents and radiation while not affecting the toxicities associated with these treatments could improve both the therapeutic index and potential success rate of established cancer therapies.

The studies reported here provide evidence that the PARP-1 inhibitor, CEP-8983, delivered as an aqueous soluble prodrug (CEP-9722) attenuates PARP-1 activity in U251MG human glioblastoma xenografts in response to administration of temozolomide and sensitizes both temozolomide- and irinotecan-resistant tumor xenografts to these agents. In vitro and in vivo pharmacodynamic data confirm previous data showing inhibition of irinotecan- and temozolomide-induced PARP-1 activity in colon carcinoma xenografts by CEP-6800 (7). The use of PARP inhibitors to interfere with PARP-1–mediated base excision repair mechanisms induced by temozolomide and PARP-1–mediated ADP-ribosylation of topoisomerase I in response to camptothecin exposure sensitizes resistant tumor cells to the cytotoxic effects of these agents (1, 5, 10). The pharmacodynamic and in vivo data reported here with CEP-8983 support these observations in that the pharmacologic effects of CEP-8983 are directly associated with its PARP inhibitory activity. No significant inhibitory activity of CEP-8983 was observed against a panel of 100 tyrosine and serine-threonine kinases at a concentration of 1 μmol/L (Upstate). In a panel of 146 receptors, transporters, and ion channels (MDS Pharma Services), only 10 displayed >50% inhibition by the prodrug of CEP-8983 at a screening concentration of 10 μmol/L (adenosine transporter and adenosine A1, A2a, and A3; bombesin BB3; muscarinic M1, M3, and M4; serotonin 5-HT1A; and thromboxane A2 receptors). These data indicate that CEP-8983 is a relatively selective compound and that its chemosensitization activity is most likely due to its low nanomolar inhibition of PARP-1 (2) activity.

The ability of CEP-8983 to sensitize tumor cells to the growth-inhibitory effects of temozolomide or SN38 as shown in the 3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assay could be due to stabilization of DNA strand breaks caused by these agents. This inhibition of proliferation could be a result of G2-M arrest while the cells were undergoing DNA damage repair. Previously, we and others have shown that incubation of chemotherapy-sensitive and chemotherapy-resistant tumor cells with PARP-1 inhibitors in combination with temozolomide, camptothecin (or SN38), or cisplatin results in significant inhibition of cell proliferation, promotes the onset and magnitude of DNA damage, and prolongs the duration of DNA repair (refs. 7, 24, 26; data not shown). In this study, CEP-8983 increased the percentage of HT29 cells with camptothecin-induced DNA damage between 24 to 28 h compared with camptothecin-treated cells. However, at the 30- and 36-h time points, the percentage of cells with DNA strand breaks decreased as the tumor cells attempt to repair DNA damage.

Using chemotherapy-resistant cell lines, the present study expanded on previous observations that PARP-1 inhibition results in the accumulation of tumor cells with DNA damage at the G2-M boundary of the cell cycle (7, 27). PARP-1 inhibition with CEP-8983 in combination with SN38 or temozolomide resulted in a shift in the onset and magnitude of tumor cell accumulation at the G2-M boundary compared with SN38 or temozolomide treatment alone using HT29 and RH18 cells, respectively. Exposure to SN38 and CEP-8983 resulted in a sustained increase in the percentage of cells accumulating at the G2-M boundary over the 72-h time course compared with SN38 alone. In contrast, CEP-8983 exposure resulted in peak accumulation at the 24-h time point with accumulation decreasing at each subsequent time point, with the percentage accumulation of the combination group almost reaching that of temozolomide alone by the end of the study. These data support our earlier findings and those of others showing that inhibition of PARP activity results in increased genomic instability (as shown by the increased DNA strand breaks observed in the comet assay) and in the accumulation of damaged cells at the G2-M boundary of the cell cycle (28).

Activation of PARP is an ATP-depleting process, and as a result, as ATP levels within cells with DNA damage fall below a critical level, cells cannot undergo apoptosis, rendering them resistant to programmed cell death or susceptible to death through necrosis. Therefore, inhibition of PARP in combination with irreparable or substantial DNA damage would conserve cellular energy levels allowing tumor cells to undergo apoptosis in response to DNA-damaging agents and thereby eliminate local toxic effects or immune responses associated with necrosis (8).
This study shows that HT29 cells arrested in the G2-M phase of the cell cycle due to coincubation with SN38 and CEP-8983 or incubation of RH18 with CEP-8983 after temozolomide exposure are undergoing apoptosis as determined by caspase-3 activity.

A variety of strategies have been developed to potentiate the potency of cytotoxic anticancer drugs with limited success. Several compounds have been shown to increase cellular susceptibility to cytotoxic drug action but have not proven any sustained therapeutic benefit (29–31). Chemosensitizing agents that do not affect xenobiotic metabolism and excretion may have greater potential to achieve selective modulation of cytotoxic drug action in tumors, without reducing drug clearance and dose tolerance. In addition, it would be highly desirable to achieve selective potentiation of drug action in tumor cells compared with normal tissues and thereby a gain in therapeutic index. Using a validated CFU-GM colony-forming assay to evaluate the myelotoxicity of PARP inhibitors, we showed that PARP modulation (by CEP-8983) in combination with topotecan or temozolomide at the recommended clinical doses of topotecan (1.5 mg/m² qd × 5) or temozolomide (200 mg/m² qd × 5; refs. 32, 33) did not enhance the myelosuppressive effects of these cytotoxic agents. Based on these data, CEP-8983 would be predicted to result in a single grade increase in neutropenia from mild to moderate in patients. In contrast, myelotoxicity data obtained using CEP-6800 showed inherent myelotoxicity, indicating a high likelihood of clinical neutropenia in patients even in the absence of chemotherapy (data not shown). These combined data show that myelotoxicity observed with CEP-6800 is a compound related phenomenon rather than due to a biological response to PARP inhibition.

The observed biochemical efficacy of the prodrug CEP-9722 on the attenuation of basal and temozolomide-induced PARP-1 activation in vivo and the effects of CEP-8983 in combination with temozolomide or camptothecin (SN38) on tumor cell proliferation, DNA damage and repair, G2-M arrest, and apoptosis would be predicted to result in significant chemosensitization of resistant tumor models in vivo. To test this hypothesis, CEP-9722 was administered to mice at a dose predicted to achieve plasma concentrations at Cmax ~ 100-fold the cellular IC50 for this compound (300 nmol/L). Administration of CEP-9722 in combination with temozolomide or irinotecan resulted in statistically significant growth inhibition in the chemoresistant RG2 glioma and HT29 colon carcinoma xenograft models, respectively, with the magnitude of the response or the tolerability of the treatment varying with respect to the dosing schedule. The magnitude of the effect of administering temozolomide before CEP-9722 using the RG2 xenograft model was greater than the effect observed when the PARP inhibitor was administered before temozolomide (26–36% CEP-9722 + temozolomide compared with 32–60% temozolomide + CEP-9722). Conversely, using HT29 colon carcinoma xenografts, the magnitude of antitumor response observed between different dosing schedules was similar; however, administration of the PARP inhibitor before irinotecan resulted in 60% mortality compared with 20% mortality when irinotecan was given before the PARP inhibitor. These combined data indicate that CEP-9722 can sensitize resistant tumor xenografts to two mechanistically distinct chemotherapeutic agents and that the efficacy and/or tolerability of this combination regimen may be schedule dependent. Additional dose- and schedule-dependent optimization of CEP-9722 and chemotherapeutic agents may be required in preclinical models to observe a maximal biological response.

In addition to potentiating the effects of temozolomide and irinotecan, administration of the CEP-9722 as a monotherapy to tumor-bearing nude mice resulted in significant tumor growth inhibition and marked inhibition of PAR accumulation as determined by PAR ELISA. This “stand alone” phenomenon is plausible as many tumors have enhanced basal PARP activity in the absence of exogenous genotoxic stimuli; thus, PARP inhibition alone could potentially effect tumor growth (8). In recent reports show the antitumor efficacy of PARP inhibitor monotherapy in select tumor cell populations (BRCA1 and BRCA2 breast carcinomas and ovarian carcinomas) with inherent defects in homologous recombination repair (34, 35). Alternatively, because the in vitro data presented here indicate that CEP-8983 alone had no effect on DNA damage/repair, G2 arrest, or cell proliferation, the effects of CEP-9722 monotherapy observed could be due to inhibition of angiogenesis as it has been shown recently that PARP inhibition reduces angiogenesis (36). Studies are currently under way to address this possibility.

In conclusion, the studies reported here show that the PARP-1 inhibitor, CEP-8983, a 4-O-methoxy-carbazole (and its prodrug, CEP-9722), can sensitize chemotherapy-resistant cell lines and subcutaneous xenografts to the effects of temozolomide and camptothecin (SN38 or irinotecan) as evaluated by inhibition of cell proliferation, modulation of DNA damage/repair, G2-M arrest, apoptosis, and in vivo antitumor efficacy. In addition, “stand alone” antitumor activity with this PARP inhibitor was observed in vivo, expanding its potential application against select tumor types. These pharmacologic data along with results showing that CEP-8983 does not potentiate temozolomide- or topotecan-associated human myelotoxicity suggest its potential clinical application as an adjunct chemosensitizing agent with a favorable therapeutic index in a wide variety of cancers resistant to these genotoxic agents.

References

Chemosensitization by PARP Inhibition


Molecular Cancer Therapeutics

The selective poly(ADP-ribose) polymerase-1(2) inhibitor, CEP-8983, increases the sensitivity of chemoresistant tumor cells to temozolomide and irinotecan but does not potentiate myelotoxicity

Sheila Miknyoczki, Hong Chang, Jennifer Grobelny, et al.


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